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INHIBITION OF SV40 DNA REPLICATION IN VITRO BY 1-N-ACYL-3"-N-(TRIFLUOROACETYL)KANAMYCIN

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A new antiviral aminoglycoside antibiotic, 1-N-eicosanoyl-3"-N-(trifluoroacetyl)kanamycin, was found to inhibit SV40 DNA replication in vitro. Several other aminoglycoside antibiotics examined did not exhibit a significant inhibition of SV40 DNA replication. The elongation of the SV40 DNA strand was profoundly affected by this agent. The degree of inhibition was decreased by increasing the amount of DNA template, but not by increasing the amount of enzyme. The inhibition of SV40 DNA replication is attributed to the interaction between the agent and the DNA template.

Recently, the antiviral effect of 1-N-acyl-3"-N-(trifluoroacetyl)kanamycin derivatives has been reported (1,2).

To observe the mode of antiviral action of 1-N-acyl-3"-N-(trifluoroacetyl)kanamycin derivatives, we chose 1-N-eicosanoyl-3"-N-(trifluoroacetyl)kanamycin (ETK)(Fig.1) and an in vitro SV40 DNA replication system. The effect of other aminoglycoside antibiotics (AGs) on this system was also examined. The reasons for our selection of this approach to analyze the mechanism of antiviral action of ETK by using SV40 DNA replication system are that the agent has shown a high chemotherapy index and that the initiation and subsequent elongation of SV40 DNA replication can take place in vitro in a manner similar to that in vivo (3,4,5,6,7,8). We have previously shown the inhibition of initiation of bacterial chromosomal DNA replication by AGs (9). In this publication, the mechanism of action of ETK on SV40 DNA replication is described.

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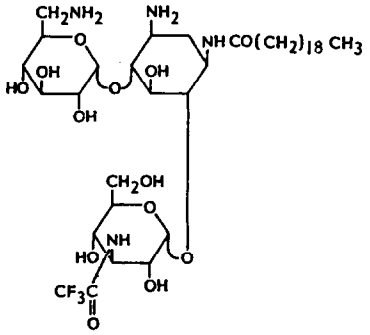

Fig. 1. 1-N-eicosanoyl-3"-N-(trifluoroacetyl)kanamycin (ETK)

MATERIALS AND METHODS

The 1-N-eicosanoyl-3"-N-(trifluoroacetyl)kanamycin (ETK) was kindly supplied by Fujisawa Pharmaceutical Co. Ltd.

Cells and virus: The SV40-transformed monkey cell CosI (10) was kindly supplied by Y.Gluzman. SV40 was propagated in the monkey cell line GC7, and SV40 DNA was extracted from purified virions by CsCl-ethidium bromide equilibrium centrifugation (3).

Preparation of nuclear and cytoplasmic extracts: FM3A nuclei were prepared by Dounce homogenization of cells suspended in hypotonic buffer consisting of 20 mM HEPES (pH 7.5), 5 mM KCl, 0.5 mM $MgCl_2$ and 0.5 mM dithiothreitol. The nuclei, which were rapidly frozen in liquid nitrogen, were thawed and extracted with 100 mM NaCl at 0°C for 5 min. The nuclei extract was freed of insoluble material by centrifugation at 20,000 x g for 20 min. Cytoplasm from SV40 infected CosI cells was prepared by Dounce homogenization (3,4). The crude cytoplasm was centrifuged at 100,000 x g for 30 min. The supernatant was precipitated with $(NH_4)_2SO_4$. The precipitate was dissolved in a solution of 25 mM Tris (pH 7.5), 0.1 mM EDTA, 7 mM 2-mercaptoethanol, 10% glycerol and 50 mM NaCl, and dialyzed against the same buffer.

Preparation of pSVO DNA: The pSVO containing the origin and early promoter region of the SV40 genome spanning the HindIII - PvuII site of 0.64 to 0.71 map units was constructed and propagated in Escherichia coli HB101 (3). Form I of pSVO was purified by CsCl-ethidium bromide equilibrium centrifugation (3).

Reaction in vitro: The reaction mixture (100 µl) for pSVO replication contained 25 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.05 mM each dATP, dGTP and dTTP, 1.5 µM [α-^{32}P]-dCTP (410 Ci/mmol) or 0.01 to 0.02 mM [^{3}H]dCTP (1 Ci/mmol), 2 mM ATP, 10 to 20 µl of FM3A nuclear extract (protein concentration, 15 mg/ml), and 3 to 5 µl of CosI cytoplasm (protein concentration, 4 mg/ml) and 0.4 to 10 µg of form I pSVO DNA. The reaction mixture was incubated at 37°C for the time indicated in the legend (Tab.1., Fig.2., Fig.3.) The radioactive DNA was precipitated with 5% TCA and counted, or processed for the product analysis.

Product analysis in pSVO replication: After incubation for various times at 37°C the reaction was terminated by the addition of final 25 mM EDTA, and subsequently digested with Pronase (10 µg) and sodium dodesyl sulfate (0.1%) for 20 min at 37°C. The DNA was extracted with phenol and precipitated by ethanol, dissolved in water, digested with HindIII, PvuII and PstI for 1.5 hr at 37°C and electrophoresed on a 1.4% agarose gel containing 40 mM Tris (pH 7.8), 1 mM EDTA and 5 mM sodium acetate. The gels were dried and autoradiographed on Kodak X-ray film (XO mat).

Spectral differences: Spectral differences between DNA and the DNA-drug complex was observed in a solution of 15 mM NaCl, 1.5 mM sodium citrate (pH 7.5), 10 μg/ml of the DNA and drug in a Hitachi 200 spectrophotometer.

RESULTS

Effect on pSV0 replication in vitro.

We have reported previously that DNA replication in vitro using an SV40 origin-containing plasmid (pSV0) as template can place in a similar manner to that in vivo (3,4). The replication is initiated at a unique origin and proceeds bidirectionally. The effect of various agents, including ETK, other AGs, aphidicolin, nalidixic acid and novobiocin, on pSV0 DNA replication was investigated. A significant inhibition of [^{3}H]dCMP incorporation was observed using ETK and aphidicolin, but not by other AGs, nalidixic acid or novobiocin (Table 1). The IC_{50} of ETK was 10 μM, and the degree of inhibition caused by the agent was decreased by the addition of increasing amounts of pSV0 DNA to the reaction mixture (Fig.2). However, the degree of inhibition was not dependent upon the concentration of the enzyme

Table 1 : Effect of Several Aminoglycoside Antibiotics and Some Inhibitors of DNA Synthesis on pSV0 Replication in vitro

Compound	(μM)	% Incorporation
omission	-	100
Streptomycin	100	100
Kanamycin	100	86
Amikacin	100	111
Habekacin	100	84
Dibekacin	100	90
Destomycin	100	115
ETK	100	9
ETK	10	54
ETK	1	93
Novobiocin	100	74
Nalidixic Acid	400	93
Aphidicolin	30	28

The reaction mixture containing 1 μg of pSV0 DNA was incubated at 37°C for 60 min, and the acid-insoluble radioactivity of [^{3}H]dCMP incorporated into the DNA template was counted.

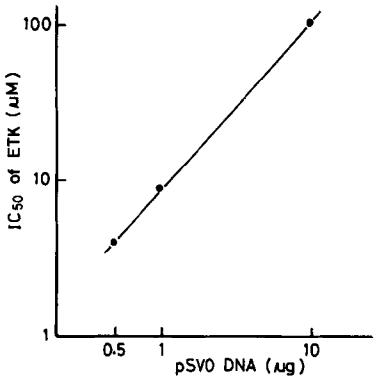

Fig. 2. Inhibition of pSV0 replication in vitro by ETK : The reaction mixture containing the amounts of pSV0 DNA as indicated was incubated at 37°C for 60 min, and the radioactivity of [^{3}H]dCMP incorporated into the DNA template of acid-insoluble fraction was counted. The degree of inhibition was expressed by IC_{50} (50% inhibitory concentration).

(data not shown). Therefore, the results suggest that the target of ETK is the DNA template. On the other hand, 72% inhibition was observed by using 30 μM aphidicolin, showing that the elongation of the DNA strand is carried out by DNA polymerase α, not by β, as reported previously (3,11). In order to understand whether ETK acts on the initiation or the elongation process of pSV0 replication, kinetic analyses were performed (Fig.3B). The experiments were divided into two groups. The reaction of one experimental group was terminated at 0, 10, 20 and 60 min after the start of incubation (Fig.3B lanes 1,2,3 and 4), and in another group ETK was introduced into the reaction mixture at intervals, at 0, 10 and 20 min, and further incubated up to 60 min (Fig.3B lanes 5,6 and 7). The DNA products were extracted from the mixtures, digested with PvuII, HindIII and PstI, and visualized on an agarose gel (Fig.3B). These three restriction enzymes give rise to three fragments, the sizes of them are 370, 784 and 1543 nucleotides in length (Fig.3A). In the first 10 min of the reaction (lane 2), almost same amount of radioactivity was incorporated into each fragment. After 20 min, the larger fragments were strongly labeled (lane 3), indicating that the DNA strand was elongated from the origin containing region to the opposite site, and the DNA replication is synchronized, as shown previously (4). The sample treated with ETK after the initial incubation (lane 5), however, showed a small amount of the incorporation compared to the 60 min control sample (lane 4). In the sample treated with ETK after the first

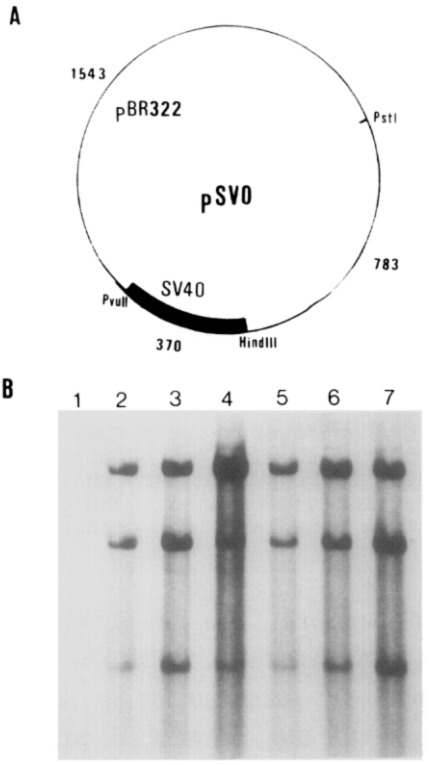

Fig. 3. Kinetic analyses of pSV0 replication in vitro and of the inhibition of replication by ETK : The reaction mixture contained 0.4 μg of pSV0 DNA, and the incubation was carried out at 37°C. The reaction was terminated at 0 min (lane 1), 10 min (lane 2), 20 min (lane 3) and 60 min (lane 4). The reaction mixture was added by 10 μM of ETK after the incubation at 0 min (lane 5), 10 min (lane 6) and 20 min (lane 7), and further incubated up to 60 min. DNA products were analyzed as described in materials and methods.

10 min of the reaction, followed by 50 min of further incubation (lane 6), the intensities of the radioactive bands were almost the same as that of the sample from 10 minutes' incubation (lane 2). A similar phenomenon was observed in the samples treated with ETK after a reaction time of 20 min (lane 7, compare to lane 3). These results show that ETK acts on the elongation step of SV40 DNA replication.

Interaction of ETK with DNA.

The interaction of ETK with pSV0 or calf thymus DNA was studied by examination of spectral difference. The change in ultraviolet absorbance of pSV0 DNA was markedly observed in the presence of ETK (Fig.4A). The amount of spectral change was less with calf thymus DNA than that of change with pSV0 DNA (Fig. 4B). Kanamycin (KM) did not cause a significant change.

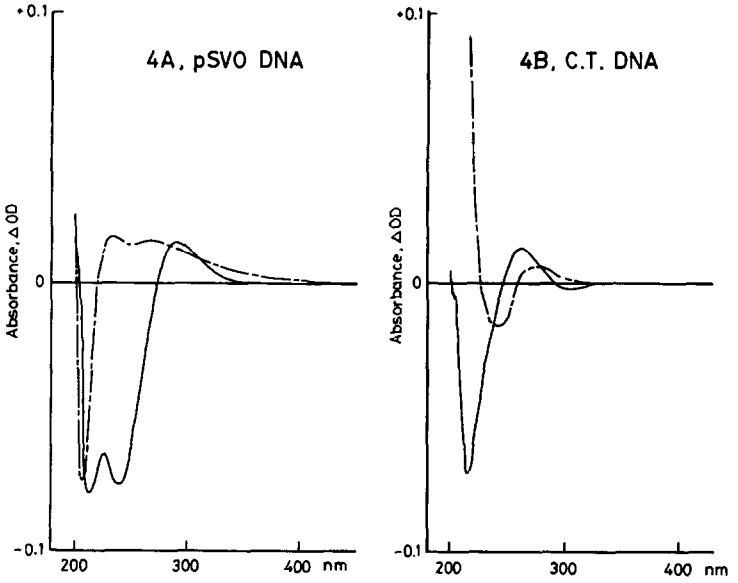

Fig. 4. Spectral differences of the pSVO DNA-ETK complex (4A, pSVO DNA) and the calf thymus DNA-ETK complex (4B, C.T.DNA) : The changes in the absorbance spectrum of DNA in the presence of ETK 10 μM (– – –) and 100 μM (————) were observed.

DISCUSSION

These experiments have demonstrated that ETK inhibits SV40 DNA replication *in vitro* by interacting with the DNA template. KM did not show any significant interaction with DNA. Therefore, the trifluoroacetyl or eicosanoyl portion of ETK can be identified as the cause of inhibition of pSVO replication *in vitro* and the source of the interaction with DNA. It is known that AGs interact with polyanions such as DNA or RNA (12). The mechanism of this phenomenon is believed to be that the cationic portion of AGs interacts with an internucleotide phosphate of a nucleic acid (13). This interaction is reversible by increasing the concentration of salts (14). However, the inhibition of the DNA polymerase reaction by ETK was not reversed by increasing the concentration of salts (data not shown), suggesting that the trifluoroacetyl or eicosanoyl portion of the agent interacts with another part of the DNA. The spectral differences suggest that the change in ultraviolet absorbance is due to the interaction of ETK with either a purine or a pyrimidine base of DNA. The assumption can be made that ETK links base to internucleotide phosphates in an intra- or inter-molecular manner. It is likely that ETK binds more tightly to form I pSVO DNA than to calf thymus DNA, which

was shown by spectral differences. The inhibition of DNA replication, as presented here, may be one of the mechanism of antiviral action of ETK.

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