ANTIPOLIOVIRUS ACTION OF ETHINE COMPOUNDS

Zen-ichi Yamazaki, Atsuko Totsuka, Kenya Ohtaki and Yujiro Yamada*

Department of Enteroviruses, National Institute of Health,
Murayama, Tokyo, 190-12, Japan
and
*Central Research Laboratories, Meiji Seika Kaisha, Ltd.,
Yokohama, Japan

Received October 1, 1970

SUMMARY: The four ethine compounds possessing anti-Qβ phage activity inhibited the replication of poliovirus in HeLa cells at the concentrations which were not toxic to the host cells. The induction of the viral RNA polymerase was suppressed at the concentrations of the compounds effective for antiviral activity. It was also shown that the compounds inhibited the activity of the viral RNA polymerase but did not inhibit the activity of HeLa cell RNA polymerase *in vitro*.

Several ethine compounds have been synthesized at the Central Research Laboratories, Meiji Seika Kaisha, Ltd. It has been shown that these compounds inhibit the growth of Qβ replicase. These compounds, however, do not suppress RNA synthesis by the DNA dependent RNA polymerase of *E. coli*[1].

To ascertain whether or not these compounds have a similar effect on an animal RNA virus, experiments have been carried out with poliovirus. In this communication, we report that poliovirus replication and viral RNA polymerase activity are inhibited by these ethine compounds.

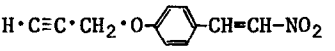
Poliovirus type 1, Mahoney strain and the monolayer and suspension cultures of HeLa cells were used.

The preparation of viral RNA polymerase and assay of the enzyme activity were carried out by the method of Baltimore and Franklin[2].

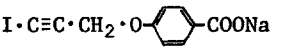
The methods of preparation of DNA dependent RNA polymerase from HeLa cells and assay of the enzyme activity were, for the most part, based on those of Goldberg[3].

The ethine compounds tested in this study are as follows:

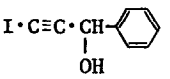
M-1124



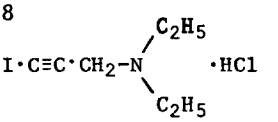
M-1164



M-1063



M-1368



M-1164 and M-1368 are water-soluble and aqueous stock solutions were prepared. M-1124 and M-1063 are hardly soluble in water, and methanol was used to prepare stock solutions. In the experiments employing the latter two compounds, an equivalent volume of methanol without the drug was added to the control samples.

To examine the cytotoxic effect of the ethine compounds, nutrient medium containing 1.5 per cent agar, neutral red (1:50,000) and the drugs at various concentrations was overlaid on HeLa cell monolayers. These were incubated at

Table 1. Cytotoxic effect of the four ethine compounds on HeLa cells

	Concentration (μg/ml)	Uptake of neutral red	Morphological changes of the cells
M-1164	50	+	-
	75	±	∓
	100	±	∓
M-1368	25	+	-
	50	±	∓
	75	-	+
M-1063	12.5	+	-
	25	-	+
M-1124	5	+	-
	10	-	+

The stock solutions of each ethine compound were prepared at 100-fold concentration of the test doses and added to the overlay medium in one hundredth volume respectively. When methanol was used as solvent of the drugs (M-1063, M-1124) there were additional control cultures in which only methanol was added, but there was no difference between the results with methanol-added and methanol-free cultures. Results are expressed as follows. Uptake of neutral red, +: taken up by almost all the cells, ±: taken up by some proportion of the cells, -: not taken up by most part of the cells. Morphological changes of the cells, -: the greater part of the cells appears normal, ∓: some proportion of the cells more or less granulated and shrank, +: most of the cells granulated and shrank.

35°C for 48 hours and the extent of the uptake of neutral red by the cells as well as the morphological changes of the cells were compared. The results are shown in Table 1.

When poliovirus was kept at 35°C for 17 hours with the drugs at 100 μg/ml, per cent survivals were the same with or without the drugs. This indicates that the drugs have no direct inactivating effect on poliovirus infectivity.

To see the effect of drugs on poliovirus replication one step growth of poliovirus in HeLa cells with or without the drugs was examined. From the results shown in Table 2, it can be concluded that virus replication was considerably inhibited at drug concentrations of 50 μg/ml of M-1164, 25 μg/ml of M-1368, 12.5 μg/ml of M-1063, and 5 μg/ml of M-1124. These concentrations were

Table 2. Growth inhibition of poliovirus in HeLa cells by the four ethine compounds.

	Concentration (μg/ml)	Virus yield (PFU/ml)	Growth inhibition rate (per cent)
M-1164	0	$10^{8.4}$	
	25	$10^{7.4}$	90
	50	$10^{6.2}$	>99
M-1368	0	$10^{8.5}$	
	12.5	$10^{8.3}$	37
	25	$10^{6.5}$	99
M-1063	0	$10^{8.5}$	
	12.5	$10^{8.1}$	60
M-1124	0	$10^{8.5}$	
	2.5	$10^{8.1}$	60
	5	$10^{6.5}$	99

HeLa cell suspension at a concentration of about 10^7 cells per ml was inoculated with poliovirus at an input multiplicity of about 10 PFU/cell. After adsorption at 35°C for one hour the cells were centrifuged, washed and resuspended at a concentration of 10^6 cells/ml in Eagle's spinner medium containing the drugs at various concentration. After 6 hours incubation at 35°C samples were removed and their infectivity was measured. The growth inhibition rate was calculated as follows.

$$\text{Growth inhibition rate} = \left(1 - \frac{\text{yield at 6 hours after infection with drug at a specified concentration (PFU/ml)}}{\text{yield at 6 hours after infection without drug (PFU/ml)}}\right) \times 100$$

all below those exerting observable cytotoxicity.

The effect on the induction of viral RNA polymerase was then examined. Poliovirus-infected HeLa cell suspension cultures containing M-1124 and M-1368 at various concentrations were harvested 4 hours after infection. From each culture, a viral RNA polymerase preparation was made, and the enzyme activity was measured. Results are shown in Table 3. It is concluded that the induction of viral RNA polymerase was suppressed almost completely at a minimum concentration of these drugs at which virus replication was significantly inhibited.

Next, it was examined whether or not the drugs inhibit the activity of poliovirus RNA polymerase as is the case with phage Qβ replicase. Viral RNA polymerase preparation was made from poliovirus-infected HeLa cells 4 hours after infection. The drugs were added to the polymerase reaction mixtures at

Table 3. Suppression of the induction of viral RNA polymerase in poliovirus infected HeLa cells by the compounds M-1124 and M-1368

		Concentration (μg/ml)	^{14}C-GMP incorporated (cpm)
Exp. 1	Control	0	3,122
	+ M-1124	2.5	989
	"	5	0
	"	10	0
Exp. 2	Control	0	1,522
	+ M-1368	25	29

As stated in the legend to Table 2, HeLa cells were infected with poliovirus and suspended in medium containing the drugs. After 4 hours incubation, infected cell cultures were harvested, centrifuged and resuspended in hypotonic medium. After homogenization and mild centrifugation to remove nuclei and cell debris, the supernatant was centrifuged at 40,000 rpm for one hour to precipitate microsomal fraction. The microsomal fraction was resuspended in 0.25 M sucrose containing 0.001 M $MgCl_2$ and the suspension was used as an enzyme preparation. The assay mixture contained in a final volume of 0.5 ml: enzyme preparation containing about 2 mg of protein; ATP, CTP, UTP 50 mμ moles respectively; ^{14}C-GTP (4,000 counts per minute per mμ mole) 20 mμ moles; Tris buffer (pH 8.0) 30μ moles, Mg-acetate 5 μ moles; actinomycin D 1 μg; phosphoenol-pyruvate 5 μ moles; phosphoenol pyruvate kinase 20 μg. After 15 minutes incubation at 37°C, the ^{14}C incorporated into the acid-insoluble fraction was measured by a windowless gas flow counter.

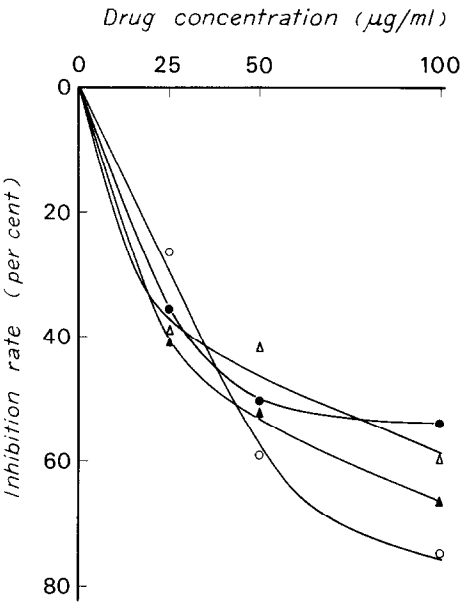


Fig. 1. Effect of the four ethine compounds on the activity of viral RNA polymerase.

The reaction mixture contained viral RNA polymerase preparation and the other reagents exactly as given in the legend to Table 3. Each stock solution of drugs was added to the reaction mixture at the beginning of reaction. Inhibition rate was calculated as follows;

Inhibition rate =

$$\left(1 - \frac{\text{incorporated } {}^{14}\text{C-GMP at each drug concentration}}{\text{incorporated } {}^{14}\text{C-GMP in drug-free control}}\right) \times 100$$

—○— M-1164, —●— M-1124, —△— M-1368, —▲— M-1063.

concentrations of 25, 50, 100 μg/ml. The results are shown in Fig. 1. Taking experimental errors into consideration, it may be given as a conclusion that these four drugs exerted almost the same effect.

The absence of any inhibitory effect of the ethine compounds on host cell RNA polymerase was shown by the following experiment. HeLa cell RNA polymerase was prepared by the method of Goldberg[3]. The ethine compounds were added to the enzyme reaction mixtures at a concentration of 100 μg/ml respectively. It may be said that there was no inhibition of the incorporation of ^{14}C-GMP into the acid insoluble fraction, though the actual ^{14}C counts incorporated were very low (Table 4).

Table 4. Effect of the four ethine compounds on HeLa cell RNA polymerase

	^{14}C-GMP incorporated (cpm)
Complete	114
- ATP, - CTP, - UTP	10
- Mg^{++}, - Mn^{++}	6
+ DNase 20 µg/ml	20
+ M-1368 100 µg/ml	111
+ M-1164 100 µg/ml	114
+ Methanol	92
+ M-1124 (methanol sol.) 100 µg/ml	84
+ M-1063 (methanol sol.) 100 µg/ml	77

HeLa cells were separated from the culture fluid by centrifugation, disrupted gently in VirTis homogenizer, and the "nuclear" fraction was isolated by centrifugation for 6 minutes at 600 x g. The nuclear fraction was resuspended and homogenized in a teflon homogenizer, and then the salt concentration was raised to 0.4 M by the addition of 2 M KCl. The resulting "aggregate" was scooped up by glass spatula and washed, The aggregate suspended in buffer was dispersed in a homogenizer, and the resulting suspension was used as an enzyme preparation.

The reaction mixture contained in a final volum of 0.5 ml: HeLa cell RNA polymerase preparation containing about 3 mg protein; ATP 500 mµ moles; UTP 50 mµ moles; CTP 50 mµ moles; ^{14}C-GTP (4,000 counts per minute per mµ mole) 20 mµ moles; Tris buffer (pH 7.9) 50 µ moles, mercaptoethanol 2.5 µ moles; Mg-acetate 5 µ moles, $MnCl_2$ 0.75 µ moles; ammonium sulfate 0.2 m moles. Each drug was added to the mixture at a final concentration of 100 µg/ml respectively. After 15 minutes incubation at 37°C, the incorporated ^{14}C into the acid-insoluble fraction was measured by a windowless gas flow counter.

All four ethine compounds examined inhibited poliovirus replication in HeLa cells. Thus the compounds are able to prevent the replication of RNA phage[1] and also of an animal RNA virus. These compounds inhibited the induction of poliovirus RNA polymerase and also suppressed the *in vitro* reaction of the enzyme as was observed with Qβ replicase[1]. In this connection, 2-(α-hydroxylbenzyl)-benzimidazole (HBB) and guanidine which are specific inhibitors for multiplication of poliovirus inhibit the induction of viral RNA polymerase but do not suppress the *in vitro* reaction of viral RNA polymerase[4].

While the extent of the inhibitory action of the four ethine compounds on poliovirus replication was different, the intensity of the effect on viral RNA polymerase was very similar. This apparent discrepancy may stem from dif-

ferences between these compounds in their rate of penetration in HeLa cells. An alternative possibility would be that the viral RNA polymerase is not necessarily the only enzyme blocked by these antiviral agents.

It is relevant that the antibiotic, gliotoxin, inhibits cytopathic effects of poliovirus, herpes simplex virus and influenza A virus[5], and has its primary site of action at viral RNA synthesis, while having no effect on cellular RNA synthesis even at a one thousand-fold higher concentration than the minimal dose required to inhibit viral RNA synthesis[6]. It is not known whether gliotoxin has also a differential effect on cellular and viral RNA polymerases in vitro.

Recently we learned that N-methylisatin-β-4': 4'dibutylthiosemicarbazone (busatin) inhibits the replication of poliovirus[7] and also viral RNA polymerase activity in vitro. This drug and the ethine compounds have similarity in their action, in spite of the dissimilarity of their chemical nature.

ACKNOWLEDGEMENT

The authors wish to express their appreciation to Prof. I. Watanabe and Dr. I. Haruna, Department of Molecular Biology, School of Medicine, Keio University, for providing them with suggestions and advice in the present study. We are indebted to Dr. I. Tagaya, Chief, Department of Enteroviruses, National Institute of Health, for his support during the course of this work and for his critical reading of the manuscript.

REFERENCES

1. Watanabe, I., Haruna, I., Yamada, Y., Nagaoka, K. and Seki, S., Proc. Japan Acad., 44, 1038 (1968)
2. Baltimore, D., and Franklin, R. M., J. Biol. Chem., 238, 3395 (1963).
3. Goldberg, I. H., Biochim. Biophys. Acta, 51, 201 (1961).
4. Baltimore, D., Eggers, H. J., Franklin, R. M., and Tamm, I., Proc. Natl. Acad. Sci. U.S., 49, 843 (1963).

5. Rightsel, W. A., Schneider, H. G., Sloan, B. J., Graf, P. R., Miller, F. A., Bartz, Q. R., and Ehrlich, J., Nature, 204, 1333 (1964).

6. Miller, P. A., Milstrey, K. P., and Trown, P. W., Science, 159, 431 (1968).

7. Pearson, G. D., and Zimmerman, E. F., Virology, 38, 641 (1969).