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Inhibition of herpes simplex virus type 2 replication in vitro by 1-*N*-pentadecanoyl-3″-*N*-trifluoroacetyl kanamycin A

M.M. Rahman[1,2], N. Yamamoto[1], T. Morishima[2], K. Maeno[1] and Y. Nishiyama[1]

[1]*Laboratory of Virology, Research Institute for Disease Mechanism and Control and* [2]*Department of Pediatrics, Nagoya University School of Medicine, Nagoya, Japan*

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Summary

The in vitro antiviral activity as well as the mechanism of action of a new antiviral agent, a kanamycin analogue, 1-*N*-pentadecanoyl-3″-*N*-trifluoroacetyl kanamycin A (PTKA) against herpes simplex virus type 2 (HSV-2) was investigated. The drug showed excellent antiviral action with negligible cytotoxic effect on the culture cells. Based on plaque reduction assays the 50% inhibitory dose (ID_{50}) of the drug was 1 μg/ml, and at 20 μg/ml plaque formation was totally suppressed. The compound inhibited viral protein synthesis in infected cells without affecting RNA and DNA synthesis, when added to the cultures after virus adsorption. Moreover, pretreatment of the cells with PTKA before HSV-2 infection, increased the antiviral activity significantly. Dot-blot hybridization analysis revealed that the drug reduced the level of immediate early viral mRNA if applied before infection. There was no detectable action at the level of virus adsorption, penetration or uncoating. These results indicate that PTKA exerted its antiviral action at the early stage of viral replication as well as at the level of viral protein synthesis.

Kanamycin analogue; HSV-2

Correspondence to: Dr. Mizanur Rahman, Laboratory of Virology, Research Institute for Disease Mechanism and Control, Nagoya University School of Medicine, 65, Tsurumai-Cho, Showa-Ku, Nagoya-466, Japan.

Introduction

The antiviral activity of some compounds having higher acyl or alkyl groups was first reported by Hoffman et al. (1973). And in 1982 it was shown by Ghendon and Mikhailovskaya that kanamycin A sulphate had antiviral activity against influenza viruses in tissue culture, but the activity was very weak. Kanamycin A itself is an aminoglycoside antibiotic which is bactericidal in nature and has been widely used for the treatment of a number of human bacterial infections. This drug interferes with bacterial protein synthesis through codon misreading at the level of translation. Kanamycin A also alters the bacterial cell membrane, either indirectly via inhibition of protein synthesis or directly by combining with membrane anionic groups (Bevan et al., 1983). Various derivatives of kanamycin A were synthesized and were found to have effective antiviral activity against herpes simplex virus type 1 and influenza virus (Matsuda et al., 1985). 3″-*N*-trifluoroacetyl kanamycin A derivatives having a palmitoyl, pentadecanoyl or hexadecanoyloxicarbonyl group at the *N*-1 position showed excellent antiviral activity both in vivo and in vitro, but the mechanism of action was not studied (Matsuda et al., 1986).

In this study we have determined the antiviral activity of 1-*N*-pentadecanoyl-3″-*N*-trifluoroacetyl kanamycin A (PTKA) against HSV-2 in vitro and have also investigated the mechanism of action of this compound.

Materials and Methods

Drug and chemicals

The drug PTKA was obtained from Fujisawa Pharmaceutical Co. Ltd., Osaka, Japan. Chemical structure of the compound is shown in Fig. 1. It was initially solubilized in dimethyl sulfoxide (Me_2SO) up to a concentration of 5 mg/100 μl. Final dilutions were made in cell culture media. L-[^{35}S]methionine (600 Ci/mmol), [*methyl*-^{3}H]thymidine (25 Ci/mmol) and [α-^{32}P]dCTP (410 Ci/mmol) were from Amersham Laboratories, U.K. 5-[^{3}H]Uridine (26.7 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Cycloheximide was purchased from Sigma Chemical Company.

Cells and viruses

African green monkey kidney cells (Vero) were used throughout all experiments. Cells were grown in Eagle's minimum essential medium (MEM) supplemented with 5% calf serum (CS), 100 units/ml of penicillin and 100 μg/ml of streptomycin. Cells were routinely passaged every 2 or 3 days. For experiments and assays, cells were plated in 35 mm diameter plastic tissue culture dishes. HSV-2 strain 186 was obtained from Dr. Fred Rapp, Pennsylvania State University College of Medicine, Hershey, U.S.A. Virus stock was prepared in human embryonic fibroblast (HEF) infected at low multiplicities (0.01–0.1 PFU per cell) as described previously (Nishiyama and Rapp, 1981).

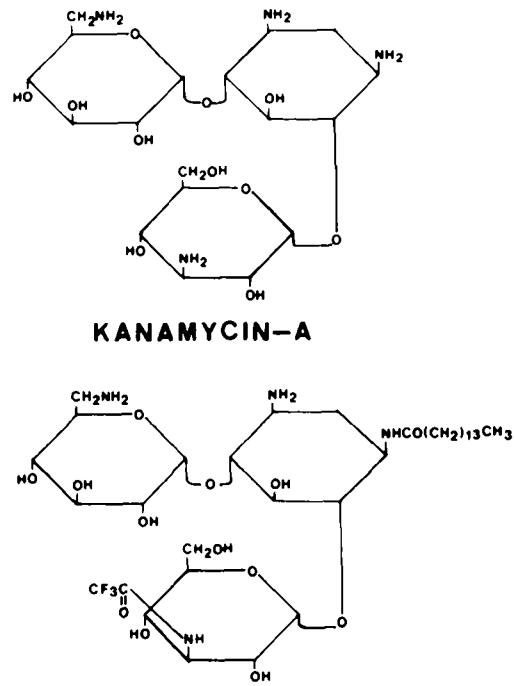

Fig. 1. Chemical structure of kanamycin A and 1-*N*-pentadecanoyl-3″-*N*-trifluoracetyl kanamycin A (PTKA).

Virus plaque reduction and yield reduction assays

For plaque reduction assay, confluent monolayers of Vero cells were infected with HSV-2, and after 1 h adsorption at 37°C, the cultures were overlaid with 2 ml of 0.5% agarose in MEM containing appropriate concentrations of PTKA. Plaques were counted on the following day after fixation with 5% aqueous formaldehyde and staining with 0.1% crystal violet. For yield reduction assay, Vero cell monolayers were infected with HSV-2 at a multiplicity of infection of about 10 PFU per cell and treated with the drug after 1 h virus adsorption at 37°C. After 24 h, the virus was harvested by freezing-thawing followed by low speed centrifugation. The supernatant was titrated in Vero cell monolayers by the plaque assay method.

To assess the effects of PTKA on Vero cell growth, the cells were counted daily for up to 3 days following treatment of the cells with various concentrations of the drug.

Isotope incorporation to study viral protein, DNA and RNA synthesis

[^{35}S]Methionine, [*methyl*-^{3}H]thymidine and [^{3} H]uridine uptake assays were performed to assess the effect of PTKA on the synthesis of protein, DNA and RNA,

respectively, in infected as well as uninfected cells. Vero cell monolayers were infected with HSV-2 at a multiplicity of infection of about 10 PFU per cell. After 1 h virus adsorption, maintenance medium without any drug was added for 3 h. PTKA diluted at various concentrations in MEM (methionine-free MEM for [^{35}S]methionine) was applied and isotope labelling (5 μCi/ml) was done between 4 and 5 h postinfection. Cells were then processed for acid-precipitable radioactivity which was measured in a liquid scintillation counter (Nishiyama et al., 1983).

Polyacrylamide gel electrophoresis (PAGE)

To determine HSV-2 induced protein synthesis, PAGE after labelling with [^{35}S]methionine (10 μCi/ml) was done according to Laemmli et al. (1970). Samples were treated with 0.0625 M-Tris HCl (pH 6.8) containing 5% SDS, 2% 2-mercaptoethanol (2-ME), 10% glycerol and 0.001% bromophenol blue followed by heating at 100°C for 1 min. After SDS-PAGE, the gels were fixed, dried and then exposed to Kodak Royal X-Omat film at −80°C.

Dot-blot hybridization

To assess viral mRNA synthesis in infected cells with or without the addition of drug, dot-blot hybridization was performed. Cytoplasmic RNA was prepared according to White et al. (1982). In brief, confluent Vero cells (2×10^6) were pretreated with PTKA and cycloheximide (100 μg/ml) for 1 h, then infected with HSV-2 and maintained for 4 h (total 5 h postinfection) in the presence of both drugs. Cells were pelleted at 600 × *g* for 5 min, washed twice with PBS and pelleted. After they had been resuspended in 45 μl of ice-cold TE buffer, the cells were lysed twice on ice by 5% NP 40 with 5 min interval. Pelleting of nuclei was done at 15,000 × *g* for 2.5 min and 50 μl of the supernatant was transferred to 1.5 ml tube containing 30 μl of 20 × SSC plus 20 μl of 37% formaldehyde. This mixture was incubated at 60°C for 15 min and stored at − 70°C. For analysis of mRNA, 2.5–20 μl of each sample was diluted with 15 × SSC to get a final volume of 100 μl and then applied with suction to a 4 mm diameter spot on Gene Screen Plus (New England Nuclear) membrane. Cytoplasmic macromolecules were fixed to the membrane, then prehybridization and hybridization were done according to the protocol as described by the supplier. Hybridization was done with plasmid (pACYC 186) containing HSV-2 *Hin*dIII digested D fragment that did not cross-react with cellular RNA sequences and that was shown to code for immediate early (IE) proteins (Tsurumi et al., 1986). After autoradiography, the membrane was exposed to Kodak Royal X-Omat film at −80°C.

Preparation of radiolabeled HSV-2

Vero cell monolayers were infected at 5–10 PFU per cell. Following a 1 h adsorption period the cells were incubated with maintenance medium for 2 h at 37°C. [*Methyl*-^{3} H]thymidine (20 μCi/ml) was added, and after a labeling period of 18–20 h supernatant was centrifuged in 50 ml centrifuge tubes at about 1000 × *g* for 10 min. Virus was pelleted by centrifugation for 60 min at approximately 90,000 × *g* and purified by continuous sucrose gradient (10–50% [w/v]) centrifugation for 60

min at 20,000 rpm in a Beckman SW 27 rotor. Peak fractions of radioactivity were collected and centrifuged again. Pellets were then suspended in PBS and served as the source of radiolabeled HSV-2.

Results

Effect of PTKA on the growth of HSV-2 and Vero cells

Antiviral activity of PTKA against HSV-2 was assessed by both plaque and yield reduction assays. As shown in Fig. 2A, plaque formation by HSV-2 was inhibited by more than 50% at a PTKA concentration of 1 μg/ml. At 20 μg/ml plaque formation was completely suppressed. In the yield reduction assay, treatment with 5 μg/ml of PTKA showed little inhibitory effect although virus production in cultures treated with 50 μg/ml was inhibited by more than 95% (Fig. 2B). Regarding the cytotoxic effect of PTKA, we could not find any significant morphological change in cultures treated with even 200 μg/ml of the drug. The growth of the cells was observed to be 3-fold in the presence of 100 μg/ml of PTKA, as compared to about 5-fold in the absence of the compound, and at a PTKA concentration of 20 μg/ml cell growth was not hampered at all (data not shown). The selectivity (therapeutic) index of the drug thus proved to be > 100.

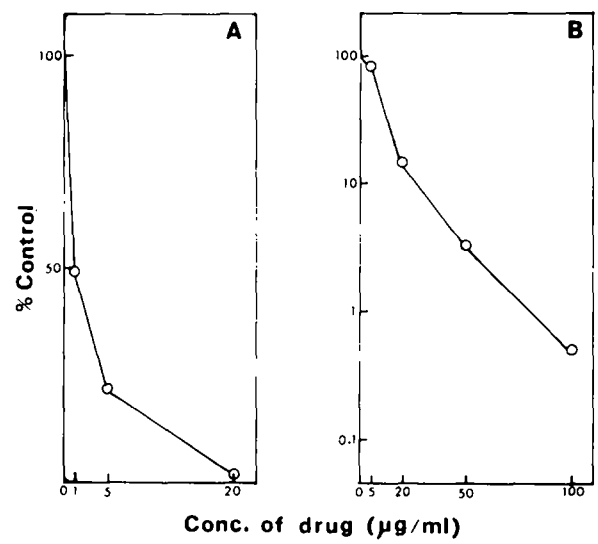

Fig. 2. Effect of PTKA on the replication of HSV-2. The sensitivity of the virus was measured by (A) plaque reduction assays and (B) yield reduction assays on monolayers of Vero cells with virus adsorption for 1 h at 37°C and maintenance medium containing various concentrations of the drug. Results are expressed as a percentage of the values obtained in control cultures without any drug.

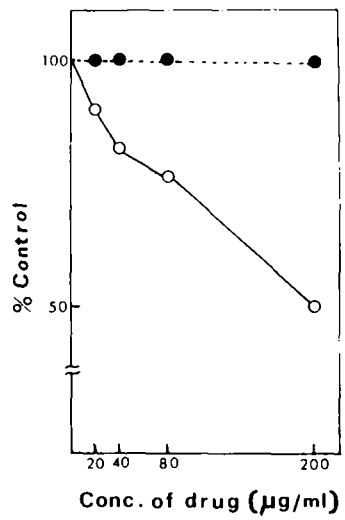

Fig. 3. Effect of PTKA on the synthesis of macromolecules in HSV-2 infected and uninfected cells. Vero cells were infected at a multiplicity of infection of about 10 PFU/cell and adsorption for 1 h was followed by incubation in maintenance medium for 3 h in the absence of the drug. Various concentrations of PTKA were then added and [^{35}S]methionine (10 μCi/ml) labeling was done for 1 h. Cells were removed with SDS, dried, and washed with 10% TCA and ethyl alcohol, and radioactivity was measured in a liquid scintillation counter. Results are expressed as a percentage of the untreated controls. The dotted line (---) represents isotope uptake by the uninfected cells, and the continuous line (———) represents uptake by the infected cells.

Effect of PTKA on the synthesis of macromolecules in HSV-2 infected and uninfected cells

Preliminary experiments indicated that if Vero cells were infected with HSV-2 at a multiplicity of about 10 PFU per cell and incubated at 37°C, most of cellular macromolecular synthesis including DNA synthesis was replaced by viral macromolecular synthesis within 4 h. Therefore, we examined the effect of PTKA on the synthesis of macromolecules in infected cells between 4 and 5 h postinfection. Results of isotope incorporation are shown in Fig. 3. A decrease in [^{35}S]methionine incorporation was found at a PTKA concentration of $\geq$ 20 μg/ml; the ID_{50} amounted to 200 μg/ml. No inhibition of protein synthesis was observed in uninfected cells. PTKA (200 μg/ml) showed little or no effect on the incorporation of [*methyl*-^{3}H]thymidine and [^{3}H]uridine in either infected or uninfected cells (data not shown), suggesting that the drug had no direct effect on DNA or RNA synthesis.

The results of isotope incorporation indicated that viral protein synthesis could be suppressed at a PTKA concentration of 200 μg/ml. This concentration was used for the PAGE analysis experiments. After 1 h virus adsorption the drug was added, and the cells were pulse-labeled with [^{35}S]methionine for 1 h at various times postinfection. As shown in Fig. 4, there was suppression of virus-induced protein synthesis after drug treatment, though the extent of suppression was incomplete. In the uninfected control cells, the drug did not suppress cellular protein synthesis at all.

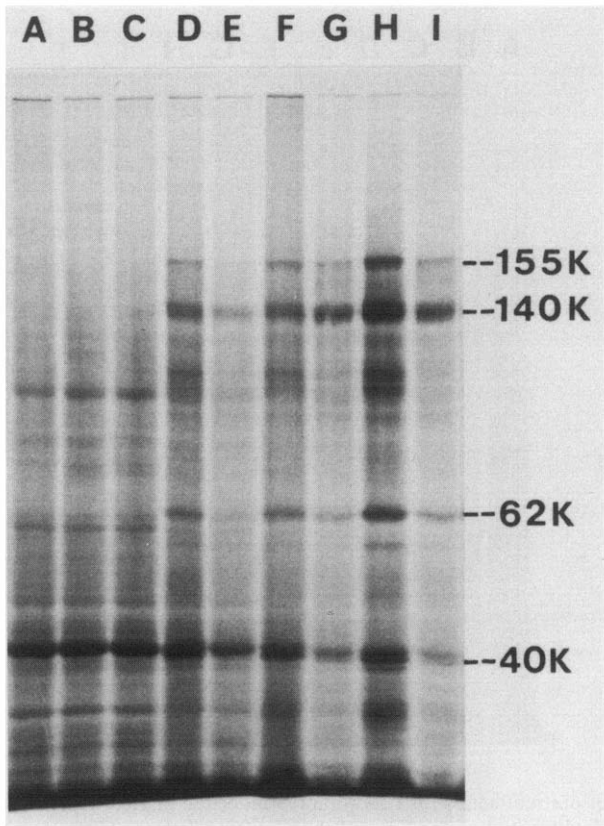

Fig. 4. Effect of PTKA on the synthesis of HSV-2-induced and host cellular proteins. Confluent monolayers of Vero cells were infected (D, E, F, G, H, I) or not (A, B, C) with HSV-2 at a multiplicity of infection of about 10 PFU/cell. After a 1 h virus adsorption period, some cultures (B, C, E, G, I) received the drug at a final concentration of 200 μg/ml and were maintained at 37°C. Labeling with [^{35}S]methionine (10 μCi/ml) was done for 1–2, 2–3 and 3–4 h. Radioactive polypeptides were analysed by SDS-PAGE followed by autoradiography as described in the text. Lanes A through I indicate: A, uninfected cells; B and C, uninfected drug-treated cells labeled for 1–2 and 3–4 h, respectively; D and E, 1–2 h labeling in the absence or presence of PTKA; F and G, 2–3 h labeling in the absence or presence of PTKA; H and I, 3–4 h labeling in the absence or presence of PTKA, respectively. Numbers on the right indicate molecular weight of the proteins.

Effect of pretreatment with PTKA on HSV-2 induced protein synthesis

The above results suggested that the high sensitivity of HSV-2 to PTKA in plaque reduction assays might be due to the inhibitory action of PTKA at an early step of infection in addition to an inhibitory effect on protein synthesis. To examine this possibility, we tested the effects of pretreatment with PTKA on the induction of HSV-2 protein synthesis. As shown in Fig. 5, viral protein synthesis was totally suppressed if the cells were treated with PTKA at 200 μg/ml for 1 h before infection and further incubated in presence of the drug. However, the inhibitory action on viral protein synthesis was markedly reduced if the drug was added 15 min after

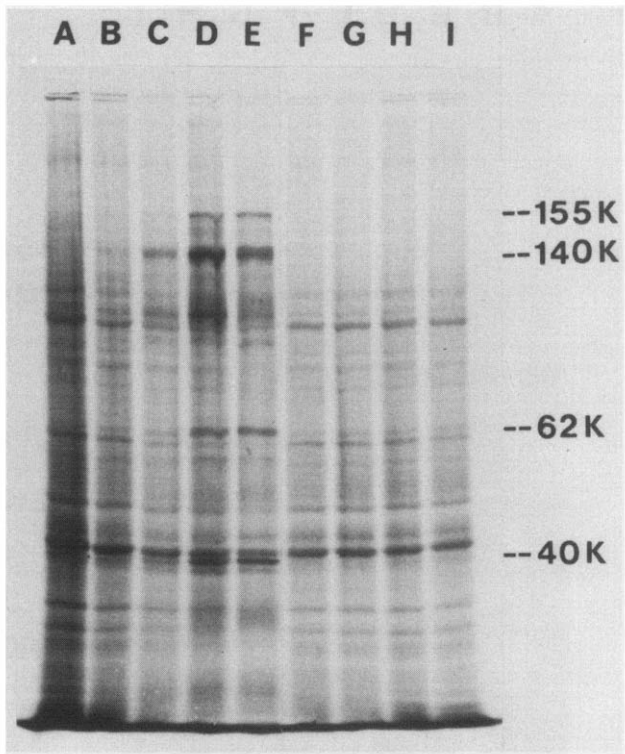

Fig. 5. Effect of pretreatment with PTKA on the induction of HSV-2 proteins. Vero cell monolayers were pretreated with the drug for 1 h and then infected with the virus at about 10 PFU/cell. One h adsorption was followed by incubation with maintenance medium in the absence or presence (200 μg/ml) of the drug for 1, 2, 3, 4 h. Then [^{35}S]methionine (10 μCi/ml) labeling was done for 1 h. SDS-PAGE followed by autoradiography was done for polypeptides. Lanes indicate: A, uninfected cells; B, C, D, E, infected non-treated cells maintained for 1, 2, 3, 4 h, respectively; F, G, H, I, infected treated cells maintained for 1, 2, 3, 4 h, respectively. Numbers on the right indicate molecular weights of the proteins.

infection (Fig. 6). These results suggested that PTKA had an inhibitory action at an early step of infection, as well as at viral protein synthesis.

Effect of PTKA on initial events in HSV-2 replication

We then examined the level of immediate early mRNA in PTKA-treated cells. Cells were pretreated with PTKA and cycloheximide (100 μg/ml) for 1 h and infected with HSV-2 at a multiplicity of infection of 10 PFU per cell in the presence of both drugs. After 1 h virus adsorption, cells were further incubated for 4 h in presence of the drugs. Cytoplasmic RNA was then extracted and examined by dot-blot hybridization using the *Hin*dIII D fragment as a probe which included immediate early (α4 and α0) genes. As shown in Fig. 7, it was found that PTKA remarkably reduced the level of immediate early mRNA.

We further evaluated the effect of PTKA on adsorption, penetration and un-

coating by using radiolabeled HSV-2. Table 1 depicts the results in terms of radioactivity. Cell-associated activity indicates the amount of adsorption or binding of HSV-2. Count remaining after trypsinization of cells indicates the amount of penetration or internalization of the virus. We could not find any difference in count between the control and drug treated samples. To assess the effect of the drug on uncoating of HSV-2, we measured the cytoplasm-associated radioactivity as well as the nucleus-associated activity in both untreated and drug-treated samples (Table 2). There was no significant difference in counts which means that PTKA had no effect on HSV-2 uncoating. These results indicate that PTKA acted at the primary transcription step without affecting adsorption, penetration or uncoating of HSV-2.

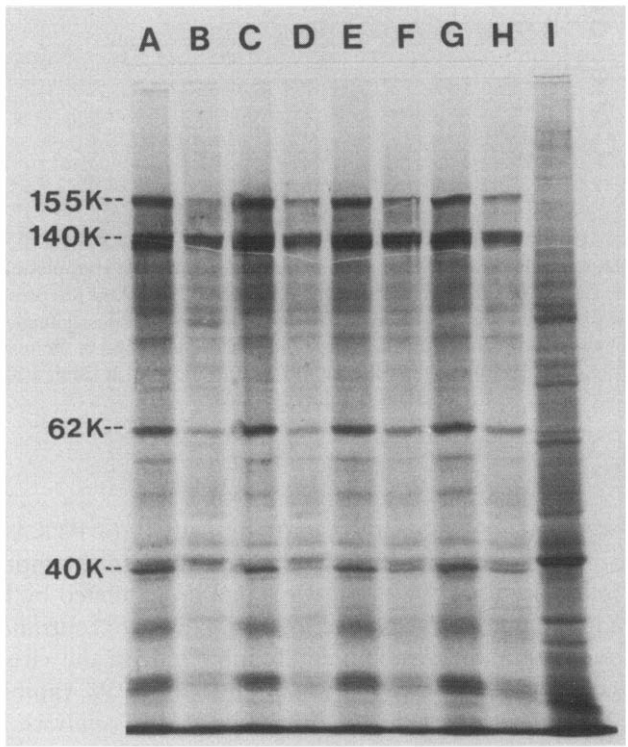

Fig. 6. Effect of PTKA on HSV-2-induced protein synthesis with change in adsorption time of the virus. Vero cell monolayers were infected with the virus (about 10 PFU/cell). After 15, 30, 45 or 60 min adsorption in the absence of the drug, some cultures received PTKA (200 μg/ml), and labeling with [^{35}S]methionine (10 μCi/ml) was done between 4 and 5 h postinfection in the absence (lanes A, C, E, G) or presence (lanes B, D, F, H) of the drug. SDS-PAGE followed by autoradiography was done as described in the text. Lanes indicate: A and B, 15 min adsorption; C and D, 30 min adsorption; E and F, 45 min adsorption; G and H, 60 min adsorption; I, uninfected cells. Numbers on the left indicate molecular weight of the proteins.

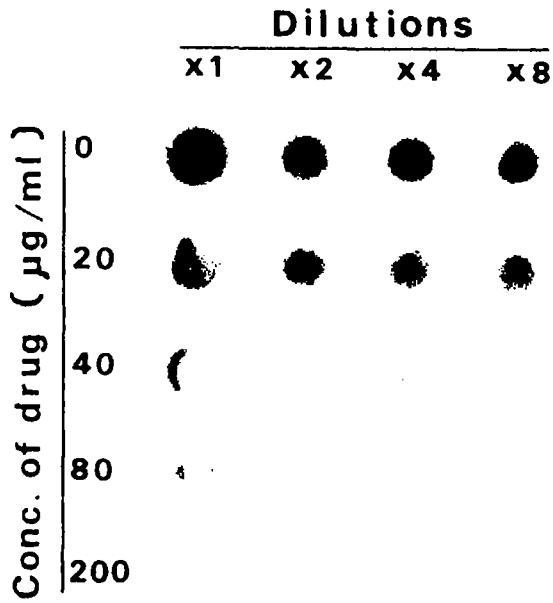

Fig. 7. Effect of PTKA on the level of HSV-2 immediate early mRNA. Cytoplasmic RNA prepared from Vero cells after pretreatment with various concentrations of PTKA in the presence of cycloheximide (100 μg/ml) was placed on a Gene Screen Plus membrane at 5 h post infection with suction at 2-fold dilutions. Prehybridization and hybridization were done as described in the text. The membrane was then exposed to Kodak Royal X-Omat film at −80°C for about 24 h using a Kodak intensifying screen (Cronex Hi-plus).

Discussion

In this study we tried to establish the antiviral activity of PTKA, a new kanamycin A derivative active against HSV-2 in vitro. We also attempted to identify the steps in the multiplication cycle of the virus that are inhibited by PTKA. PTKA achieved 50% reduction in HSV-2 plaque formation at a concentration of 1 μg/ml; at 20 μg/ml plaque formation was completely inhibited. In the virus yield reduction assays a concentration of 50 μg/ml was required for 95% inhibition.

From isotope incorporation studies, followed by PAGE analysis, it was evident that the synthesis of viral proteins was suppressed by PTKA at a concentration of 200 μg/ml. This was particularly evident when the cells were pretreated for 1 h. At 200 μg/ml PTKA selectively inhibited viral protein synthesis in the virus-infected cells, the amounts of host cellular protein synthesized in the infected and treated cells being almost the same as those in uninfected cells. In the uninfected cells no change was noted in protein synthesis upon drug treatment.

It is a characteristic of HSV-infected cells that there is rapid shutoff of host macromolecular synthesis early in infection, and, as a result, there is decline in host

TABLE 1

Effect of PTKA on the adsorption and penetration of HSV-2

Drug dose (μg/ml)	Counts before trypsinization	Counts after trypsinization	% of counts persisting after trypsinization
0	349.4	217.0	62.0
20	327.0	235.5	72.0
40	337.0	227.5	67.5
80	321.0	224.5	69.9
200	325.5	215.0	66.0

Results are expressed as the counts per minute (cpm) after [*methyl*-^{3}H]thymidine-labeled HSV-2 infection and PTKA treatment.

TABLE 2

Effect of PTKA on the uncoating of HSV-2

Experiment number	Drug dose (μg/ml)	Cytoplasm-associated counts	% of total counts	Nucleus-associated counts	% of total counts
1	0	161.5	62.8	95.5	37.2
	80	165.0	56.4	127.5	43.6
	200	182.0	57.8	132.5	42.1
2	0	139.5	56.4	108.0	43.6
	200	162.0	61.1	103.0	38.8

Results are expressed as the counts per minute (cpm) after [*methyl*-^{3}H]thymidine-labeled HSV-2 infection and PTKA treatment.

protein synthesis (Roizman et al., 1965). Here we found that pretreatment with PTKA led to the blockade of this shutoff mechanism and host cell protein synthesis remained almost unchanged. Without pretreatment there was incomplete suppression of virus-induced protein synthesis by PTKA. Drug addition after 15 min adsorption did not block the induction of viral proteins, leading us to assume that PTKA may also act at an early stage of viral replication.

HSV-2 primary transcription was measured by using dot blot hybridization to detect immediate early mRNA after pretreatment with PTKA in presence of cycloheximide. The level of immediate early mRNA was markedly reduced by pretreatment with PTKA at 200 μg/ml. This finding indicated that the antiviral action of PTKA is also situated at the stage of primary transcription or prior. To investigate the stages prior to primary transcription, we used [*methyl*-^{3}H]thymidine-labeled HSV-2, and found that the drug did not inhibit virus adsorption, penetration or uncoating. These results suggest that PTKA acts at the stage of primary transcription or at an unspecified stage between uncoating and primary transcription, as well as at the level of protein synthesis.

Kanamycin A sulphate has no effect on protein synthesis in uninfected cells but is capable of penetrating into orthomyxovirus-infected cells and inhibiting viral protein synthesis as well as formation of infected virions (Ghendon and Mikhailovskaya, 1982). Virus infection itself is the cause for modification of plasma membrane leading to leakiness (Carrasco, 1978). It was presumed that chemical mod-

ification of kanamycin A molecule to make it more lipophilic would help the compound to penetrate through the membrane of a virus-infected cell. The addition of an acyl group to kanamycin A led to be hydrophilic and the introduction of trifluoroacetyl group at the *N*-3″ position was aimed at reducing its cytotoxicity (Matsuda et al., 1986) and these modifications resulted in the loss of antibacterial activity of the compound. The exact mechanism by which PTKA inhibits HSV-2 replication still remains unresolved.

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