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STUDIES ON THE ANTIVIRAL ACTIVITY OF GUANYLHYDRAZONES ESPECIALLY AGAINST ~~ARB~~ ARBO- AND MYXOVIRUSES

by

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March, 1967

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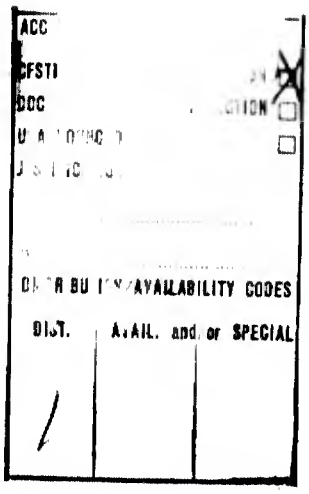

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ABSTRACT

Antiviral and antitumor activities of 4,6-dichloro-2-aminophenol hydrochloride (#178) were studied.

Non-lethal dosis of #178 in vivo was 500mg, 350mg, 25mg, and 1.5mg per Kg. of mouse by intragastric, intraperitoneal, intranasal, and intracerebral administration, respectively. In ovo, one mg. per egg of #178 was non-lethal.

The minimal toxic concentration of #178 in chorio-allantoic menbrance culture in vitro was 25r/ml in final and the minimal inhibitory concentration against influenza virus was observed as 3.2r/ml when #178 was not authoclaved and 0.2r/ml when autoclaved. Therefore, the ratio of the minimal toxic to the minimal inhibitory concentration was 8 with non-autoclaved and 125 with autoclaved solution of #178. The #178 dissolved in 50% glycerol was more effective than its distilled water solution.

Three types of influenzavirus, PR8 strain of type A, Adachi strain of type A_2 and Lee strain of type B, and Japanese B encephalitis virus were inactivated by #178 solution. However, #178 did not inactivate polio virus containing RNA in virus capside and herpes simplex virus containing DNA.

Cultured HeLa cell was very sensitive to #178 and was completely dettached from glass with maintenance medium containing 25r/ml. Ehrlich ascitic cells were relatively resistance to #178 and 1.5 mg/ml of #178 was needed to kill the cells in vitro.

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INTRODUCTION

It has been reported that guanidine and its derivatives were inhibitory against polio, coxakie, measles, influenza and para-influenza viruses (Lwoff & Lwoff 1 Crowther and Molnick 2, Tamm et al 3, Ueda 4, 5, Loddo 6).

Amantadine hydrochloride was reported to inhibit influenzavirus penetration into the host cells (Hoffmann et al. 6) and it was recently reported that piperazine-carboxylate protected mice against influenzavirus infection (Lindh & Forbes 8) and in vitro tests (Fletcher et al 9).

In the course of screening compounds for antiviral activity against Myxo-and Arboviruses, 4,6-dichloro-2-aminophenol hydrochloride was found to be inhibitory to influenzavirus (Nagaki 10). Effects of this compound on tumor cells, tissue culture cells, and major DNA and RNA containing viruses were investigated. The experimental results will be presented here.

MATERIALS AND METHODS

1. Egg and Chorio-allantoic membrane culture

A group of 4 eggs which were eleven day old fertile ones was used for each dilution except noted in text. Eggs inoculated were kept in an egg-incubator at 36°C with 60% humidity.

A group of 4 chorio-allantoic membrane cultures per dilution was used throughout experiments. Chorio-allantoic membranes (CAM) were harvested from 11 to 12 day fertile eggs. Membranes washed with PBS were cut into pieces about a size of 5 x 10 mm.. The content of chorio allantoic membrane tube culture was as follows:

chorio-allantoic membrane	1 piece
Egg-shell (5 x 10 mm.)	1 piece
Hanks' BSS (pH 7.2 - 7.4)	1.0 ml.

All membrane cultures were rubber-stoppered and incubated at 36°C on shaking machine (stroke distance 120 mm; 110 strokes per minute).

2. Viruses

Influenzavirus PR8 strain of type A, Adachi strain of type A2, and Lee strain of type B were used. Experiments were mostly performed with infection of Adachi strain otherwise noted in text. Three types of influenzavirus has been serially passed for a number of years. Infected chorioallantoic fluid (CAF) was ampouled in an amount of 1 ml. each and stored in a dry-ice box until use.

Japanese encephlitis virus, JaTH 160 strain was kindly obtained from Dr. A. Oya of National Institute of Health in Tokyo by courtesy of Dr. I. Yoshioka of Kitasato Institute. One-hundredth ml of a 10^{-3} virus dilution was inoculated intracerebrally into 2 day old dd-mice. The infected brains from paralized mice were harvested aseptically. Each brain was kept separately in a small test tube with a rubber stopper and stored at -70°C until use.

Mahoney strain of poliovirus type 1 and herpes simplex virus were used. Both were serially passed in HeLa cells. The stock viruses were kept at -70°C until used.

3. Mouse

DD strain of random inbred male albino mouse with an average weight of 18 to 20 grams was used for pharmatheutical experiments. For Japanese encephalitis virus experiments, weaning dd mouse, anaverage weight about 10 grams, was employed. All mice were purchased from a comercial farm.

4. Influenzavirus titration in membrane cultures and in eggs

One-tenth ml. of serial ten-fold dilution of the stock virus was inoculated into test tube containing a piece of membrane and egg shell in Hanks' solution (Iwasaki et al., 11). After shaking culture of 4 tubes each dilution for 48 hours at 36°C, hemagglutination (HA) titer of each culture fluid was determined by the standard HA test. The maximal dilution showing HA positive in all four tubes was detected. Serial dilution of the stock virus between this maximal dilution showing HA positive in all four tubes and the next ten-fold dilution were retested to estimate exact titer of HA inducing dilution of the stock virus. Thus determined maximal dilution inducing HA in all 4 tubes is named one MID_{100}. Ten MID_{100} was used throughout experiments.

Two-tenths ml. of ten-fold serial dilution of virus was inoculated into eggs. After 48 hours incubation, EID_{50} was culculated by hemagglutinin production which was determined by HA test. Infectious titers of each virus were as follows:

Type A PR8 strain: $10^8 EID_{50}$/0.2 ml. and
$10^6 MID_{100}$/0.1 ml.

Type A_2 Adachi strain: 10^8 EID_{50}/0.2 ml. and
10^7 MID_{100}/0.1 ml.

Type B Lee strain: 5×10^6 EID_{50}/0.2 ml. and
10^2 MID_{100}/0.1 ml

(EID_{50} = 50% egg infective dosis: MID_{100} = 100% maximal HA inducing dosis)

5. Chemical compound

One compound, sorial No. 212, synthesis No. 178, 4,6-dichloro-2-aminophenol hydrochloride was synthesized at the Department of Organic Chemistry, Kitasato University College of Hygienic Sciences. This compound (#178) was dissolved in double distilled water or 50% glycerol solution, and autoclaved at 121°C for 15 minutes except mentioned in text.

6. Lethal dosis test in ovo and in vivo

The determination of lethal dosis in ovo and in vivo was based on the death of inoculated embryos and mice. The observation periods were 48 hours in ovo and 7 days in vivo test. Eggs and mice inoculated with lethal dosis of #178 were practically died within 24 hours after inoculation.

Methods of hemagglutination test (HA test) of influenza virus, toxicity and antiviral tests of #178 in CAM in vitro were described elsewhere (10).

RESULTS

1. pH of #178

One mg./ml. #178 solution was made with 50% glycerol solution, and divided into two groups. One group of #178 solution was autoclaved at 121°C for 15 minutes. The other was kept at room temperature for 15 minutes. The two-fold serial dilutions of #178 autoclave and non-autoclaved solutions were made respectively with Hanks BSS prior to determination of pH. As shown in Table 1, autoclaved and non-autoclaved #178-50% glycerol solutions had similar pH ranges. pH of 1 mg./ml. solution which was not diluted was 2.5, and that of 100 r/ml solution was 7.1.

2. Lethal dosis of #178 with different administration routes in vivo.

Mice of dd strain were inoculated with #178 solution by different routes. The inocula were 0.02 ml., 0.03 ml., and 0.5 ml. per mouse by intranasal, intracerebral, and intraperitoneal injection. Two tenths ml. and 0.4 ml. of #178 solution were used for intragastric administration with a plastic needle and cylinge.

As listed in Table 2, 0.03 ml. of 10 mg./ml. #178-50% glycerol solution, and controls of 50% glycerol, pH 2.0 HCl, and water were non-lethal by intracerebral injection. Five-tenths of 50% glycerol solution was lethal and the minimal lethal dosis of #178 solution was 7.5 mg. per mouse (375 mg./kg. of mouse) by intraperitoneal inoculation. It was found that non-lethal dosis of #178 was 10 mg./mouse (500 mg./kg.), 7 mg./mouse (350 mg./kg.), 500 r/mouse (2.5 mg./kg.) and 300 r/mouse (1.5 mg./kg.) by intragastric, intraperitoneal, intranasal, and intracerebral administration respectively.

3. Lethal dosis of #178 in ovo

Eleven day old fertile eggs were inoculated with 0.2 ml. of various concentrations of #178 solution. It was shown that 0.2 ml. of 50% glycerol and 0.2 ml. of 5 mg./ml. #178 solutions were not lethal. One experimental result in repeated tests was presented in Table 3.

4. Toxicity and inhibitory concentration against influenzavirus of #178 in CAM. system.

For the determination of toxic concentration of #178 to membranes, two-fold serial dilution of autoclaved compound #178 was made with Hanks BSS and 0.1 ml. of dilution was inoculated into membrane culture containing 0.9 ml. of Hanks, 4 tubes each. After 18 hour incubation, the membrane cultures were washed with PBS 3 times and inoculated 0.1 ml. of 10 MID_{100}/0.1 ml. of influenzavirus type A_2, Adachi strain after adding 0.9 ml. of Hanks to each culture. Then HA production of each infected culture after 48 hour incubation was tested by HA test with 0.5% chicken blood cell suspension. The minimal toxic concentration was constantly a final concentration of 25 r/ml. of autoclaved #178 solution. For the inhibition test, two-fold serial dilution was made with Hanks and mixed with an equal volume of 10 MID_{100}/0.1 ml. of Adachi strain of influenzavirus type A_2. The #178 and virus mixture were kept at room temperature for 30 minutes and 0.2 ml. of the mixture inoculated into cultures which were incubated for 18 hours prior to use. 48 hours after infection, HA production was tested by HA test. The minimal inhibitory concentration of autoclaved #178 against the virus was observed as low concentration as 0.2 r/ml. in final. The compound solution was always autoclaved at 121°C for 15 minutes in order to abolish mold contaminations. However, the solution was colored and formed ferruginous precipitates by autoclaving when the solution concentration was 20 mg./ml. or higher in 50% glycerol. Non-autoclaved #178 solution was tested whether it was inhibitory and/or toxic in the same degree as autoclaved solution. Virus inhibition and toxicity tests were performed with both of autoclaved and non-autoclaved #178 solutions, and results were presented in Table 4. Toxic concentration of autoclaved and non-autoclaved solutions were the same and a final concentration of 25 r/ml.

However, non-autoclaved solution was found less virus inhibitory. The ratio of the minimal toxic to the minimal inhibitory concentration was 8 with non-autoclaved and 125 with autoclaved solutions. Thereafter, autoclaved solution was used unless otherwise noted.

5. Inhibition test in ovo

Autoclaved and non-autoclaved solutions of 1,000 and 500 r/ml. #178 were prepared and mixed with an equal volume of 10 EID_{50}/0.2 ml. of Adachi strain of influenzavirus. A group of 3 eggs was inoculated with 0.4 ml. of

each mixture after storage at room temperature for 30 minutes, and incubated the same manner as in the case of inoculated eggs. As presented in Table 5, a final concentration of 2,000 r/egg of autoclaved #178 solution completely inhibited HA production of virus.

Eggs inoculated with 200 r, 100 r of non-autoclaved and 100 r of autoclaved #178 produced the same titer of HA as control eggs.

6. Effects of solvents and autoclaving of #178 on virus inhibitory concentration in CAM.

#178 was dissolved in double distilled water and 50% glycerol solution. A half of each solution was autoclaved and the remainders of each were kept at room temperature. A two-fold serial dilution of each #178 solution was made with Hanks and mixed with an equal amount of 10 MID_{100}/0.1 ml. of virus. The mixtures were allowed to stand at room temperature for 30 minutes, and 0.2 ml. of each mixture inoculated into membrane culture which had been already incubated for 18 hours and contained 0.8 ml of Hanks. Forty-eight hours after infection, HA production was determined by HA test. It was found that autoclaved #178-glycerol solution was more virus inhibitory than others. Non-autoclaved #178 solutions inhibited HA production with a final concentration of 3.2 r/ml. On the contrary, autoclaved #178 in water or in 50% glycerol inhibited virus at concentrations of 0.8 r/ml. and 0.2 r/ml. in final, respectively. The inhibitory concentrations of each solution were listed in Table 6.

7. Effect of glycerol concentration on #178 virus inhibition

It was observed that autoclaved 50% glycerol solution enhanced that virus inhibitory activity of compound #178. The following tests were performed to determine the minimal concentration of glycerol at which the marked increase in virus inhibition of #178 can be observed. #178 was dissolved in two series of glycerol solution from 100% to 0% and autoclaved for 15 minutes. The result in two tests was shown in Table 7: The #178 solution in at least 10% glycerol inhibited HA production the same as in the 50% glycerol.

8. Effect of heating of #178 solution on virus inhibition

Compound #178 was dissolved in 50% and 100% glycerol solution, and heated in an oil-bath for 15 or 60 minutes. Control solutions were allowed to stand at room temperature. The minimal virus inhibitory concentrations of each solution were listed in Table 8. Heating at 150°C and 180°C for 60 minutes reduced remarkably the virus inhibitory activity of #178. The activity was not reduced by heating at 120°C for 60 minutes.

9. Node of action of #178 virus inhibition in ovo

The mode of action of #178 virus inhibition was investigated with following two sets of experiments. The first experiments were performed by administrating 500 r or 800 r of #178 per egg before or after virus infection. Controls of virus only and the mixture of the same amount of #178 and 10 EID_{50}/0.2 ml. of virus and 0.2 ml. of #178 solution. The second experiments were done by the following method; eggs were infected with 1) #178 and virus mixed just before inoculation, 2) simultaneously #178 and virus, 3) the mixture of #178 and virus stored at room temperature for 30 minutes, and 4) only virus suspension. All eggs were inoculated with 0.1 ml. of 10^7 EID_{50}/0.2 ml. virus and 0.1 ml. of 10 mg./ml. #178 solution. The inhibition of HA production was tested by HA test after 48 hour incubation at 36°C. As seen in Table 9, HA production in the first experiments was inhibited by both administration of #178 on virus infection time zero, and inoculation of the mixture stored for 30 minutes. In the second experimenta, HA production was inhibited by only the inoculation of the mixture of #178 and virus suspension which was stored at room temperature for 30 minutes.

10. Effect of diluents on #178 virus inactivation

In ovo test, 500 r and 800 r per egg of #178 could not inhibit HA production of influenzavirus. Those concentrations were sufficient enough to inhibit HA production when #178 and virus were mixed in a tube and stored at room temperature for 30 minutes. A question arises whether #178 inoculated into chorio-allantoic fluid (CAF) which is alkaline, was interfered by CAF or its alkaline pH. The following experiments were performed

to answer the question. Autoclaved and non-autoclaved #178 solutions were diluted by two-fold serial dilution with Hanks or freshly harvested CAF, and mixed with the same amount of virus for 30 minutes. Two-tenths of the mixture was used for infection of membrane cultures. After 48 hour inoculation of the mixture, the minimal virus inhibitory concentration of each inoculum was determined by HA test.

As can be seen in Table 10, the maintenance medium, Hanks and CAF, of membrane cultures did not affect virus inactivation by #178 solution. The minimal inactivity concentration of #178 diluted with Hanks was a final concentration of 0.1 r/ml. to 0.2 r/ml., and when diluted with CAF it was 3.2 r/ml. to 6.3 r/ml. in final. #178 solution diluted with Hanks was almost 32 times more inhibitory than that of CAF dilution. This result indicates that CAF interferred with the virus inactivation of #178 and the virus inactivation could be done within 30 minutes inactivation period after virus and #178 solution were mixed.

11. Virus inactivation experiment in vitro

Autoclaved #178 4 mg./ml. solution was mixed with an equal volume of 10^4 MID_{100}/0.1 ml. and 10^5 MID_{100}/0.1 ml. virus suspension.

For virus control, virus was mixed with 50% glycerol solution at pH 2.0 acidefied with HCl instead of #178-50% glycerol solution. The mixtures were diluted with Hanks by ten-fold serial dilution after 30 minutes incubation. Membrane cultures were inoculated with 0.2 ml. of each dilution. The result in experiments indicates that virus, even 10^5 MID_{100}/0.1 ml. was completely inactivated with 4 mg./ml. of #178 solution.

12. Effect of virus inactivation temperature on the inhibitory activity of #178

Autoclaved 12 mg./ml. of #178 solution was mixed with 10^7 EID_{50}/0.2 ml. of virus, and the mixtures were allowed to stand at 37°C, 25°C and 4°C for 2 hours, respectively. For virus control, 10^7 EID50/0.2 ml. of virus suspension was mixed with glycerol pH 2.0 acidefied with HCl and kept under the same condition of as in treating samples.

Then, all the mixtures were diluted by ten-fold dilution with Hanks, and 0.4 ml. of dilution was inoculated into eggs. Virus inactivation was determined by HA test. The results were presented in Table 12, and indicate that virus inactivation could be done by different incubation temperature described above.

13. Inactivation experiment of three types of influenzavirus in OVO

Influenzavirus PR8 strain of type A, Adachi strain of type A_2, and Lee strain of type B were used. The infectious titers of PR8, Adachi, and Lee strains were 10^8 EID_{50}.10^8 EID_{50}, and 5 x 10^6 EID_{50} per 0.2 ml. each. Ten-fold dilution of each virus and 10 mg./ml. of #178 solution were mixed at room temperature for one hour, and for the virus control, diluted virus and pH 2.0 HCl glycerol solution were mixed at room temperature for one hour. Each mixture was then diluted by 10-fold serial dilution with Hanks. A group of 4 eggs were inoculated with 0.2 ml. of the mixture, respectively, and incubated at 36°C for 48 hours. The results in Table 13 indicated that three types of influenzavirus were equally inactivated with #178 solution.

14. Effect of #178 on Japanese encephalitis virus, JaTH160.

It is obvrious that #178 inactivates completely the infectivity of influenza virus. Thus, it is of interest to test its ability on other DNA and RNA containing viruses. JaTH160 was used for the first experiments. Frozen brain was quickly thawed and its 10% homogenate was prepared with a glass homogenizer. The infectivity of JaTH160 was titrated as in Table 14. One-tenth ml of appropriate dilution was inoculated intraperitoneally into a group of five mice. One group of mice inoculated with 3 x 10^{-4} homogenate was died within 9 days after infection. Thereafter, a 10^{-4} dilution was used for virus inoculum. Ten mg/ml of #178, 50% glycerol solution was prepared and autoclated. The solution was diluted to make solutions of 4,000 to 250 r per ml in Hanks' BSS. and the same volume of a 10^{-4} JaTH160 dilution was mixed. The mixtures were stood at room temperature for 30 minutes. Five to ten mice each group were inoculated with 0.2 ml of the each mixture. As controls, 2 x 10^{-4}, 2 x 10^{-5} virus dilution, and an equal volume of 10^{-4} virus and 50% glycerol at pH 2.5 adjusted with HCl were inoculated into mice as the same condition as

experimental groups. All mice died within 12 days after inoculation, if they showed a typical paralysis.

The results in virus inactivation experiments shown in Table 15, indicate that in a final concentration of #178, 750 r/ml was enough to inactivate completely the lethal dosis of JaTH160. The minimum lethal dosis of #178 alone was found to be 7.5 mg. per mouse by intraperitoneal administration. Therefore, the ratio of minimal virus inactivation to minimal lethal dosis is 10.

The following experiment was tried to cure the mice inoculated with 7 daily injections of 1 mg/0.4ml per mouse of #178 solution. As a control, mice were injected with 0.4 ml of acid glycerol solution. As shown in Table 16, there is no significant different regards to munbers of dead mice between treatments with and without #178 solution. This result indicates that #178 is a virucidal but not therapeutic compound, as previously demonstrated in experiments with influenza virus.

15. Relationship between ascitic tumor formation and #178

Autoclaved #178 was diluted to make solutions containing 4,000 r/ml to 200 r/ml, and each solution was mixed with an equal volume of Ehrlich ascitic cell suspention. Two-tenth ml of each mixture was intraperitoneally injected into adults mice. Each mouse was administrated finally with 10^7 cells. As controls, cell suspension only and cell suspension acidified to pH 2.5 were employed. The inoculum of 10^7 cells per mouse contained sufficient cell numbers to give a balooning abdomen within 12 days after cell inoculation as shown in Table 17.

Complete inhibition of ascitic tumor formation was observed by the administration of the mixture with 1,500 r/ml in final. A half of mice inoculated with the mixture of 1,000 r/ml of #178 was not suffered from ascitic tumor and the rest of half finally took tumor, however, prolonged period was taken to observe balooning abdomens. As presented in Table 18, both of control groups inoculated cells only and acidified cell suspension were formed big balooning ascitic retention within 11 days after cell administration. 750 r/ml or less amount of #178 did not affect the formation of tumor. Table 19 shows the result of treatment experiment of mice.

Adult mice were divided into three groups. Mice of group A were administrated with 1.5 mg/0.4 ml/mouse of #178 solution one day before tumor cell injection and recieved 7 daily injectiin of 1.5 mg/0.4 ml/mouse of #178. Group C, a control, was injected

glycerol solution acidified by HCl instead of #178 solution. Group B of mice was inoculated simultaneously with cells and #178 and thereafter, was kept under the same condition as group A. Here again, therapeutic effect of #178 on tumor formation was not observed.

16. Cytotoxicity of #178 on HeLa cells.

The first experiments were performed to determine the effect of #178 on attachment of HaLa cells onto glass-tube surface and its growth. The cell suspension was adjusted to 2 x 10^5 cells/ml and an equal volume of appropriately diluted #178 solution was mixed. The mixtures of cell suspension and #178 solution were prepared in the cell growth medium of YLH supplemented with 10% calf serum. Tube cultures were prepared by inoculation of 1 ml per tube of cell suspension into a group of 4 tubes. Growth medium was changed every 3 to 4 day interval and the effect of #178 on HeLa cells was examined on the 10th day after seeding. It was found that a final concentration of 10 r/ml or more of #178 was very cytotoxic, and 5 r/ml was a little, and 1 r/ml or less was non-cytotoxic to HeLa cell attachment onto glass-surface and its growth.

The second experiments were carried out to compare the cytotoxicity of #178 to monolayers of HaLa cells to the results in the experiments on cell attachment and its growth. Test tubes were seeded with 1 ml of 10^5 cell suspension per ml of HeLa cells. Two days after seeding, tubes were fed 1 ml of growth medium containing various concentrations of #178. The toxicity of #178 was examined 10 days after cell seeding with changing medium every 3 to 4 days. The following results were obtained; 25 r/ml or more of #178 was toxic and all cell sheets were dettached completely, 10 r/ml was a little toxic and approximately a half of cells came off, however, cell mitosis was observed in survived cells. 5 r/ml was far less toxic and cell growth of cells which were left on glass was more delayed than control cells, 1 r/ml or less concentration of #178 was appearently non-toxic.

17. Does #178 affect polio and herpes simplex viruses replication in vitro?

HeLa cell cultures were prepared in tubes, and infected with 0.1 ml per tube of 1 to 10^3 TCID50 of polio virus type 1. One group of tube culture cells was fed with maintenance medium containing 2.5 r/ml of

#178 18 hours before virus inoculation. The second group was allowed to adsorb virus first at 37°C for 30 minutes and fed with the maintenance medium. As a control, cells were infected with poliovirus without #178 in maintenance medium. No effect of #178 on polio virus replication was observed.

The same volume of poliovirus ($2 \times 10^{5.5}$ $TCID_{50}$/0.2 ml) and #178 (2 mg/ml) were mixed and allowed to stand at room temperature for 30 minutes. The infective titers of the mixture and a control of virus suspension only were compared. There was no significant differente infectivity between poliovirus suspension with and without #178. These results indicate that 1 mg/ml of #178 does not inactivate poliovirus.

The similar experiments were performed with #178 and herpes simplex virus, and identical results to polio virus were obtained.

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Table 1. pH of #178 solution

Solvent	Glycerol (%) 50	25	12.5	6.3	3.2	1.6	A_q Dest.
Concentration (r/ml) Heating	1,000	500	250	100	50	25	20,000
Non-autoclaved #178	2.50	5.10	6.18	7.15	7.30	7.40	1.8
Autoclaved #178	2.53	4.70	6.06	7.15	7.38	7.45	

Table 2. Lethal dosis of #178 with different administration routes in vivo

Route	Inoculum	Total Concentration of #178	Remark
Intranasal	0.02 ml of 25 mg/ml #178	500 r	Non-lethal
Intragastric	0.2 ml of 25 mg/ml #178	5 mg	"
"	0.4 ml of 25 mg/ml #178	10 mg	"
Intracerebral	0.03 ml of 10 mg/ml #178	300 r	"
	0.03 ml of 50% glycerol	0	"
	0.03 ml of HCl, pH 2.0	0	"
	0.03 ml of H_2O	0	"
Intraperito-neal	0.5 ml. of 50% glycerol	0	Lethal
	0.5 ml. of 25% glycerol	0	Non-Lethal
	0.5 ml. of 15 mg/ml #178	7.5 mg	Lethal
	0.5 ml. of 14 mg/ml #178	7.0 mg	Non-lethal
	0.5 ml. of 12 mg/ml #178	6.0 mg	"
	0.5 ml. of HCl, pH 2.0	0	"

Table 3. Lethal dosis of #178 in ovo

	#178 administrated concentration mg per egg					Control
	1.0	1.3	1.6	1.9	2.2	25% glycerol
No. of Inoculated eggs	3	3	3	3	3	3
No. of dead eggs	0	1	3	3	3	0
Percent of dead eggs	0	33.3	100	100	100	0

Table 4. Toxic and inhibitory concentration of #178 in CAM

		Final concentration of #178 (r/ml)										
Test	Heating	100	50	25	12.5	6.3	3.2	1.6	0.8	0.4	0.2	0.1	Ratio**
Toxicity	Autoclaved	0	0	0	25*	50	100	100	100	100	NT	Nt	Autoclaved #178
	Non-Autoclaved	0	0	0	25	25	25	50	50	100	NT	NT	25/0.2=125
Inhibition	Autoclaved	0	0	0	0	0	0	0	0	0	0	50	Non-autoclaved #178
	Non-Autoclaved	0	0	0	0	0	0	25	25	50	100	100	25/3.2 = 8

* Percent of HA titer in comparison with control

** Ratio = minimal toxic dosis/minimal inhibitory dosis.

Table 5. Inhibitory concentration of #178 in ovo

Heating	Final Concentration of #178 (r/egg) 200	100	Virus control
Autoclaved	0	100*	100
Non-autoclaved	100	100	100

* Percent of HA titer in comparison with control.

Table 6. Effects of solvents and autoclaving of #178 on virus inhibitory concentration in vitro

	Minimal inhibitory concentration (r/ml)	
Solvents	Autoclaved #178	Non-autoclaved #178
50% glycerol	0.2	3.2
H_2O	0.8	3.2

Table 7. Effect of glycerol concentration on #178 virus inhibition in vitro

Expt. I		Expt. II	
Percent of glycerol	Minimal inhibitory concentration(r/ml)	Percent of glycerol	Minimal inhibitory concentration(r/ml)
100	0.2	100	0.1
50	0.2	50	0.1
25	0.2	25	0.1
12.5	0.2	10	0.1
6.25	0.4	5	0.2
0	1.6	0	1.6

Table 8. Effect of heating of #178 on virus inhibition in vitro

Expt. #	Original glycerol concentration	Heating Temperature	Period (minute)	Minimal inhibitory concentration of #178 (r/ml)
I	50%	120°C	15	0.2
		Non		3.2
II	100%	120°C	15	0.1
		"	60	0.1
		150°C	15	0.1
		"	60	1.6
		180°C	15	0.4
		"	60	1.6
		Non		3.2

Table 9-1. Mode of action of #178 virus inhibition in ovo

Expt.#	Inoculum of #178	#178 administration post virus infection (minute) -60	-30	0	15	30	60	90	120	Virus Control	Mixed ****
I	500r/egg	100	NT*	0**	100	100	100	100	100	100	0
		100	NT	0	100	100	100	100	100	100	0
		100	NT	0	100	100	100	100	100	died	0
II	800r/egg	100	100	100***	100	100	NT	NT	NT	100	0
		100	100	100	100	100	NT	NT	NT	100	0
		100	100	100	100	100	NT	NT	NT	100	0
		100	100	100	100	100	NT	NT	NT	100	0

* Not tested, ** Inoculated just after mixing of #178 and virus.

*** #178 and virus inoculated simultaneously.

**** Stored at room temperature for 30 minutes.

Table 9-2.

Expt. #	Inoculum	Percent of HA of each egg			
III	Mixed just before inoculation	100	100	100	100
	Simultaneously	100	100	100	100
	Stored at room temperature for 30 min.	0	0	0	0
	Virus control	100	100	100	100

Table 10. Effect of diluents on #178 virus inactivation

Expt.#	#178 solution	Diluent	Maintenance medium	Minimal inactivating concentration of #178(r/ml)
I	Autoclaved	Hanks	Hanks	0.2
	1 mg/ml	CAF*	Hanks	6.3
II	Autoclaved	Hanks	Hanks	0.1
	1 mg/ml	"	CAF	0.1
		CAF	Hanks	3.2
		"	CAF	3.2
III	Non-autoclaved	CAF	Hanks	6.3
	1 mg/ml			
	Control. pH 2.0-50% glycerol was non-inhibitory			

*CAF Chorio-allantoic fluid.

Table 11. Virus inactivation experiment of Adachi strain in vitro

Inoculum	Dilution of virus 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
10^5 MID_{100} Virus+#178, 4 mg/ml glycerol	0	0	0	0	0
10^4 MID_{100} Virus+#178, 4 mg/ml glycerol	0	0	0	0	0
10^5 MID_{100} Virus+Glycerol, pH 2.0	50	100*	100	25	0
10^4 MID_{100} Virus+Glycerol, pH 2.0	100	100	50	0	0

* HA percent of control.

Table 12. Effect of virus inactivation temperature on the inhibitory activity of #178 in ovo.

Temperature	37°C		25°C		4°C	
Dilution of virus	Treated	Non-Treated	Treated	Non-Treated	Treated	Non-Treated
10^{0}	0	NT	0	NT	0	NT
10^{-2}	0	NT	0	NT	0	NT
10^{-3}	0	NT	0	NT	0	NT
10^{-4}	NT	50	NT	100	NT**	100*
10^{-5}	NT	0	NT	100	NT	100

* Control: 100 indicates 1:6,400 HA of CAF, and 0 complete negative HA.

** NT: Not-tested.

Table 13. Virus inactivation experiment of three types of influenza virus in ovo.

Inoculum	10^{0}	10^{-1}	10^{-2}	Virus Control
Inf.type A, PR8 10^{7} EID_{50}+#178 10mg/ml	0	0	0	100
Inf.type A_2, Adachi 10^{7} EID_{50}+#178 10 mg/ml	0	0	0	100
Inf.type B, Lee 5 x 10^{5} EID_{50}+10mg/ml	0	0	0	100

Table 14. Titration of JaTH160 in vitro

Virus d.l.	$3x10^{-1}$	$3x10^{-2}$	$3x10^{-3}$	$3x10^{-4}$	$3x10^{-5}$	control
No.of dead mice	4	2	4	5	5	0
No.of inoculated mice	5	4*	5	5	5	5
Mice dead days within	10	17	10	9	17	

* Initially, five mice were inoculated, and one was accidently died within 24 hours after virus inoculation.

Table 15. JaTH160 virus inactivation with #178 solution

Final cons. of #178 (r/ml)									cotrol
	2,000	1,000	750	500	250	125	$2x10^{-4}$ Low pH	$2x10^{-4}$	$2x10^{-5}$
No.of dead m.	0	0	0	9	5	5	7	9	5
No.of inoc. m.	5	10	5	10	5	5	10	10	5

The paralized mice were first observed at 10 days and died by 12 days after virus inoculation.

Table 16. Treatment of infected mice with #178

	Inoculated virus		
Treated with	3×10^{-3}	3×10^{-4}	3×10^{-5}
#178	5/5	2/5	0/5*
Control pH 2.5 gly	4/5	2/5	1/5

* Numbers of dead mice per numbers of inoculated mice.

Table 17. Ascitic tumor incident with Ehrlich ascitic cells.

	10^6	10^5	10^4
No. of tumor taken mice	4	4	4
No. of inoculated mice	4	4	4
Days taken	12	12	25

Table 18. Inhibition of Tumor Formation with #178

	Final concentration of #178 (r/ml)								
	2,000	1,500	1,000	750	500	250	100	pH 25 of gly.	control cell control
No. of tumor formed	0	0	7	5	10	5	9	14	4
No. of inoculated	5	5	14	5	10	5	9	14	4
Days taken			28	20	13	13	13	11	11

All mice were inoculated with 0.2 ml of the cell and #178 mixture.
The cell numbers inoculated were 10^7 per mouse.

Table 19. Treatment of inoculated mice with #178

Treated with		
A (#178)	B (#178)	C (pH 25 gly)
6/7	4/7	5/6

All mice were inoculated with 10^7 cells per mouse.
A group of mice was treated one day before and after cell injection with 15 mg/0.4ml of #178 solution.

B group of mice was inoculated simultaneously with cells and #178 solution,

C group was a control mice treated with low pH 2.5 glycerol solution.

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13. ABSTRACT

Antiviral and antitumor activities of 4,6-dichloro-2-aminophenol hydrochloride (#178) were studied.

Non-lethal dosis of #178 in vivo was 500mg, 350mg, 25mg, and 1.5mg per Kg. of mouse by intragastric, intraperitoneal, intranasal, and intracerebral administration, respectively. In ovo, one mg. per egg of #178 was non-lethal.

The minimal toxic concentration of #178 in chorioallantoic membrane culture in vitro was 25r/ml in final and the minimal inhibitory concentration against influenza virus was observed as 3.2r/ml when #178 was not autoclaved and 0.2r/ml when autoclaved. Therefore, the ratio of the minimal toxic to the minimal inhibitory concentration was 8 with non-autoclaved and 125 with autoclaved solution of #178. The #178 dissolved in 50% glycerol was more effective than its distilled water solution.

Three types of influenzavirus, PR8 strain of type A, Adachi strain of type A_2 and Lee strain of type B, and Japanese B encephalitis virus were inactivated by #178 solution. However, #178 did not inactivate polio virus containing RNA in virus and herpes simplex virus containing DNA.

Cultured HeLa cell was very sensitive to #178 and was completely detached from glass with maintenance medium containing 25r/ml. Ehrlich ascitic cells were relatively resistance to #178 and 1.5 mg/ml of #178 was needed to kill the cells in vitro. (Author)

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