THE ANTIVIRAL EFFECT OF GUANIDINE AND ITS DERIVATIVES. IV

Antiviral Effect of 2-Morpholinyl-4-amino-s-triazine-6-carboxyguanidide K-799

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Our group[1,2], RIGHTSEL, *et al*[3]. CROWTHER and MELNICK[4] and TAMM and EGGERS[5] reported independently that guanidine inhibits the multiplication of poliomyelitis virus in tissue culture and the effect probably is due to the inhibition of intracellular viral replication. Guanidine, however, has a rather small chemotherapeutic index and when doses over 30 mg/kg/day of guanidine are administered to human beings, such side effects as rash and paralysis are observed[6]. Thus, more effective and less toxic antiviral agents are desired to provide highly effective chemotherapeutic drugs against viral diseases. Following a program of synthesis of many types of compounds related to guanidine and their screening for antiviral activity, the present authors found that 2-morpholinyl-4 amino-s-triazine-6-carboxyguanidide (K-799) exerted a greater inhibitory effect than guanidine. This report concerns the antiviral effect of this compound on several viruses and its site of action.

MATERIAIS AND METHODS

A new compound, K-799, was synthesized by our group[7]. This compound is readily soluble in water, but sparingly soluble in organic solvents such as methanol and ethanol, *etc.* The i. p. LD_{50} of this compound was found to be 1.5 g/kg in mice.

The following viruses were employed: poliomyelitis virus (MAHONEY, MEF_1 and SAUKETT), ECHO virus (type-6 and type-9), Coxackie virus (A-9 and B-5 strains), adeno virus (from type-1 to type-7), vaccinia virus (DV-96 strain) and Japanese B Encephalitis (NAKAYAMA strain).

Host cells: HeLa(wild type), FL, KB and HEP-2 cells were used.

Media: for the growth medium, the YLA medium supplemented with 15 % bovine serum and for the maintenance medium both of the YLA medium supplemented with 5 % bovine serum or 5 % horse serum were employed in the end-point estimating dilution method. For plaque assay, the two-fold YLA medium supplemented with 10 % bovine serum was mixed with 2.2 % agar suspension in ratio of 1:1 and neutral red solution was added to the mixture in the final concetration of 1:40000. This agar overlay medium was kept at 43°C for 20 minutes prior to use. The K-799-containing agar overlay medium was prepared by adding K-799 to the above ordinary agar overlay medium. The maintenance medium supplemented with 5 % horse serum was used for the cultivation of adeno virus. while the other viruses were cultivated by using the maintenance medium with 5 % added bovine serum. The basal medium of both growth and maintenance media, YLA medium, contains the following components: NaCl 7.18 g, KCl 0.4 g, $CaCl_2$ 0.2 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, NaH_2PO_4 0.125 g, glucose 4.5 g, phenol red 1.5 cc of a solution of 10 mg/cc, $NaHCO_3$ 1.1 g, lactalbumin hydrolysate 5.0 g, yeast extract 1.0 g and distilled water, q.s. 1000 cc. Streptomycin (20 γ/cc) and penicillin (100 units/cc) were added to YLA medium.

Animals: for the experiments of Japanese B Encephalitis, D. D. strain mice, 10 g in body weight, were employed.

For the determination of viral infectivity, both the end-point estimating dilution method (tissue culture infective $dose_{50}$ -- it is abbreviated as $TCID_{50}$ in the following description) and the plaque forming unit (PFU) estimating techniqne were employed. In the former method, a host cell level

of 2×10^5 cells/cc of the host cell-line used was employed in each tube. These assay tubes had been preincubated at 37°C for 4 days to obtain a monolayer cell sheet. After the establishment of the cell sheet, the growth medium was removed from the tubes, and the cells were washed 3 times with phosphate buffered saline (PBS pH 7.4). In plaque technique, a small bottle was used for the culture. After the establisment of the monosheet of the host cell line, the cell sheet was washed 3 times with PBS. The viral material (0.3 cc) was introduced into the bottle and the bottle was incubated at 37°C for one hour. There was then added 4.0 cc of agar overlay medium; the bottle stood at 22°C for 15 minutes, and was then incubated at 37°C for 4 days. After incubation, the number of plaques was counted.

A detailed description of the experimental procedures will be given in the respective sections below.

EXPERIMENTAL RESULTS

Inhibitory Effect of K-799 on the Growth of Selected Viruses. K-799 was added to tubes in which a cell sheet had been established to give a final concentration of 10^{-3} M (270 γ/cc). These tubes were inoculated at once with various dilutions of the viruses to be tested. After incubation at 37°C for 10 days without any exchange of maintenance medium, the effectiveness of K-799 against all of the tested viruses was evaluated. In the control group, the maintenance medium without the addition of K-799 was added. Besides daily microscopic observation during the incubation periods, the median tissue culture infective doses ($TCID_{50}$) of both the control and the treated group were calculated by the method of REED and MEUNCH[8]. In the experiments of Japanese B Encephalitis, 0.3 cc of 1/2 LD_{40} of the NAKAYAMA strain was injected intraperitoneally into a mouse, followed 72 hours later by a single dose of 1/3 LD_{50} of K-799 by the same route. After the observation for two weeks, the numbers of the mice which showed symptoms and then died were recorded. X^2 was calculated from the ratio between the non-surviving mice of the control group and those of the treated group. If X^2 value was over 3.8, it was recognized as a significant one.

The experimental results are shown in Table 1.

Table 1-a Effect of K-799 on Several Viruses

Virus Strain	Host Cells	Results ($TCID_{50}$/01 cc* Control	Treated
Poliomyelitis			
MAHONEY	KB	7.0	3.0
MEF_1	KB	7.0	3.5
SAUKETT	KB	7.5	2.5
ECHO			
Type-6	HeLa	5.5	3.5
Type-9	HeLa	5.0	5.5
Coxsackie			
A-9	FL	3.5	2.5
B-5	HeLa	7.5	7.0
Adeno			
From type-1 to type-7	KB	5.5	5.5
Vaccinia			
DV-96	KB	5.0	5.0

*$TCID_{50}$ expressed as negative log.

Table 1-b Effect of K-799 on Japanese B Encephalitis in Mice

Virus Strain	Single dose of K-799 (mg/kg)	Time of Administration after viral inoculation (hrs)	Results C*	T**
Nakayama	500	72	6/6 (5.7)	6/6*** (6.2)****

*Control group. **Treated group. ***Non-surviving mice/total mice used. ****Average survival days.

The results of these experiments indicate that K-799 markedly inhibited the growth of all of three types of poliomyelitis virus. The effectiveness of K-799 on ECHO and coxsackie viruses was less than that on polio virus, while it was ineffective on adeno, vaccinia and Japanese B Encephalitis viruses. Among the three types on polio virus employed, no significant difference in the drug-sensitivity was found.

Minimum Inhibitory Concentration (MIC) of K-799 on the Growth of MAHONEY Strain of Poliomyelitis Virus. The following four dilutions of K-799 were prepared; 10^{-2} M (2700 γ/cc), 2×10^{-3} M (540 γ/cc), 10^{-3} M (270 γ/cc), and 5×10^{-4} M (135 γ/cc). For the determination of the MIC of K-799 plaque techniques were employed. The various dilutions of the MAHONEY strain were prepared

with PBS. Each of these dilutions (0.3 cc) was introduced into a bottle in which a monosheet of the Hep. No. 2 cell had been established. The cell sheet was washed 3 times with PBS after removal of the growth medium and these tubes were incubated at 37°C for one hour. After incubation, the cell sheets were washed 3 times with PBS to remove unadsorbed virus and then in minutes, the agar overlay medium, in which each of the K-799 solutions was added at a volume of the one-tenth, was added to the bottles. Thus, the concentration of K-799 was finally 270 γ/cc, 54 γ/cc, 27 γ/cc and 13.5 γ/cc, respectively. In the control group, the same amount of PBS as that of the K-799 solution was added to agar overlay medium. After standing at 22°C for 15 minutes, these bottles were incubated at 37°C for 4 days. After incubation, PFU of both of the control and the treated groups was calculated.

The experimental results are shown in Table 2.

Table 2. Minimal Inhibitory Concentration of K-799 on MAHONEY Strain

Final concentration of K-799	PFU/cc
10^{-3} M (270 γ/cc)	4.8×10^{2}
2×10^{-4} M (54 γ/cc)	10^{4}
10^{-4} M (27 γ/cc)	2.6×10^{7}
5×10^{-5} M (13.5 γ/cc)	3.6×10^{7}
0	4.2×10^{7}

The results of these experiments indicate that K-799 did not produce any inhibitory effect at a concentration of less than 10^{-4} M (27 γ/cc), while in the group in which 2×10^{-4} M (54 γ/cc) of K-799 was present, the appearance of plaques was clearly inhibited. Accordingly, the MIC of K-799 on polio virus was 2×10^{-4} M (54 γ/cc).

Inactivating Effect of K-799 on the MAHONEY Strain. It can be assumed that the direct inactivating effect of K-799 against virus might participate in the inhibition of the growth of virus ih host cells. To clarify this point, the inactivating effect of K-799 against the MAHONEY strain of polio virus was determined as follows. Dilutions of the MAHONEY strain in 10-fold steps, and 10^{-3} M (270 γ/cc) of K-799 in final concentration were added to tubes containing no host cells. These drug-virus mixtures were incubated at 22°C for 24 hours. At this time, 0.1 cc of each of the incubated mixtures was added to a tube in which a monolayer of Hep. No. 2 cells had been established (0.9 cc of the maintenance medium had been added to each tube). Four tubes were used for one viral 1ilution. All tubes were then incubated at 37°C for 10 days. The $TCID_{50}$ of both the control and the K-799 treated group was calculated by the method of REED and MUENCH[8]. The results showed that the $TCID_{50}$ of both the control and treated groups was $10^{-7.5}$/0.1 cc. These results indicate that K-799 does not possess any direct inactivating effect against virus when 10^{-3} M (270 γ/cc) of the compound, an optimal concentration for viral growth inhibition, was employed.

Inhibitory Effect of K-799 on the Viral Adsorption on Host Cells. Tubes in which a monosheet of Hep. No. 2 cells had been developed were divided into two groups, a control and a K-799 treated group. In the latter group, to each of the tubes was added 0.1 cc of 10^{-2} M (2700 γ/cc) and 0.8 cc of the maintenance medium, and in the former group 0.1 cc of PBS was employed instead of K-799 solution. Next, 0.1 cc of 10 input multiplicity of a viral material was inoculated into these tubes. For the cell-free control group, 0.1 cc of the above input multiplicity of the viral material and 0.9 cc of the maintenance medium were added to a tube in which no host cells were present. These tubes were then incubated at 37°C for one hour. After incubation, the culture fluid of both the control and the K-799 treated groups was decanted, and the cells of both groups was washed 3 times with the

Table 3. Influences of K-799 on Viral Adsorption on Host Cells

	Total PFU in medium	Adsorbed virus (PFU)	Percentage of viral adsorption (%)
Control-1*	10^{4}		
Control-2**	10^{3}	9×10^{3}	90
Treated***	5×10^{3}	5×10^{3}	50

* The group in which virus was incubated in a tube without any cells.

** The group in which virus was incubated in a tube containing host cells, but without any addition of K-799.

*** The group in which virus was incubated with K-799 in a tube containing host cells.

maintenance medium. The amount of virus of the mixtures of the culture fluid and the maintenance medium employed for cell-washing was estimated by using plaque technique.

As can be seen in Table 3, the adsorption rate of the control group was 93 % of the inoculated viral amount, while that of the K-799 treated group was 91 %. These results indicate that K-799 did not exert any inhibitory effect on the viral adsorption on host cells.

Influences of K-799 on the Release of the MAHONEY Strain from Host Cells. The inhibitory influences of K-799 on the viral release from host cells is one on the remaining possibilities with regard to the site of action of this compound. To clarify this point, the following experiments were carried out.

MAHONEY strain was inoculated using a multiplicity of 1.0 infectious unit per cell and incubated at 37°C for one hour. The medium was removed from the tubes and the cells were washed 3 times with PBS. Anti-MAHONEY monkey serum was then added to a final dilution of 5 % and the tubes were kept at 37°C for 10 minutes. After incubation, anti-serum was removed from the tubes, the cells were washed 3 times with PBS and fresh maintenance medium was added to each of the tubes. All the tubes were further incubated at 37°C. Five hours after the viral inoculation, the medium was removed from the tubes, the tubes were washed 3 times with PBS and an aliquot of anti-serum was added to the tubes. After the tubes were kept at 37°C for 10 minutes, the cells were washed 3 times with PBS. The tubes were then divided into a control and a K-799 treated group. In the latter group, an aliquot of the 10^{-3} M (270 γ/cc) of K-799 containing maintenance medium was added. In the former group, the same amount of the ordinary maintenance medium was employed. All of the tubes were further incubated at 37°C for 2 hours. At the end of the incubation, the culture fluid of the two groups was collected and the amount of virus was estimated using plaque technique.

Table 4 indicates that the total amount of virus in the culture fluid of the control was the same as that of the treated group. It suggests that K-799 does not have any influence on the viral release from host cells.

Table 4. Influences of K-799 on Viral Release from Host Cells

	Viral amunt released from host cells from 6 hrs to 8 hrs after inoculation (PFU/cc)
Control	5.2×10^5
Treated	6.4×10^5

The Inhibition of K-799 on the Intracellular Multiplication of the MAHONEY Strain. The remaining possibility for the site of action of K-799 must be the inhibition of the intracellular multiplication of virus. To confirm this hypothesis the following experiments were carried out. The MAHONEY strain was inoculated using a multiplicity of 1.0 infectious units per cell into tubes in which a monosheet of Hep. No. 2 cells had been established. After incubation for 1 hour at 37°C, the medium was removed and the cells were washed 3 times with PBS. To the K-799 -treated group was added maintenance medium containing 10^{-3} M (270 γ/cc) K-799 and to the control group was added the maintenance medium with PBS instead of the K-799 solution. After 24 hours' incubation of the tubes at 37°C, the culture fluid of both the control and the treated groups was collected for the estimation of the extracellular viral amount. The cells were washed 3 times with PBS and and aliquot of the maintenance medium was added to each of the tubes. The cells were scraped down with a rubber policeman and were frozen and thawed 5 times. After centrifuging at 3000 rpm for 10 minutes, the supernatant was employed for estimating the intrecellular virus.

Table 5. Influence of K-799 on Intracellular Multiplication of MAHONEY Strain

	$TCID_{50}$ $(-\log_{10})/0.1$ cc	
	Intracellular virus	Extracellular virus
Control	8.0	6.5
Treated	5.0	3.5

Table 5 indicates that the amount of virus of both the extracellular and the intracellular fractions of the treated group was clearly lower than that of the control. As described above, K-799 does not inactivate the infectivity of virus and does not

inhibit the viral release from host cells. Thus, the low amount of virus in the culture fluid of the treated group should be due to the decrease of the intracellular multiplication of virus in the treated group. From the results, it may be said that L-799 inhibits the intracellular viral multiplication.

DISCUSSION

As described in the experimental section, K-799 was found to show the inhibitory effect on all three types of polio virus and some of ECHO and coxsackie viruses, while it was ineffective on adeno, vaccinia and Japanese B Encephalitis viruses. Among the above viruses sensitive to L-799, palio virus was most clearly inhibited by K-799, while both of ECHO and coxsackie viruses were not as sensitive as polio virus.

The effect of K-799 is considered more remarkable than that of guanidine, and this eompound was found not to liberate any guanidine in the culture fluid as indicated by chromatographic detection[9]. It is therefore evident that the effect of K-799 should not be ascribed to guanidine liberation.

As to the site of action of K-799, it was shown that the effect of this compound is not due to the viral inactivating effect. Neither the viral adsorption on host cells nor the viral release from the cells are inhibited by this compound. These findings suggest that the site of action of K-799 should be intracellular. The experiments concerning the influences of this compound on the intracellular multiplication of the MAHONEY strain confirmed such a possibility, *i. e.* the intracellular growth of the MAHONEY strain was markedly inhibited by K-799 and the extracellular amount also was lower than that of the control when K-799 was added at the latent period of the viral multiplication. These results suggest that the mechanism of the inhibitory effect of K-799 must involve the process of the replication of viral particles, and further study will clarify the details of the mode of action of K-99.

SUMMARY

On the basis of the finding that guanidine exerts an inhibitory effect on the multiplication of polio virus in tissue culture, 2-morpholinyl-4-amino-s-triozine-6-carboxyguanidide, K-799, was found to be inhibitory on such entero viruses as polio, ECHO type-6 and coxsackie A-9 from the screening tests of many compounds related to guanidine. Among these entero viruses, polio virus was most sensitive to K-799, both ECHO and coxsackie viruses were less sensitive than polio virus, while adeno, vaccinia and Japanese B Encephalitis viruses were insensitive to this compound. The effect of K-799 is due neither to the direct viral inactivating effect nor to the inhibition of viral adsorption on host cells or viral release from the cells. The site of action of this compound is intracellular, particularly in the latent phase of viral multiplication.

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