Inhibition of Poliovirus by Effect of a Methylthiopyrimidine Derivative (34542)

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(Introduced by F. L. Black)

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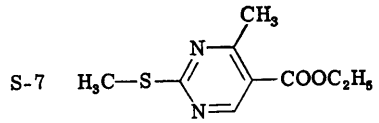
We have previously described the inhibitory effect of several substituted pyrimidine compounds on poliovirus (1–3). Of 38 compounds tested, 4 were found to inhibit the cytopathic effect of the virus, and ethyl-2-methylthio-4-methyl-5-primidinecarboxylate (S-7) was most effective. The earlier studies also demonstrated that the effect was not due to direct virus inactivation, inhibition of virus adsorption or inhibition of cell growth.



In this study the antiviral spectrum of S-7 has been explored and drug-resistant and drug-dependent strains have been isolated and characterized. Comparisons have been made between the effect of S-7 and of 2-(α-hydroxybenzyl)-benzimidazole (HBB) and guanidine as determined by Eggers and Tamm (4–6) and by Melnick *et al.* (7).

Materials and Methods. Trypsinized monkey kidney cells (MK) were grown in 0.5% lactalbumin-Earle's BSS (LE) supplemented with 2% calf serum. HeLa cells were grown in 0.1% yeast extract, 0.5% lactalbumin hydrolyzate, Earle's BSS (YLE) supplemented with 20% calf serum. Vero cells were grown in reinforced Eagle's MEM (2-fold concentration of amino acids and vitamins) supplemented with 10% calf serum.

Virus strains were poliovirus type 1 (Mahoney), type 2 (MEFl), type 3 (Saukett), Sabin's attenuated vaccine strains type 1, 2, and 3, ECHO 1 (Farouk), ECHO 5 (Noyce), ECHO 13 (Hamphill 2-188), ECHO 22 (Harris), ECHO 23 (Williamson), Coxsackie A7 (Parker), Coxsackie A9 (Bozek), Coxsackie B3 (Nancy), and Coxsackie B5 (Faulkner). Polioviruses were propagated in either Hela or MK cells; other viruses were grown in MK cells. Infectivity was measured as described previously, and titers were expressed in $TCID_{50}$ in primary MK or in HeLa, or in plaque-forming units (PFU) on HeLa or Vero cell cultures (1, 3).

S-7 and guanidine-HCl were obtained from Toyama Kagaku Kogyo Company, Tokyo, and HBB from Eisai Company, Tokyo.

To measure viral cytopathic effect (CPE), tube cultures were changed with 0.9 ml of Eagle's MEM containing the specified concentration of S-7 and inoculated with 50–300 $TCID_{50}$ virus in 0.1 ml PBS. A supplement of 3% calf serum was included in medium for HeLa cell cultures. Tubes were kept at 36° and examined daily for CPE. Four cultures were used for each point.

Inhibition of the virus yield was tested on MK tube cultures (4.5×10^4 cells per tube). MK cultures were inoculated with various doses of virulent poliovirus. Drug was added at the time of inoculation and left unreplenished until completion of the experiment. When CPE involved more than 90% of the cells in untreated cultures, all cultures were frozen and thawed. The suspension was clarified by low-speed centrifugation, and the supernatant fluid was titrated on Vero cell cultures.

Results. Inhibition of virus-induced CPE. Earlier studies have shown that compound S-7 does not inhibit the multiplication of HeLa cells (2); in undertaking the present study, it was shown that S-7 did not affect the growth of MK cells at the concentration of 100 μg per ml.

Cytopathic effects of poliovirus on MK

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TABLE I. Percentage of Cells Showing CPE at Time of Complete CPE in Control.

Cell culture	Virus	Challenge dose ($TCID_{50}$)	Day of complete CPE in control	μg S-7/ml medium (%)	
				10	100
HeLa	Mahoney	100	3	25	ND
	MEF1	180	5	0	0
	Saukett	180	3	0	0
	Sabin's 1	320	6	35	0
	2	56	5	30	0
	3	320	5	35	0
MK	Mahoney	320	3	70	5
	MEF1	56	4	0	0
	Saukett	320	3	ND	5
	ECHO 1	100	3	100	100
	5	56	4	90	80
	13	180	5	ND	90
	22	180	5	90	80
	23	180	3	ND	100
	Coxsackie A7	100	4	ND	100
	A9	100	3	100	100
	B3	100	4	85	50
	B5	100	4	ND	90

cells were reduced by 10 μg/ml of S-7 and greatly delayed by 100 μg/ml. This protection was greatest in MEF1 and rather less in Mahoney strain (Fig. 1, Table I), but these differences in sensitivity were not consistent when different stocks of nominally the same strains were used. Sabin's attenuated strains in HeLa cell cultures were unambiguously inhibited by S-7, though the degree of protection was lower than with the virulent ones (Table I). The ECHO and Coxsackie viruses were not sensitive to S-7, and cytopathic effects attributable to them were not modified by the drug.

Inhibition of virus yield. The yield of virus from cultures infected by virulent poliovirus strains was greatly reduced when 50 μg/ml of S-7 was incorporated into the growth medium. Results are given in Table II. No infectious virus was found in drug-treated cultures inoculated less than 30 $TCID_{50}$. There was also a marked suppression of virus yield by the drug when larger inocula were used. (Table II).

Inhibition of plaque formation. Plaque titrations of MEF1 virus were carried out on HeLa cells under agar containing various concentration of S-7 (Fig. 2). A concentration of 1.5 μg/ml was sufficient to give 50% plaque inhibition, but even 100 μg/ml did not give total inhibition. At drug concentration of 3 μg/ml or more, the size of many, but not all, of plaques was reduced.

TABLE II. Inhibition by S-7 of Poliovirus Yield.

Strain	Dose ($TCID_{50}$)	Day of harvest	Yield/ml (PFU)	
			Untreated cultures	50 μg/ml S-7
Mahoney	3000	2	1.5×10^8	2.4×10^3
	300	3	2.3×10^7	1.6×10^3
	30	4	1.2×10^7	<10
	3	4	9.3×10^7	<10
MEF1	3000	2	8.0×10^8	5.4×10^3
	300	3	1.8×10^7	1.3×10^2
	30	3	2.5×10^7	<10
	3	4	4.8×10^7	<10
Saukett	30	3	7.3×10^7	<10
	3	3	1.1×10^8	<10

Similar studies were carried out with the attentuated poliovirus and 10 μg/ml was found to reduce the plaque titers of all types two or more log units.

S-7 resistant and dependent clones. The emergence of S-7 resistant strains after the passage of MEF1 in the presence of these drugs was reported previously (8). There was cross resistance between S-7 and structurally

TABLE III. Tests of S-7-Resistance and S-7-Dependence in Clones Developed in the Presence of S-7.

Concentration of S-7 (μg/ml):		Effect of S-7 on titer[a]	
In cloning culture	In passage culture	10 μg/ml	30 μg/ml
0	0	—1.2, —2.0, —2.2, —4.0, —2.2	
30	0		—0.5, —2.2, —3.8
10	10	—0.8, +2.0, +3.0	
30	30		+0.2, +2.8, +3.2

[a] (log $TCID_{50}$ with S-7) — (log $TCID_{50}$ of control).

related compounds. The earlier study did not make use of cloning techniques, so new lines have been established for further investigation.

A freshly cloned strain of MEFl was plaqued on HeLa cell cultures under overlays containing 0, 10, and 30 μg/ml of S-7. Selected plaques were passaged in HeLa cell cultures with or without S-7 and the progeny titrated with and without drug (Table III).

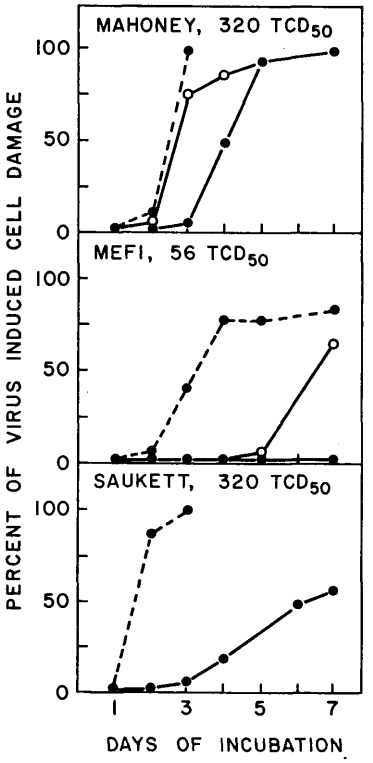

FIG. 1. Inhibition by S-7 of cytopathic effect of virulent polioviruses in monkey kidney cell cultures. ●—●: 100 μg/ml, ○—○: 10 μg/ml, ●- - -●: 0 drug.

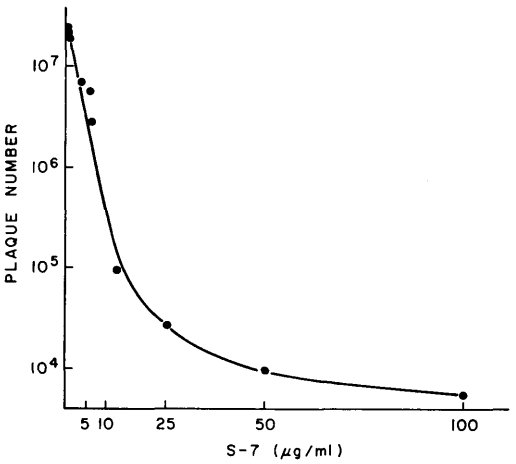

FIG. 2. S-7 dosage curve measured by plaque count. Compound S-7 was contained in overlay agar.

All of five control clones that had not been exposed to S-7 were sensitive when tested with the compound. One of three clones plaqued in the presence of S-7 but passaged in the absence of it was resistant to the compound. Two of six clones plaqued and passaged in the presence of the compound were resistant, and the titer of the other four was depressed in the absence of S-7. Titers of these four clones in the presence of S-7 were 6.2–7.2 logs, the normal range for parental virus without drug.

The resistant strains retained this property on the further passage in the absence of drug, but two of the four drug-dependent strains only became drug-resistant on further passage in a low (10 μg/ml) concentration of drug.

TABLE IV. Lack of Cross-Resistance and Dependence between S-7 and Guanidine and HBB.

Virus clone[a]	Control titer	Titers when grown with drug (log)			
		S-7		Guanidine,	HBB,
		10 μg/ml	30 μg/ml	200 μg/ml	100 μg/ml
O-1	7.0	4.5	4.0	3.5	3.0
R-1	7.0	ND	6.5	3.5	3.25
R-4	6.75	6.0	ND	3.5	3.5
R-5	6.75	ND	7.0	3.5	3.25
D-1	3.75	6.75	ND	1.0	1.5
D-2	5.25	7.25	7.25	1.5	1.5
D-3	3.5	ND	6.25	≤0.5	≤0.5
D-4	3.5	ND	6.75	1.0	≤0.5
G-1	6.5	ND	4.0	6.75	ND
G-2	7.0	ND	4.5	6.75	ND

[a] O = original; R = S-7-resistant; D = S-7-dependent; G = guanidine-resistant.

Lack of cross resistance or dependence between S-7 and guanidine and HBB. The seven modified strains described above were titrated in HeLa cell tubes in the presence of 200 μg/ml of guanidine or 100 μg/ml of HBB. Results were summarized in Table IV. All strains tested were sensitive to guanidine and HBB.

Two guanidine-resistant clones were obtained from plaques developed under overlay containing guanidine. The selected strains were titrated in HeLa cell tubes with S-7. Results are shown in Table IV. The guanidine-resistant strains showed the same sensitivity to S-7 as control strains.

Discussion. The foregoing studies demonstrate a marked specificity in the action of the drug S-7. It is active against both virulent and attenuated strains of all three types of poliovirus but not against any of several ECHO and Coxsackie viruses. It is not toxic to HeLa or MK cells at the same concentration. An earlier study showed no effect of the drug on measles or adenovirus (2). Drug-resistant and drug-dependent strains very like those observed with guanidine (7) and HBB (4) have been observed, but the strains modified with respect to S-7 were not also modified with respect to the other drugs. The modified strains seem to be stable and may prove to be useful genetic markers.

The mechanism of action of S-7 remains to be defined. Earlier studies (1–3) indicate that both the methylthio and the ethylcarboxylate groups are essential for maximum activity.

Summary. Ethyl-2-methylthio-4-methyl-5-pyrimidinecarboxylate (S-7) inhibited the cytopathic effect and multiplication of poliovirus in primary monkey kidney cultures but not that of ECHO 1, 5, 13, 22, 23, Coxsackie A7, A9, B3, or B5. It did not inhibit cell multiplication under these conditions. Passage of poliovirus type 2 in the presence of S-7 gave rise to S-7-resistant and S-7-dependent strains. There was no cross resistance between S-7 and guanidine or HBB.

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