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Suppression of BK virus replication and cytopathic effect by inhibitors of prokaryotic DNA gyrase

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Summary

Nalidixic acid and oxolinic acid, two antibacterial agents known to inhibit bacterial DNA gyrase, are shown to suppress the replication, as well as the cytopathic effect, of BK virus in Vero cell cultures. The inhibition of virus replication was detectable at day 4 post infection in cultures which had been continuously exposed to drugs at concentrations as low as 0.02 to 0.04 mM of nalidixic acid and 0.2 mM of oxolinic acid. These active concentrations are inferior to plasma levels attained in the course of clinical use of the drugs for antibacterial chemotherapy. Also, under these circumstances, no cytotoxicity occurred. The inhibition of development of cytopathology and of virus-induced cell death was demonstrable in cultures treated for 12 days with the drugs. Under these circumstances of prolonged action, oxolinic acid proved to be slightly cytotoxic in that virus inhibitory doses reduced the viability of normal cells. No alterations in the topological conformation of the viral genome or accumulation of end products of viral DNA replication were detected. However, accumulation of viral DNA form I at 48 h post infection suggests that the drugs act through a mechanism involving DNA topoisomerase.

BK virus; Papovavirus; Nalidixic acid; Oxolinic acid; DNA topoisomerase

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Introduction

DNA topoisomerases are enzymes involved in topological variations of DNA conformation (Gellert, 1981; Wang, 1985). They are distinguished in type I and type II according to whether they produce single-stranded or double-stranded breaks on DNA. Gyrase (Cozzarelli, 1980; Gellert et al., 1976) is the most investigated among prokaryotic type II topoisomerases. It introduces negative supercoiling into relaxed DNA molecules and is necessary for specific initiation and elongation of nascent DNA strands during DNA replication of *Escherichia coli* and coliphage T4 (Liu et al., 1979). Eukaryotic DNA topoisomerases have been described (Baldi et al., 1980; Liu and Miller, 1981; Liu et al., 1981; Miller et al., 1981) and shown to participate in the replication complex during DNA synthesis. Both prokaryotic and eukaryotic topoisomerases are involved in several additional functions, such as transcription, recombination, transposition, DNA repair and assembly of chromatin (Vosberg, 1985).

Some aspects of topoisomerase function were elucidated by development of temperature-sensitive and deletion mutants as well as by the use of specific inhibitors. Several such inhibitors have been discovered. The quinolone antibiotics nalidixic and oxolinic acids inhibit prokaryotic gyrase subunit A (Cozzarelli, 1980; Dhalluin, 1980) endowed with nicking-closing activity, whereas the coumarin antibiotics novobiocin and coumermycin A1 competitively inhibit gyrase subunit B (Gellert et al., 1976) which displays ATPase and energy-transducing activity. Indirect experimental evidence suggests that topoisomerases are involved in the replication cycle of mammalian viruses, such as adenoviruses (Dhalluin et al., 1980; Goding and Russell, 1983), herpesviruses (Francke and Margolin, 1981; Palù et al., 1986) and retroviruses (Varnier et al., 1984, 1985). Since papovaviruses have a circular DNA genome, topological transitions in DNA formation are required during virus replication and transcription. Recently, a DNA topoisomerase II was found associated with polyomavirus minichromosomes. This activity cosediments with replicative intermediate forms (Krauss et al., 1984). Moreover, in a cell-free system topoisomerases were required for simian virus 40 (SV40) replication (Yang et al., 1987). Either topoisomerase I or topoisomerase II from HeLa cells can mediate progression of the replication fork during SV40 DNA synthesis, whereas only topoisomerase II is able to resolve the catenated dimers formed at the end of replication leading to segregation of the newly synthesised daughter molecules. Following these indications, we studied the possible involvement of DNA topoisomerase II in the replicative cycle of BK virus (BKV), a human papovavirus ubiquitous in human populations (Yoshiike and Takemoto, 1986), by examining its replication in cells treated with nalidixic and oxolinic acids.

Materials and Methods

Virus and cells

BKV was grown in Vero cells (Maraldi et al., 1975) and titrated by hemagglu-

tination (HA) of human type O erythrocytes (Portolani et al., 1974). Vero cells were employed in all experiments by infection with 1000 hemagglutinating units (HAU) of BKV. Growth and maintenance medium was Eagle's minimum essential medium supplemented with 10 and 5% fetal bovine serum respectively.

Chemicals

Nalidixic and oxolinic acids (Sigma Chemical Co., St. Louis, MO) were dissolved in 0.01 N NaOH at 4 mM concentration as a stock solution and added to the cultures by diluting the stock solution in maintenance medium. (*Methyl*-^{3}H)-thymidine (spec. act. 43 Ci/mMole) was purchased from the Radiochemical Centre, Amersham, U.K.

Cytotoxicity tests

Cytotoxicity of nalidixic acid was investigated by adding the drug to Vero cells seeded 6 or 24 h earlier. Cell growth was measured by cell counting, protein determination and uptake of labelled thymidine. Viable cells were counted in a hemocytometer by the trypan blue exclusion method. For protein determination, cell monolayers were harvested and sonicated three times for 30 s at full power in an MSE ultrasonic oscillator. Protein concentration was measured according to Lowry et al. (Lowry et al., 1951). For incorporation of (*methyl*-^{3}H)thymidine, Vero cells were labelled for 15 h and treated with trichloroacetic acid. The radioactivity of trichloroacetic acid-precipitable material was measured by liquid scintillation counting.

Determination of BKV growth

Cells were infected with BKV 6 or 24 h after seeding. The viral inoculum was removed after 2 h of adsorption at 37°C. Cell monolayers were washed once with PBS and fed with maintenance medium containing the drugs. At different time intervals, infected cultures were frozen and thawed three times to release intracellular virus and centrifuged at 800 × *g* for 10 min to sediment cell debris. BKV growth was calculated by titration of the hemagglutinating activity of the supernatants (Portolani et al., 1974).

Detection of BKV tumor (T) and viral coat protein (VP) antigens

Cells grown on cover slips were infected with BKV 6 h after seeding and maintained in medium containig 0.04 mM nalidixic acid. T and VP antigens were detected in infected cultures by indirect immunofluorescence (IF) (Portolani et al., 1975).

Analysis of BKV DNA synthesis

Cells were infected with BKV 24 h after plating and cultured in medium containing nalidixic or oxolinic acid. For blot hybridization, DNA was extracted from infected cultures by the method of Hirt (Hirt, 1967) and volumes of Hirt supernatants corresponding to 10^6 cells were migrated in 0.8% agarose gels, transferred to nitrocellulose sheets according to Southern (1975) and hybridized, as described

(Meneguzzi et al., 1981), to ^{32}P-labeled BKV-DNA probes (specific activity 1 to 3 $\times$ 10^8 cpm/μg) produced by nick translation (Rigby et al., 1977). Quantitative analysis was carried out by densitometric scanning of hybridization bands with an LKB Ultroscan laser densitometer.

Results

Antiviral acitivity

Results of a series of experiments on the antiviral effect of nalidixic acid on BKV growth at 4 days after infection are shown in Fig. 1. Significant antiviral activity of 0.02, 0.04 and 0.08 mM nalidixic acid was detected (t = 7.86, $P < 0.0001$; t = 13.82, $P < 0.001$ and t = 14.17, $P < 0.0001$ respectively). The rate of inhibition of BKV growth calculated by measuring the ratio of the geometric means of HA titers was as follows: 5.62 equal to 82.21% reduction, 13.18 equal to 92.42% reductions and 13.86 equal to 92.79% reduction for 0.02, 0.04 and 0.08 mM nalidixic acid, respectively. Antiviral activity of 0.04 mM nalidixic acid was significantly higher than that of 0.02 mM nalidixic acid (t = 9.001, $P < 0.001$) but not significantly different from that of 0.08 mM nalidixic acid (t = 1.80, $P < 0.095$).

A typical experiment on the effect of nalidixic acid on BKV growth at different times after infection is shown in Fig. 2. Since a reduction in HA titer equal to or greater than fourfold is significant, the inhibition of BKV replication appears to be related to drug concentration in both time of appearance and magnitude of effect. Table 1 shows reduction of BKV growth in cultures infected 6 or 24 h after seeding and kept in contact with nalidixic acid for different time periods. The

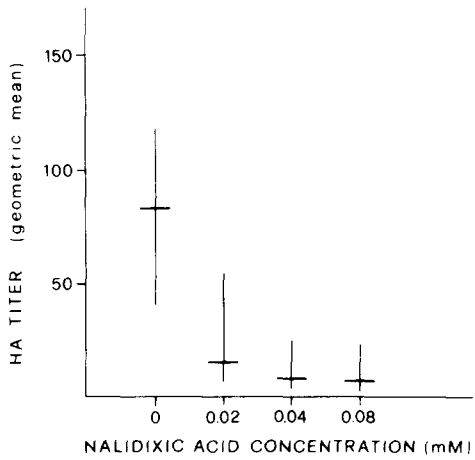

Fig. 1. Antiviral activity of nalidixic acid. Experiments were performed in Vero cultures infected 24 h after plating. BKV growth was calculated by hemagglutination 4 days after infection. Values are the average of 10 experiments. Horizontal and vertical bars represent geometric means and ranges respectively.

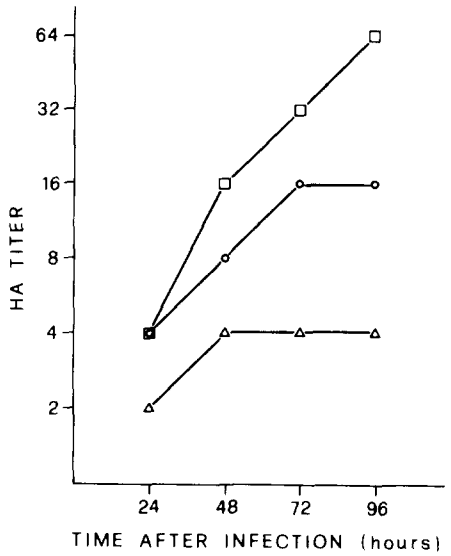

Fig. 2. Growth of BKV in the absence (□) and in the presence of 0.02 mM (○) and 0.04 mM (△) nalidixic acid. Experiments were performed with cultures infected 24 h after plating.

highest inhibition was obtained with cells infected 24 h after seeding, where a significant reduction in HA titer was detected even after a short time (48 h) of exposure to the drug.

Cytotoxicity

An important characteristic to judge the efficacy of an antiviral drug is its toxicity for the host cells. We therefore studied the inhibitory effect of nalidixic acid on cell growth, as measured by inhibition of protein synthesis. The results are reported in Table 2. In cells treated with the drug 6 h after plating a significant inhibition was evident only with 0.04 mM nalidixic acid starting from 96 h of ex-

TABLE 1

Reduction of BKV growth in Vero cells after different time periods of exposure to nalidixic acid

Time of exposure to the drug (h)	Cultures infected 6 h after plating[a]		Cultures infected 24 h after plating[a]	
	Nalidixic acid		Nalidixic acid	
	0.02 mM	0.04 mM	0.02 mM	0.04 mM
24	1.00 (100.00)	1.00 (100.00)	1.00 (100.00)	1.41 (70.92)
48	1.41 (70.92)	1.00 (100.00)	2.00 (50.00)	2.83 (35.33)
72	2.83 (35.33)	4.00 (25.00)	2.00 (50.00)	5.66 (17.07)
96	4.00 (25.00)	11.31 (8.85)	8.00 (12.50	11.31 (8.85)

[a]Virus yield was titrated by HA 4 days p.i. Rate of inhibition was calculated by measuring the ratio of the HA geometric mean titers obtained in the absence and in the presence of nalidixic acid. In parentheses: percent of control values.

TABLE 2

Effect of nalidixic acid on cell protein synthesis

Time of exposure to the drug (h)	Drug concentration (mM)	Drug added 6 h after plating μg protein/ 300 μl total cell lysate[a]		Drug added 24 h after plating μg protein/ 300 μl total cell lysate[a]	
24	0	146.59 ± 4.8		288.60 ± 65.3	
	0.02	146.50 ± 11.2	(99.93)	359.60 ± 35.3	(124.6)
	0.04	147.70 ± 3.2	(100.7)	336.50 ± 51.9	(116.6)
48	0	209.00 ± 3.1		318.00 ± 4.2	
	0.02	202.27 ± 8.0	(96.77)	347.00 ± 49.0	(109.1)
	0.04	142.03 ± 7.4	(67.95)	322.00 ± 91.0	(101.0)
72	0	244.85 ± 31.3		419.20 ± 65.2	
	0.02	268.95 ± 4.7	(109.8)	395.00 ± 55.3	(94.2)
	0.04	160.75 ± 4.8	(65.6)	342.50 ± 0.7	(81.6)
96	0	307.00 ± 4.6		382.00 ± 84.8	
	0.02	265.00 ± 7.7	(86.3)	361.50 ± 44.5	(94.5)
	0.04	201.00 ± 0.0[b]	(65.4)	342.50 ± 3.5	(89.5)
120	0	312.00 ± 23.6		500.10 ± 14.2	
	0.02	269.30 ± 5.4	(86.2)	313.30 ± 34.8[b]	(62.6)
	0.04	205.60 ± 27.2[b]	(65.7)	322.00 ± 7.9[b]	(64.4)

[a]Each value represents the mean from four Petri dishes ± standard deviation. In parentheses: percentage of control values.
[b]Significant difference ($P<0.05$). All the other values were not significant.

TABLE 3

Effect of nalidixic acid on ^{3}H-thymidine incorporation into cellular DNA

Time of exposure to the drug (h)	Drug concentration (mM)	Drug added 6 h after plating Incorporation (dpm/μg protein)[a]	Drug added 24 h after plating Incorporation (dpm/μg protein)[a]
24	0	570.00 ± 27.6	392.30 ± 29.8
	0.02	788.20 ± 5.4[b] (137.9)	387.60 ± 84.0 (96.9)
	0.04	605.70 ± 70.0 (105.8)	430.80 ± 96.2 (107.5)
48	0	547.70 ± 32.7	158.30 ± 21.2
	0.02	627.14 ± 71.3 (114.7)	100.25 ± 36.5 (62.1)
	0.04	794.39 ± 39.3[b] (145.3)	75.80 ± 13.5[b] (46.9)
72	0	249.30 ± 18.3	65.90 ± 12.0
	0.02	122.70 ± 1.3[b] (49.0)	27.50 ± 5.9[b] (41.2)
	0.04	125.90 ± 38.6 (50.3)	15.20 ± 6.3[b] (22.8)
96	0	94.10 ± 0.5	41.80 ± 0.1
	0.02	42.10 ± 2.3[b] (44.6)	6.40 ± 0.7[b] (15.2)
	0.04	9.60 ± 2.0[b] (10.2)	4.90 ± 2.6[b] (11.7)
120	0	81.70 ± 6.0	9.10 ± 5.4
	0.02	43.20 ± 2.3[b] (52.7)	0.34 ± 1.0[b] (9.1)
	0.04	8.50 ± 0.8[b] (10.3)	0.31 ± 0.1[b] (8.8)

[a] Figures represent mean values from four Petri dishes ± standard deviation. In parentheses percentage of control values.

[b] Significant difference ($P<0.05$). All the other values were not significant.

TABLE 4

Effect of nalidixic acid on BKV T and VP antigen synthesis

Hours of exposure to the drug (0.04 mM)	Percent synthesis[a]	
	T antigen	VP antigen
24	123.00	N.D.[b]
48	77.97	66.69
72	68.85	59.49
96	57.71	48.46

[a] Percent synthesis of T and VP antigens is referred to fluorescent nuclei in cells cultured in the presence of nalidixic acid compared to untreated cells. Values are the average from three different experiments performed in Vero cells infected with BKV 6 h after plating.
[b] N.D., not done.

posure. In cells exposed to the drug 24 h after plating both 0.02 and 0.04 mM concentrations were inhibitory, but only after 120 h of exposure. Cell counting after 96 and 120 h of exposure to the drug confirmed these results (data not shown).

The effect of nalidixic acid on incorporation of labelled thymidine is shown in Table 3. If the drug was added 6 h after plating, both drug concentrations showed significant stimulatory effect within the first 48 h of exposure. The inhibitory effect of the drug started at 72 h of exposure when the incorporation of thymidine in control cultures decreased, probably due to attainment of cell confluency, and it was more marked with the higher drug concentration. If the drug was added 24 h after culture preparation, there was no stimulatory effect, and the inhibition started earlier, at 48 h of drug exposure.

Effect of nalidixic acid on synthesis of BKV T and VP antigens

Since papovavirus T antigen is essential for the replication of the viral genome and VP antigen synthesis monitors the course of infection, we investigated the effect of nalidixic acid on BKV T and VP antigen synthesis. As shown in Table 4,

TABLE 5

Inhibition of BKV DNA replication by nalidixic and oxolinic acids

Drugs	Concentration	Per cent inhibition[a]		Ratio of BKV DNA FI to FII[a]	
		48 h	96 h	48 h	96 h
Nalidixic acid	0.02 mM	58.3	25.4	1.66	1.06
Nalidixic acid	0.04 mM	70.7	26.7	1.59	0.75
Oxolinic acid	0.2 mM	60.2	35.4	1.67	0.79
None	–	–	–	0.76	1.25

[a] Percent inhibition and amount of BKV DNA supercoiled form I (FI) and relaxed form II (FII) were calculated by comparing densitometric values of hybridization band in drug-treated and untreated Vero cells infected with BKV 24 h after plating. Values are the average determinations from two different experiments.

the antibiotic added at 0.04 mM concentration to Vero cells infected with BKV 6 h after seeding stimulated the expression of T antigen at 24 h after infection, while the inhibitory activity on T and VP antigens appeared at 48 h and increased with the time of exposure. Stimulation of T antigen synthesis was observed also after 24 h of treatment with 0.02 mM nalidixic acid in Vero cells infected 24 h after seeding (data not shown).

Inhibitory effect of nalidixic and oxolinic acids on viral DNA synthesis

Southern blot hybridization of Hirt supernatant DNA extracted from Vero cells infected 24 h after plating showed a significant reduction in the amount of viral DNA at 48 h after infection in cells treated with both 0.02 mM or 0.04 mM nalidixic acid and 0.2 mM oxolinic acid. At 96 h after infection the inhibitory effect of both drugs was less marked (Table 5). There was no evidence of oligomer accumulation and of molecules with conformational changes or with transitional topology between BKV DNA supercoiled form I and relaxed form II (Fig. 3). However, the ratio between the amounts of viral DNA form I and form II was increased at 48 h after infection compared to controls (Table 5).

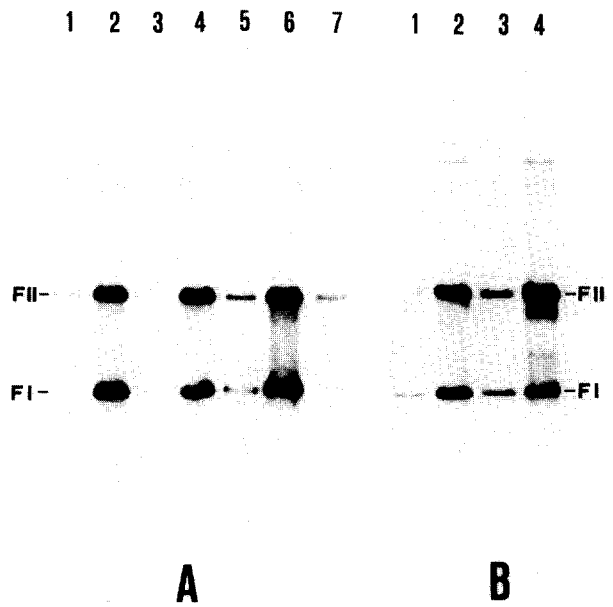

Fig. 3. Southern blot hybridization of Hirt supernatant DNA from 10^6 BKV-infected Vero cells to a ^{32}P-labelled BKV DNA probe. Cells were infected 24 h after plating. A. Cells were treated with 0.02 mM nalidixic acid for 48 h (lane 1) and 96 h (lane 2) or with 0.04 mM nalidixic acid for 48 h (lane 3) and 96 h (lane 4). Lanes 5 and 6 contain DNA from untreated cells at 48 and 96 h after infection. Lane 7 contains 400 genome equivalents per diploid cell genome of control BKV DNA. B. Cells were treated with 0.2 mM oxolinic acid for 48 h (lane 1) and 96 h (lane 2). Lanes 3 and 4 contain DNA from untreated cells at 48 and 96 h after infection. FI and FII indicate BKV DNA supercoiled form I and relaxed form II. A and B are two different experiments.

Inhibition of BKV cytopathic activity by nalidixic and oxolinic acids

To test if gyrase inhibitors are able to hinder BKV cytopathology over a long time period, BKV-infected Vero cells were treated with different concentrations of nalidixic and oxolinic acids. Viability was determined by counting cells with the trypan blue exclusion test 12 days after infection when the cytopathic effect in BKV-infected untreated controls involved approximately 75% of the cell monolayer. Maintenance medium containing the drugs was changed every 4 days. The results reported in Fig. 4 show that both drugs significantly inhibit cell death at middle and high concentrations and oxolinic acid has a better protective effect than nalidixic acid, but is more toxic for normal cells. The virus titer detected by hemagglutination in the supernatant medium of BKV-infected cells was reduced fourfold or more in all drug-treated cultures.

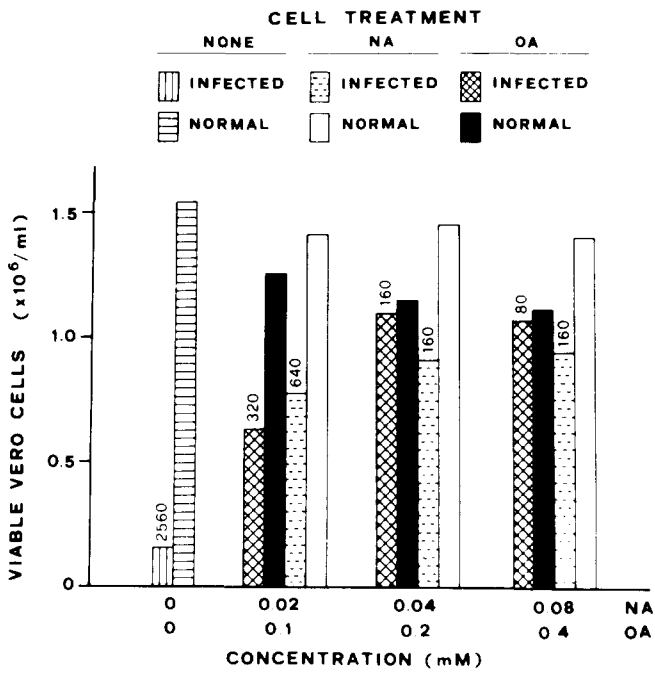

Fig. 4. Protection of Vero cells from BKV cytopathology by nalidixic acid (NA) and oxolinic acid (OA). Cell counts are the average of 3 determinations. Numbers over columns refer to virus titers in HAU/ml detected in the culture medium of BKV-infected cells.

Discussion

Results on reduction of both virus production and viral DNA synthesis show that nalidixic and oxolinic acids inhibit BKV growth. With nalidixic acid this effect is obtained at drug concentrations (0.02–0.04 mM) lower than plasma concentrations (0.08–0.2 mM) achieved during antimicrobial chemotherapy (Goodman and Gilman, 1975). It is worth noting that inhibitory concentrations of coumermycin A1 for herpes simplex virus type 1 (Palù et al., 1986) and of novobiocin for SV40 (Edenberg, 1980) are more than 50 times higher than those effective against *Escherichia coli* DNA gyrase (Higgins et al., 1978). In spite of substantial inhibition of virus yield, BKV DNA synthesis was resumed at 96 h after infection, suggesting that the activity of the inhibitors on viral DNA replication is reversible. This is in agreement with the reversibility of the effect of nalidixic acid on mammalian cell DNA synthesis (Mattern and Scudiero, 1981). By renewing the culture medium with drugs every 4 days, cells were protected from cytopathology over a long time period. However, under these conditions oxolinic acid had a toxic effect.

Apparently a correlation between inhibition of thymidine incorporation and antiviral activity is detectable in 6 and 24 h cell cultures. The inhibition of cellular protein synthesis and thymidine incorporation by nalidixic acid is higher in resting cells. This may be the reason why inhibition of virus yield, up to 48 h of exposure to the drug, was lower in experiments performed with 6 h cultures as compared to 24 h cultures. However, inhibition of viral DNA synthesis by nalidixic and oxolinic acids was clearly dissociated from inhibition of cellular DNA synthesis. In fact, viral DNA replication was mostly inhibited at 48 h after infection, when thymidine incorporation was normal or enhanced. On the other hand, viral DNA synthesis was resumed at 96 h after infection, when maximum reduction in thymidine incorporation was observed, suggesting that both drugs have different effects on cellular and viral DNA synthetic pathways. Also, absence of cytotoxicity during short treatment as well as the low and late inhibitory activity on cellular protein synthesis indicate that the two drugs have a relatively high selectivity index. These results confirm previous data on the low cytotoxicity of nalidixic and oxolinic acids for mammalian cells (Francke and Margolin, 1981; Mattern and Scudiero, 1981). In these studies drug concentrations 10 to 150 times higher than those inhibiting BKV replication were necessary for 50% reduction of cell growth rate. This is in contrast with the marked cytotoxicity of other topoisomerase II inhibitors, such as coumermycin A1 (Palù et al., 1984; Varnier et al., 1984).

BKV DNA synthesized in cells treated with nalidixic and oxolinic acids did not show topological alterations. Nevertheless, accumulation of viral DNA form I was detected at 48 h after infection, when the maximum inhibitory effect on virus DNA replication was observed. This suggests that the drugs may specifically inhibit the unlinking activity of topoisomerase II which is necessary for propagation of the replication fork during papovavirus DNA synthesis (Yang et al., 1987). Although it was directly demonstrated that nalidixic acid inhibits in vivo DNA topoisomerase II in mammalian cells (Mattern et al., 1982), it cannot be excluded that the drug blocks BKV replication by acting on a target other than DNA gyrase. This

assumption is supported by studies on other inhibitors of DNA gyrase. Thus, novobiocin reduces negative supercoiling of SV40 DNA (Edenberg, 1980), while coumermycin A1 does not (Edenberg, 1980). Nevertheless, both drugs inhibit DNA, RNA and protein synthesis in mammalian cells by mechanisms independent from interaction with topoisomerase II (Cotten et al., 1986; Gottesfeld, 1986; Masotti et al., 1984; Palù et al., 1984). In *E. coli* nalidixic and oxolinic acids have been shown to induce genome fragmentation by introduction of double-stranded breaks into cellular DNA (Snyder and Drlica, 1979; Sugino et al., 1977).

Papovavirus T antigen is essential for viral DNA replication (Tegtmeyer, 1980). It seems unlikely, however, that impairment of BKV DNA synthesis depends on a primary effect of the drugs on T antigen expression, since T antigen synthesis was stimulated by drug treatment in the period preceding the onset of viral DNA replication. It was shown in prokaryotes that different transcriptional promoters can be induced or inhibited by nalidixic or oxolinic acids and even the same promoter can be up- or down-regulated according to the physiological state of the cell (Vosberg, 1985). In our system a similar effect of the two drugs on transcription directed by cellular and viral promoters would explain stimulation or inhibition of cellular protein synthesis and thymidine incorporation as well as of viral T antigen synthesis, according to the different functional state of the host cells.

Suppression of BKV replication by inhibitors of prokaryotic DNA topoisomerases prompts to test on the same model system the more specific inhibitors of eukaryotic topoisomerases. Furthermore, it would be interesting to analyse whether replicative steps sensitive to topoisomerase inhibitors can be detected in the replication cycle of other papovaviruses, e.g. papillomaviruses. An in vitro system of bovine papillomavirus-transformed mouse or rat cells, harboring episomally replicating viral genomes (Howley and Schlegel, 1987), is available to test the effects of topoisomerase inhibitors on both papillomavirus DNA replication and papillomavirus-induced transformation.

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