Alkylated Pyrimidine Derivatives as Antiviral Agents. II.¹⁾
Inhibition of Early Stages of Viral Multiplication
by 5-Alkyluracil and 5-Alkyluracil Nucleoside

MASAKO MURAOKA,^{2a)} YOSHIKO SETO^{2b)}
and TAKEO UEDA^{2c)}

Department of Chemistry, Japan Women's University,^{2a)}
Pharmaceutical Institute, Keio-Gijuku University^{2b)}
and College of Pharmaceutical Sciences,
Kitasato University^{2c)}

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The site of the inhibition of 5-alkyluracils and 5-alkyluracilnucleosides on intracellular multiplication of Mahoney strain of poliomyelitis virus and type-12 of adeno virus were investigated in Hep. No. 2 cells.

Among these compounds 5-ethyluracil (EU), 5-butyluracil (BU), 5-ethyluracil-1- β -D-glucopyranoside (EUG), 5-ethyluracil-1- β -D-ribofuranoside (EUR) and 5-butyluracil-1- β -D-ribofuranoside (BUR) possessed inhibitory effect on the early phase of intracellular multiplication of Mahoney strain. Especially, BUR was more effective than the other compounds.

It was suggested from the experimental results, that the site of action of BUR is the inhibition on the early phase of viral reproduction and the inhibition of viral release from host cells is probably the secondary phenomenon.

As described in the preceding paper,¹⁾ the authors found that 5-ethyluracil (EU), 5-butyluracil (BU), 5-ethyluracil-1- β -D-glucopyranoside (EUG), 5-ethyluracil-1- β -D-ribofuranoside (EUR) and 5-butyluracil-1- β -D-ribofuranoside (BUR) exerted the inhibitory effects on viruses of type-1 and type-12 strain of adeno viruses, DV-96 strain of vaccinia virus, K-2211 strain of ECHO virus and Mahoney strain of poliomyelitis virus. Among these agents especially BUR showed the most marked effect on the viruses. Most of antiviral agents have, hitherto, been found to possess comparatively narrow antiviral spectra, inhibiting biosynthesis of viral nucleic acid in cells. On the contrary it is of interest that 5-alkylpyrimidinenucleoside was found to have a comparatively wide antiviral spectrum, active on both RNA and DNA viruses. This fact suggests that 5-alkylpyrimidinenucleoside might have a specific activity different from those inhibitory substances of viral nucleic acid biosynthesis, which have been found to date.

This report describes the investigation regarding the site of antiviral action of 5-alkylpyrimidine and its nucleoside in viral multiplication.

Materials and Methods

Host Cell—Hep. No. 2 cells are employed.

Viral Materials—Mahoney strain of poliomyelitis virus was employed as a representative of RNA virus and Hoie strain of type-12 of adeno virus, as a representative of DNA virus.

Media—For the growth medium, YLA medium supplemented with 15% of bovine serum was employed. For the maintenance medium to cultivate polio virus, YLA medium supplemented with 5% of bovine serum was employed, and for the cultivation of adeno virus, YLA medium supplemented with 5% of horse serum was employed.

1) Part I: M. Muraoka, A. Takada and T. Ueda, *Chem. Pharm. Bull.* (Tokyo), **18**, 261 (1970).

2) Location: a) No. 8-1, Mejirodai-2-chome, Bunkyo-ku, Tokyo; b) No. 35, Shinanomachi, Shinjuku-ku, Tokyo; c) Shirogane-Sanko-cho, Minato-ku, Tokyo.

General Procedure—The monolayer of Hep. No. 2 cell was prepared by inoculating 2×10^5 cells in 1 ml per tube. After a tube was incubated at 37° for 3 or 4 days to obtain the monolayer cell sheet, growth medium was removed from the tube. The tube was washed three times with phosphate buffered saline (pH 7.6) and 0.1 ml of the dilution of a viral material and 0.9 ml of the maintenance medium were added. Each tube was incubated at 37° . The experimental details will be described in each section.

Assay of the Infectivity of Tested Viral Materials—The two methods, the TCID₅₀ estimating dilution method for the Hoie strain of type-12 of adeno virus and the plaque assay technique to estimate plaque forming unit (PFU) for the Mahoney strain of poliomyelitis virus were used.

In the former method, 0.9 ml of the maintenance medium and 0.1 ml of each of the dilution of a tested viral material were added into each tube, in which the mono-sheet of the Hep. No. 2 cell had already been established, and then the tubes were incubated at 37° for 7 days. After the daily microscopic observation for 7 days, the TCID₅₀ of the material was determined.

In the latter method, after the growth medium was removed from a small Roux's bottle, in which the mono-sheet of the Hep. No. 2 cell had been established, the cells were washed three times with PBS and then 0.3 ml of each of the dilution of a tested viral material was added. After the incubation at 37° for 1 hr, 4.0 ml of the agar overlay medium was added into the bottle. After being allowed to stand at 24° for 15 min, the bottle was incubated at 37° for 4 days. From counting the numbers of plaque, PFU of a tested material was determined. To prepare agar-overlay medium, the doubly concentrated YLA medium supplemented with 10% of bovine serum and 2.2% purified Difco agar were mixed in a ratio of 1:1. The agar-overlay medium were kept at 43° for 15 min prior to pouring of it into a bottle, 40000 folds solution of neutral red in final dilution was added into the medium for viral staining.

Results

I) Experiments on Inhibitory Action of 5-Alkylpyrimidine and Its Nucleoside against Early Stage of Viral Multiplication

(1) At first, EU, BU, EUG and BUR were examined as to their inhibitory action on the early stage of the intracellular multiplication of Mahoney strain of poliomyelitis virus.

For this experiments, one input multiplicity of Mahoney strain was added to the tubes in which the monolayer sheet of Hep. No. 2 cells had been established, the tubes were incubated at 37° , for 1 hr and the unadsorbed virus was removed. After that the maintenance medium containing the agents in maximum non-toxic dose for the cell was added to the tubes. At this time the tubes treated above were divided into A and B groups.

For A group, after the tubes were incubated at 37° , for 6 hr, the viral amount in the host cells were determined according to the plaque assay method.

For B group, the viral amount in the cells were determined 24 hr after the viral inoculation. The other procedures were the same to those of A group. These experimental results are presented in Table I and II.

As can be seen in the tables, it may be said that all of the four agents exerted inhibitory effects on the virus 6 hr after the viral inoculation, but only BUR showed a some inhibitory effect on the virus 24 hr after the viral inoculation.

TABLE I. Effect of EU, BU, EUG and BUR on Intracellular Multiplication of Mahoney Strain of Poliomyelitis Virus (A)

Compound	Dose (M)	Intracellular viral amount ^{a)}	
		Total PFU	Inhibition % ^{b)}
Control		4.7×10^6	
EU	10^{-3}	1.7×10^6	64.2
BU	10^{-3}	2.0×10^6	57.1
EUG	10^{-3}	1.0×10^6	78.4
BUR	2×10^{-3}	6.7×10^5	84.1

a) Intracellular viral amounts were estimated 6 hr after the viral inoculation.

b) Inhibition % was calculated by $\frac{(\text{Total PFU of control}) - (\text{Total PFU of agent})}{(\text{Total PFU of control})}$

TABLE II. Effect of EU, BU, EUG, EUR and BUR on Intracellular Multiplication of Mahoney Strain of Poliomyelitis Virus (B)

Exp. No.	Compound	Dose (M)	Intracellular viral amount ^{a)}	
			Total PFU	Inhibition %
1	control		2.9×10^7	
	EU	10^{-3}	4.5×10^7	
	BU	10^{-3}	3.8×10^7	
	EUG	10^{-3}	2.8×10^7	
	BUR	2×10^{-3}	1.6×10^7	44.8
2	control		1.2×10^8	
	EUR	2×10^{-3}	7.5×10^7	34.8
	BUR	2×10^{-3}	5.0×10^7	56.5
3	control		3.0×10^7	
	EUR	2×10^{-3}	2.1×10^7	30.0
	BUR	2×10^{-3}	1.7×10^7	43.3

a) Intracellular viral amount was estimated 24 hr after viral inoculation.

(2) BUR selected as the most effective, was tested as to its effect on the virus in the time course of the viral multiplication. The time course experiments were conducted for A and B groups. For A group, one input multiplicity of Mahoney strain was added to the tubes, in which the mono-sheet of Hep. No. 2 cells had been established, and the tubes were incubated at 37°, for 1 hr. After that the medium was removed from the tubes and the cells were washed three times with PBS. After the maintenance medium containing 2×10^{-3} M of BUR was added to the tubes and the tubes were incubated at 37°, for 5 hr, the viral amounts in the cells were determined. For B group, after Mahoney strain was added to the tubes in which the mono-sheet had been established and the tubes were incubated at 37° for 1 hr, the unadsorbed virus was removed from the tubes, the maintenance medium without BUR was added to the tubes and the tubes were incubated at 37°, for 5 hr. And then the medium was removed from tubes. At this time, the tubes were added with the maintenance medium containing 2×10^{-3} M of BUR in final concentration. After the tubes were reincubated at 37°, for 18 hr, the viral amounts in the cells were determined by plaque assay method.

The experimental results for A and B groups were shown in Table III. This table indicates that BUR showed an effect of 76.1% inhibition when the agent was added 1 hr after the viral inoculation and then incubated for 6 hr, and the efficacy of the agent was lowered when added 6 hr after the viral inoculation and incubation.

TABLE III. Effect of BUR on the Time Course of the Multiplication of Mahoney Strain of Poliomyelitis Virus

	Group	Intracellular viral amount	
		Total PFU	Inhibition %
A ^{a)}	control	1.8×10^4	
	BUR	4.3×10^3	76.1
B ^{b)}	control	6.3×10^6	
	BUR	3.5×10^6	44.5

a) A group: BUR present in culture fluid from 1 hr to 6 hr after viral inoculation.

b) B group: BUR present in culture fluid from 6 hr to 24 hr after viral inoculation.

This finding suggests that BUR showed a marked effect on the virus, when added at the first stage of the viral reproduction and the efficacy was lowered, when added after the considerable progress of the viral growth cycle. At any rate, it may be said that BUR inhibits the multiplication of Mahoney strain in intracellular site and particularly at the earlier stage.

(3) The inhibitory effect of BUR on the adsorption of Mahoney strain onto host cells and the release of Mahoney strain from host cells were investigated as follows.

Tubes in which the mono-sheet of Hep. No. 2 cells had been established were added with one input multiplicity of Mahoney strain and $2 \times 10^{-3}M$ of BUR in final concentration; these tubes were incubated at 37° , for 1 hr. For the control, PBS was added to the tubes in lieu of BUR. After that, the culture fluids were removed from the tubes, and the cell sheet in the tubes was washed three times with PBS. After all of the washings were added to the fluids, the amounts of the unadsorbed virus in the mixture were determined by plaque assay method.

The result of this experiment shown in Table IV, indicates that BUR hardly affected the virus in the stage of adsorption, because the viral amount of the treated group in the culture fluid did not differ significantly from that of the control group.

Next, the inhibitory effect of BUR on the release of Mahoney strain from the host cells was examined.

The experiment was conducted in the following manner: one input multiplicity of Mahoney strain was inoculated into tubes in which the mono-sheet of Hep. No. 2 cells had been established; the tubes were incubated at 37° , for 4 hr. The fluid then was removed from the tubes and cells were washed three times with PBS to remove unadsorbed virus. Maintenance medium containing $2 \times 10^{-3}M$ of BUR in final concentration was added to the tubes, which were reincubated for 2 hr. For the control group, PBS in the same volume was used in lieu of BUR. After the 2 hr reincubation, the culture fluid was removed from the tubes and the cells were washed three times with PBS: the culture fluid was added with the washings. The viral amount in the mixture was determined by plaque assay method.

The experimental result shown in Table IV indicates that BUR inhibits the virus released from the cells, because the viral amount in the fluid of the BUR treated group was significantly less than that of the control.

TABLE IV. Effect of BUR on the Adsorption of Mahoney Strain of Poliomyelitis Virus into Host Cells and on Release of Mahoney Strain from Host Cells

Group	Compound	Unabsorbed or released virus total PFU	Adsorption % or inhibition %
Effect on absorption	control	3.4×10^4	96.3
	BUR	2.8×10^4	97.0
Effect on release	control	6.5×10^2	
	BUR	2.2×10^2	66.1

II) Actions of EUG, EUR, BUR, Noformicine and 5-Bromodeoxyuridine against Intracellular Multiplication of Type-12 of Adeno Virus

The inhibitory action of alkylpyrimidinenucleoside was investigated on type-12 of adeno virus. For the comparison, noformicine (NF) which was claimed to have inhibitory effect on adeno virus, and bromodeoxyuridine (BUDR) which was asserted to inhibit the early stage of the multiplication of adeno virus, were examined as to their effect on type-12 of adeno virus. Moreover, the synergistic action of the above agents was also surveyed.

For the experiment A, 10^{-1} dilution ($TCID_{50} = 10^{-2.5}/ml$) of type-12 of adeno virus was inoculated into the tubes, in which mono-sheet of Hep. No. 2 cells had been established. The tubes were incubated at 37° , for 2 hr. And then the tubes were added with agents in maximum non-toxic dose for the cell and reincubated at 37° , for 22 hr. After that, the viral amounts in the cells were determined in term of $TCID_{50}$.

For the experiment B, 2×10^{-1} input multiplicity of the adeno virus was inoculated into the tubes, in which the cell-sheet had been established. After the virus was adsorbed at 37° ,

for 6 hr, the maximum non-toxic dose of the agent in final concentration was added to the tubes and the tubes were reincubated at 37°, for 42 hr. After that, viral amounts in the cells were determined in term of TCID₅₀.

In the two above experiments, the CPE in the tubes were observed. The experimental results are shown in Table V.

As can be seen from the Table, it is evident that all of the agents showed inhibitory effects on the virus and more or less inhibited the generation of CPE. It is noteworthy that the effectiveness of BUR was the most marked among the five agents.

From the results of the experiment B, it may be emphasized that the effectiveness of NF and BUDR were lowered by the prolongation of the incubation time of the virus while that of BUR was not.

The synergistic effect of NF and the other agents was observed to be so significant.

TABLE V. Effect of EUG, EUR, BUR, NF and BUDR on the Intracellular Multiplication of Type-12 of Adeno Virus

Compound	Dose (M)	CPE score ^{a)} on A group	Amount of intracellular virus of A group ^{b)} -log ₁₀ TCID ₅₀	Amount of intracellular virus on B group ^{c)} -log ₁₀ TCID ₅₀
Control		4	2.5	2.5
NF	10 ⁻⁴	0	1.0	2.5
BUDR	10 ⁻⁵	2	1.0	1.5
EUG	10 ⁻³	1	1.0	1.5
EUR	10 ⁻³	2	1.0	
BUR	10 ⁻³	0	1.0	1.0
NF+EUG	10 ⁻⁴ +10 ⁻³	0	1.0	
NF+EUR	10 ⁻⁴ +10 ⁻³	0	1.0	
NF+BUR	10 ⁻⁴ +10 ⁻³	0	1.0	
NF+BUDR	10 ⁻⁴ +10 ⁻⁵	0.5	1.0	

a) CPE scores expressed as follows; 10, CPE in 1 scale; 8, CPE in 2 scales; 6, CPE in 4 scales; 4, CPE in 8 scales; 2, CPE in 16 scales; 1, CPE in 32 scales.

b) A group: Tested compound present in culture fluid from 2 hr to 22 hr after viral inoculation.

c) B group: Tested compound present in culture fluid from 6 hr to 42 hr after viral inoculation.

Discussion

On the basis of the previous findings that alkylpyrimidine (EU and BU) and their nucleoside (EUG, EUR and BUR) have broad antiviral spectra, the antiviral site of these compounds was investigated by using Mahoney strain of poliomyelitis virus and type-12 of adeno virus in tissue culture system.

As described in the experimental section, BUR was found as the most effective agent on the both of the viruses among the compounds. BUR, therefore, was used on behalf of alkyl pyrimidine derivatives, to investigate the site of action of them. The results of the experiments in which the agents were added to Mahoney strain Hep. No. 2 cells system, indicated that the effect of the agents was weaker as the incubation time was prolonged, in other words, the viral multiplication proceeded. This fact suggests that the agents inhibits the virus at the early stage of the viral multiplication. According to the experimental results that BUR was added to Mahoney strain Hep. No. 2 cells system at various intervals after the viral inoculation, the effect of the agent became weaker as the time of the administration was delayed. This finding suggests that the agent is effective on the virus at the first step of the viral reproduction and the efficacy of the agent is decreased at the prolonged stage of the viral growth cycle.

Since the above experiments were conducted by the estimation of the viral amounts in the host cells, the site of antiviral action of the agent should be intracellular.

With the experimental result that the agent was added to the cell system at the time of the viral adsorption, the agent did not exert any influence on the viral adsorption. It, therefore, may be said that the site of action of the agent is not extracellular at the stage of the viral adsorption.

On the other hand, the experiments that the agent was administered to the cell system at the stage of the viral release, resulted in the inhibition of the release. Hereupon, there is raised a question whether the agent attacks only the virus released from the cells into the culture fluid, or the agent attacks the virus in the cells and thereby the viral amount released from the cells is decreased secondarily. To elucidate this problem, more work is necessary. At any rate, it may be said that the site of action of the agent is intracellular at the early stage of the viral reproduction and extracellular at the stage of the viral release.

Next, in the experiments in which the agents were added to type-12 of adeno virus Hep. No. 2 cells system at various intervals after the viral inoculation, the agents are effective on the virus when added at the earlier stage of the viral multiplication, but their efficacy became weaker then administered at the later stage. This finding also suggests that the site of action of the agents is intracellular at the early stage of the viral reproduction.

In the experiments with the adeno virus, BUDR, NF and the mixtures of NF and one of EUG, EUR or BUR were employed. Therein, BUR was found as the most effective among these agents by the observations regarding the CPE score and the intracellular viral inhibition. It, therefore, is of interest to make clear the mode of antiviral action of BUR.

The problem of this line will be reported in the next report.