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Inhibitory effect of selected antiviral compounds on measles (SSPE) virus replication in vitro

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Summary

A variety of antiviral compounds were examined for their inhibitory effect on measles (SSPE) virus plaque formation in VERO cells. The following compounds inhibited SSPE virus (strain Niigata-1) replication at concentrations that were significantly lower than their minimum cytotoxic concentrations: neplanocin A, neplanocin C, carbocyclic 3-deazaadenosine, 9-(trans-2′, trans-3′-dihydroxycyclopent-4′-enyl)adenine, 9-(trans-2′,trans-3′-dihydroxycyclopent-4′-enyl)-3-deazaadenine, (RS)-3-adenin-9-yl-2-hydroxypropanoic acid isobutyl ester, carbodine, cyclopentenyl cytosine, 3-deazaguanine, pyrazofurin, ribavirin and 6-azauridine. As the most selective inhibitors of SSPE virus replication emerged pyrazofurin, 3-deazaguanine, 6-azauridine and ribavirin. These compounds were further examined for their relative potency against a number of measles (SSPE) virus strains. Their order of (decreasing) potency was pyrazofurin > 6-azauridine ~ 3-deazaguanine > ribavirin. Amantadine, inosiplex and glycyrrhizin, that were also included in these assays, did not show appreciable activity against any of the measles (SSPE) virus strains.

Measles; SSPE; Pyrazofurin; 3-Deazaguanine; 6-Azauridine; Ribavirin

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Introduction

Subacute sclerosing panencephalitis (SSPE) is a rare but progressive and fatal disease of the central nervous system. It results from a persistent measles (SSPE) virus infection. Since the introduction of the attenuated live measles virus vaccine, the incidence of measles and SSPE has decreased considerably in those countries where vaccination is widely applied (Modlin et al., 1977). Although the frequency of SSPE following measles vaccination is markedly reduced as compared to the frequency of SSPE following natural measles infection, a few cases of SSPE following measles vaccination have been reported (Modlin et al., 1977; Ueda, 1987). Moreover, SSPE is still prevalent in those countries where the children are not routinely vaccinated with the live measles vaccine.

A few compounds, i.e. inosiplex (Jones et al., 1982; Fukuyama et al., 1987) and amantadine (Haslan et al., 1969; Robertson et al., 1980) have been claimed to have a favorable effect, i.e. prolong the life of patients with SSPE, but these compounds are by no means able to cure the disease. In our search for potential anti-SSPE drugs, we examined a wide variety of antiviral compounds for their inhibitory effects on the replication of different SSPE virus strains in vitro.

Materials and Methods

Cells

VERO cells were used throughout all experiments. The growth medium of VERO cells consisted of Eagle's minimum essential medium (EMEM) supplemented with 10% newborn bovine serum, 100 U of penicillin G/ml, and 100 μg of streptomycin/ml. The maintenance medium consisted of EMEM supplemented with 2% fetal calf serum and antibiotics at the same concentrations as in the growth medium.

Viruses

The virus strains used were the measles virus Sugiyama strain and the SSPE virus Niigata-1, Yamagata-1 and Kitaken-1 strains. The Sugiyama strain is widely used as the standard measles strain in Japan. It was kindly provided by Dr. M. Matsumoto (Institute of Medical Science, University of Tokyo, Japan). To prepare cell-free measles virus, virus-infected cells were frozen at −80°C, thawed at 37°C, and then centrifuged at 1,600 × *g* for 10 min. After centrifugation the supernatant was used as virus stock. The three SSPE virus strains were isolated in Japan from patients with SSPE and were reported to be cell-associated (Ohuchi et al., 1979, 1980). To prepare cell-free SSPE virus, virus-infected cells were co-cultivated with uninfected VERO cells, scraped off by glass beads in 5 ml of a solution consisting of 0.0038 M KH_2PO_4, 0.0072 M K_2HPO_4, 0.0049 M L-glutamic acid, 0.128 M saccharose and 1.0% bovine serum albumin in water, and sonicated at 80

W for 15 s (Ultrasonic Disruptor Model UR-200P, Tomy Seiko, Tokyo, Japan). After centrifugation at 1600 × *g* for 10 min, the supernatant containing the cell-free SSPE virus was collected and used as the virus stock.

Antiviral compounds

The test compounds and their sources were as follows: tubercidin [4-aminopyrrolo-(2,3-d)pyrimidine-β-D-ribofuranoside], Upjohn, Fine Chemicals Division, Kalamazoo, MI; 5-chlorotubercidin, Dr. D.E. Bergstrom, University of North Dakota, Grand Folks, ND; C-c^{3}Ado (carbocyclic 3-deazaadenosine), Dr. J.A. Montgomery, Kettering-Meyer Laboratory, Southern Research Institute, Birmingham, AL; neplanocin A and neplanocin C, Dr. J. Murase, Toyo-Jozo Co., Shizuoka-ken, Japan; DHCA [9-(*trans*-2′,*trans*-3′-dihydroxycyclopent-4′-enyl)adenine] and DHCDA [9-(*trans*-2′,*trans*-3′-dihydroxycyclopent-4′-enyl)-3-deazaadenine], Dr. R.T. Borchardt, University of Kansas, Lawrence, KS; (*S*)-DHPA [(*S*)-9-(2,3-dihydroxypropyl)adenine], (*RS*)-AHPA [(*RS*)-3-adenin-9-yl-2-hydroxypropanoic acid]isobutyl ester and (*s*)HPMPA [(*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine], Dr. A. Holý, Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Science, Prague, Czechoslovakia; carbodine [carbocyclic cytidine], Dr. Y.F. Shealy and Dr. J.A. Montgomery, Kettering-Meyer Laboratory, Southern Research Institute, Birmingham, AL; cyclopentenyl cytosine, Dr. J. Murase; 3-deazaguanine [6-aminoimidazo(4,5-C)pyridine-4(5H)-one], Dr. R.K. Robins, Nucleic Acid Research Institute, Costa Mesa, CA; pyrazofurin [3-(β-D-ribofuranosyl)-4-hydroxypyrazole-5-carboxamide], Calbiochem Behring, Lucerne, Switzerland; ribavirin [1-(β-D-ribofuranosyl)-1,2,4-triazole-3-carboxamide], ICN Nutritional Biochemicals, Cleveland, OH; 3′-C-methyluridine, Dr. W. Pfleiderer, Universität Konstanz, F.R.G.; 3-deazauridine, Sigma, St. Louis, MO; 5′-deoxy-5-fluorouridine, Hoffman-La Roche, Nutley, NJ; 6-azauridine, Serva, Feinbiochemica, Heidelberg, F.R.G.; inosiplex [1:3 molar complex of inosine with demethylamino isopropanol-p-acetamido benzoate], Mochida Pharmaceutical, Tokyo, Japan; amantadine (1-adamantanamine hydrochloride), Ciba-Geigy Japan, Hyogo-ken, Japan; glycyrrhizin (1:2 molar complex of glycyrrhetinic acid with glucuronic acid), Minophagen Pharmaceutical, Tokyo, Japan.

Antiviral assay

VERO cells were seeded in 1.6 cm-diameter wells of 24-well tissue culture plates (Falcon 3047: Becton Dickinson, Oxnard, CA) at 1 × 10^5 cells per well and incubated at 35°C. After 2 days of incubation, when the cells were grown to confluency, the growth medium was withdrawn and the cell monolayers were washed once with EMEM. To each well were added 20–40 plaque forming units (PFU) in 0.2 ml maintenance medium. After a 2-h virus adsorption period at 35°C, cell cultures were washed twice with EMEM, and serial (1/5, 1/25, 1/125, ...) dilutions of compounds prepared in maintenance medium with 0.6% methylcellulose were

added to the cell cultures. The cells were then incubated for 5 days at 35°C in a CO_2 incubator, and after staining with neutral red, virus plaques were counted. The concentration of compound required to inhibit the virus plaque number by 50% was estimated as the 50% effective dose (ED_{50}).

Cytotoxicity assay

In the first preliminary experiment, we examined the cytotoxicity of the nucleoside analogues based on morphological alteration of the cell cultures. Monolayers of VERO cells in 24-well trays were exposed to various concentrations of the compounds in 1 ml maintenance medium and incubated in a CO_2 incubator at 35°C. After 5 days of incubation, morphological alteration of VERO cells was detected by microscopic examination. The minimal concentration required to alter normal cell morphology was estimated as the minimum cytotoxic dose (MCD).

As a marker of cell viability, trypan blue exclusion was examined. Monolayers of VERO cells in 24-well trays were washed once with EMEM, exposed to various concentrations of the compounds in 1 ml maintenance medium and incubated at 35°C in a CO_2 incubator for 5 days. After this incubation period, the cells floating in medium together with the cells dispersed from the monolayer with 0.05% trypsin were harvested and the viability of the cells was determined by trypan blue exclusion. The concentration of compound that reduced the viability of the VERO cells by 50% was estimated as the 50% cytotoxic dose (CD_{50}).

Cytotoxicity assays were also based upon the inhibition of deoxythymidine (dThd), uridine (Urd) or leucine (Leu) incorporation into VERO cells. Monolayers of VERO cells, incubated for 48 h in a CO_2 incubator at 35°C after being seeded in 24-well tissue culture plates, were in a resting state when incubated with maintenance medium containing 1 μCi each of [6-^{3}H]dThd, [5-^{3}H]Urd of L-[4,5-^{3}H]Leu in a CO_2 incubator at 35°C (leucine-free maintenance medium was used for the assessment of L-[4,5-^{3}H]Leu incorporation). To examine the inhibitory effects of the compounds on the incorporation of the radiolabeled precursors, serial five-fold step dilutions of the compounds were added to the cells (2 wells per dilution). After an 18-h incubation period, cells were washed three times with PBS (phosphate-buffered saline) and removed from the plates by a rubber policeman. The cells were then precipitated on glass fiber filter papers (GC-90, 26-mm diameter, Toyo Roshi, Tokyo, Japan) by using 5% trichloroacetic acid (TCA) (for the assessment of Leu incorporation, PBS was used instead of 5% TCA), followed by three washings with 95% ethanol. The filter papers were dried, and the radioactivity retained on the filter paper was analyzed in a liquid scintillation counter. The concentration of compound that inhibited the incorporation of either [6-^{3}H]dThd or [5-^{3}H]Urd into the acid-insoluble fraction of VERO cells, or L-[4,5-^{3}H]Leu into VERO cells, by 50% was estimated as the 50% inhibitory dose (ID_{50}).

Results

Comparative potency of various nucleoside analogues as inhibitors of the replication of SSPE virus (strain Niigata-1)

The antiviral activity of the nucleoside analogues was assessed based on their inhibitory effect on plaque formation by the Niigata-1 strain of SSPE virus. The ED_{50} values of the compounds required for inhibition of SSPE virus replication are shown in Table 1. In these preliminary experiments, toxicity of the compounds for VERO cells was examined by microscopic examination of cell morphology. Twelve compounds, i.e. neplanocin A, neplanocin C, C-c^{3}Ado, DHCA, DHCDA, (RS)-AHPA, carbodine, cyclopentenyl cytosine, 3-deazaguanine, pyrazofurin, ribavirin and 6-azauridine, inhibited SSPE virus replication at a concentration that was lower than the minimum cytotoxic concentration (altering normal cell mor-

TABLE 1

Comparative potency of several nucleoside analogues as inhibitors of SSPE virus (strain Niigata-1)

Compound	ED_{50} (μg/ml)[a]	MCD (μg/ml)[b]	SI[c]
Adenosine analogues			
Tubercidin	>0.64	0.64	<1
5-Chlorotubercidin	>1.6	1.6	<1
Neplanocin A	6.5	8.0	1.2
Neplanocin C	0.84	8.0	9.5
C-c^{3}Ado	2.2	16	7.3
DHCA	8.5	100	12
DHCDA	24	200	8.3
(S)-DHPA	>200	>200	><1
(RS)-AHPA	62	400	6.5
(S)-HPMPA	>200	200	<1
Cytidine analogues			
Carbodine	0.95	1.6	1.7
Cyclopentenyl cytosine	0.049	0.32	6.5
Guanosine analogues			
3-Deazaguanine	0.7	40	57
Pyrazofurin	0.11	25	230
Ribavirin	8.5	>250	>29
Uridine analogues			
3′-C-Methyluridine	>500	500	<1
3-Deazauridine	>500	>500	><1
5′-Deoxy-5-fluorouridine	>500	500	<1
6-Azauridine	0.84	40	48

[a]Fifty percent effective dose, or concentration required to inhibit virus plaque formation by 50%.
[b]Minimum cytotoxic dose, or minimal concentration required to alter normal cell morphology.
[c]Selectivity index: ratio of MCD to ED_{50}.

TABLE 2

Potency of selected compounds as inhibitors of different measles (SSPE) virus strains[a]

Compounds	ED_{50}[b] (μg/ml) for SSPE virus				ED_{50}[b] (μg/ml) for measles virus
	Strain Niigata-1	Strain Yamagata-1	Strain Kitaken-1	Average value for SSPE virus strains	Strain Sugiyama
Pyrazofurin	0.11[a] (0.08–0.14)	0.12 (0.07–0.16)	0.16 (0.09–0.20)	0.13	0.14 (0.11–0.20)
6-Azauridine	0.84 (0.58–1.1)	0.74 (0.62–0.90)	0.78 (0.50–1.1)	0.78	0.66 (0.48–0.80)
3-Deazaguanine	0.70 (0.58–0.82)	1.18 (0.65–1.70)	0.83 (0.80–0.90)	0.90	1.23 (0.90–1.70)
Ribavirin	8.5 (6.5–10.4)	8.7 (6.5–12.5)	6.8 (3.5–12.0)	8.0	7.4 (5.4–10.0)
Amantadine	85 (80–90)	90 (80–100)	130 (90–200)	100	100 (90–150)
Glycyrrhizin	760 (660–850)	ND	680 (450–900)	720	1000 (900–1100)
Inosiplex	550 (250–800)	890 (460–1400)	990 (720–1250)	810	670 (540–800)

[a]Mean value for 2 to 4 independent experiments (range of individual values indicated in parentheses).
[b]See footnote to Table 1.
ND, Not determined.

phology), and four compounds, i.e. 3-deazaguanine, pyrazofurin, ribavirin and 6-azauridine, inhibited virus replication at a concentration that was significantly (more than 25-fold) lower than the minimum cytotoxic concentration.

Inhibitory effects of 3-deazaguanine, pyrazofurin, ribavirin, 6-azauridine, inosiplex, amantadine and glycyrrhizin on the replication of several measles (SSPE) virus strains

The four nucleoside analogues (3-deazaguanine, pyrazofurin, ribavirin and 6-azauridine) that had emerged as the most selective inhibitors of SSPE virus replication from the preliminary experiments described above and three other compounds which have been claimed to prolong the life span of patients with SSPE (inosiplex, amantadine) or are known to interfere with the replication of several enveloped viruses in vitro (glycyrrhizin) were further examined for their inhibitory effects on four different measles (SSPE) virus strains. The ED_{50} values of these compounds for the measles/SSPE virus strains are presented in Table 2.

The most potent inhibitor of SSPE virus replication was pyrazofurin (average ED_{50} for the three SSPE virus strains: 0.13 μg/ml), followed by 6-azauridine, 3-deazaguanine and ribavirin (average ED_{50} values: 0.78, 0.90, 8.0 μg/ml, respectively). Amantadine, glycyrrhizin and inosiplex showed an average ED_{50} of 100, 720 and 810 μg/ml, respectively. No marked differences were noted in the susceptibility of the different measles (SSPE) virus strains to the seven compounds (Table 2).

Selectivity of pyrazofurin, 3-deazaguanine, 6-azauridine, ribavirin, amantadine, inosiplex and glycyrrhizin as inhibitors of SSPE virus

The cytotoxicity of the seven aforementioned compounds for VERO cells was determined by the following measurements: (i) trypan blue exclusion, (ii) [6-^{3}H]dThd incorporation into host cell DNA; (iii) [5-^{3}H]Urd incorporation into host cell RNA, (iv) inhibition of [4,5-^{3}H]Leu incorporation into host cell protein.

As shown in Table 3, pyrazofurin reduced cell viability at a concentration of 80μg/ml and inhibited the host cell protein synthesis at a concentration of 75μg/ml; however, pyrazofurin did not inhibit host cell DNA or RNA synthesis at concentrations up to 200 μg/ml. Neither 3-deazaguanine, 6-azauridine nor ribavirin affected the viability of Vero cells at a concentration of 200 μg/ml. 3-Deazaguanine inhibited host cell DNA and protein synthesis at a concentration of 150 μg/ml, whereas 6-azauridine inhibited host cell RNA and protein synthesis at a concentration of 120 and 56 μg/ml, respectively. Ribavirin inhibited host cell DNA and RNA synthesis at a concentration of 37 and 105 μg/ml, respectively, but did not inhibit protein synthesis at a concentration of 200 μg/ml. Inosiplex and glycyrrhizin did not reduce cell viability at concentrations up to 5 mg/ml, although they altered normal cell morphology (as detectable by microscopic examination) at 5 mg/ml. Amantadine reduced cell viability and inhibited cellular DNA and RNA synthesis at a concentration of 700, 500 and 400 μg/ml, respectively.

TABLE 3

Selectivity indexes of selected compounds as inhibitors of SSPE virus

Compound	Minimum antiviral concentration (μg/ml)	Minimum cytotoxic concentration (μg/ml)				Selectivity index			
	ED_{50}[a] (A)	CD_{50}[b] (B)	ID_{50}[c]			B/A	C/A	D/A	E/A
			dThd (C)	Urd (D)	Leu (E)				
Pyrazofurin	0.13	80	>200	>200	75	620	>1500	>1500	580
3-Deazaguanine	0.90	>200	150	>200	150	>220	170	>220	170
6-Azauridine	0.78	>200	>200	120	56	>260	>260	150	72
Ribavirin	8.0	>200	37[d]	105	>200	>25	4.6	13	>25
Inosiplex	810	>5000	>5000	>5000	ND	>6.2	>6.2	>6.2	–
Amantadine	100	700	500	400	ND	6.7	4.8	3.8	–
Glycyrrhizin	720	>5000	ND	ND	ND	>6.9	–	–	–

[a]Average value for three SSPE virus strains (data taken from Table 2).

[b] Fifty percent cytotoxic dose, or concentration required to reduce cell viability by 50%, as measured by trypan blue exclusion.

[c]Fifty percent inhibitory dose, or concentration required to inhibit [6-^{3}H]dThd incorporation into host cell DNA, [5-^{3}H]Urd incorporation into host cell RNA or L-[4,5-^{3}H]Leu incorporation into host cell protein by 50%.

[d]For ribavirin, ID_{50} determinations based on inhibition of [6-^{3}H]dThd incorporation may not provide an accurate estimation of the cytotoxicity of the drug, since ribavirin is known to interfere with the salvage pathway of dThd (Drach et al., 1981).

ND, not determined.

Selectivity indexes (SI) were calculated based on the ratio of CD_{50} for the host cells (cell viability determined by trypan blue exclusion) or ID_{50} for host cell DNA, RNA and protein synthesis to the average ED_{50} for the three SSPE virus strains. Pyrazofurin showed the highest SI as inhibitor of SSPE virus replication, followed by 3-deazaguanine, 6-azauridine and ribavirin. Inosiplex, amantadine and glycyrrhizin could not be considered as effective inhibitors of SSPE virus replication.

Discussion

In our search for effective agents against SSPE virus, we examined a broad variety of nucleoside analogues for their inhibitory effects on SSPE virus replication in VERO cell cultures. Twelve out of the nineteen nucleoside analogues that were evaluated inhibited SSPE virus replication at a lower concentration than that showing toxicity to the host cells. Particularly, pyrazofurin, 3-deazaguanine, 6-azauridine and ribavirin inhibited SSPE virus replication at concentrations that were significantly lower than the cytotoxic concentrations. The order of (decreasing) potency of these compounds was pyrazofurin > 6-azauridine ~ 3-deazaguanine > ribavirin. All four compounds have been previously shown to inhibit measles virus replication, i.e. ribavirin (Kirsi et al., 1984; Descamps and De Clercq, 1978), 3-deazaguanine (Kirsi et al., 1984), pyrazofurin (Descamps and De Clercq, 1978) and 6-azauridine (ter Meulen et al., 1972). Ribavirin (Huffman et al., 1973) and 6-azauridine (ter Meulen et al., 1972) have also been reported to be inhibitory to SSPE virus in vitro.

When we examined the cytotoxicity of the nucleoside analogues by 5 different methods (i.e. morphological alteration, trypan blue exclusion, inhibition of RNA, DNA and protein synthesis) pyrazofurin, 3-deazaguanine and 6-azauridine altered normal cell morphology at concentrations that were lower than the cytotoxic concentrations based upon trypan blue exclusion test or inhibition of DNA or RNA synthesis. They inhibited protein synthesis at concentrations that were comparable to the cytotoxic concentrations based upon morphological alteration. However, ribavirin did not alter normal cell morphology or inhibit protein synthesis at a concentration of 200 μg/ml which was higher than the concentrations of ribavirin required for inhibition of DNA or RNA synthesis. In this context it should be pointed out that in ribavirin-treated cells inhibition of [^{3}H]dThd incorporation reflects a decrease of the [^{3}H]dTTP pool levels rather than an inhibition of DNA synthesis per se (Drach et al., 1981).

Of all compounds that were evaluated for their activity against SSPE virus, pyrazofurin had the highest potency and selectivity index. Upon systemic administration in vivo pyrazofurin is more toxic (50% lethal dose for mice: approximately 5 mg/kg) than could be expected from its behavior in vitro (Descamps and De Clercq, 1978). Although pyrazofurin has been the subject of phase I clinical trials (Ohnuma et al., 1977; Cadman et al., 1978), it has not been thoroughly pursued from an antiviral chemotherapy viewpoint. If it were shown to be too toxic for systemic use in humans, pyrazofurin may well be appropriate for topical use (i.e. as an aer-

osol) in the treatment of respiratory tract virus infections and for intrathecal administration in the treatment of central nervous system (CNS) virus infections (i.e. SSPE). However, we have been unable to trace any data or references on pyrazofurin crossing the blood-brain barrier. Also, 3-deazaguanine and 6-azauridine, being inhibitory to SSPE virus replication at relatively low concentrations, offer potential as candidate antiviral drugs. 3-Deazaguanine is known not to penetrate adequately into the brain upon systemic administration but to alter the course of herpes encephalitis in mice following intracranial administration (Allen et al., 1977).

From the data shown in Table 3, ribavirin seems to be inferior to pyrazofurin, 3-deazaguanine and 6-azauridine, but far superior to inosiplex, amantadine and glycyrrhizin, as a potential anti-SSPE drug. Ribavirin has been recently assessed for its therapeutic potential in the treatment of infections due to respiratory syncytial virus (Taber et al., 1983; Hall et al., 1983; McIntosh et al., 1984), influenza virus (McClung et al., 1983; Wilson et al., 1984) and Lassa fever virus (Gilbert et al., 1986; McCormick et al., 1984). The systemic use of ribavirin in the treatment of CNS virus infections such as SSPE is compounded by its difficulty in passing the blood-brain barrier (Sidwell et al., 1988).

Inosiplex and amantadine which have both been reported to affect in a favorable manner the course of SSPE were inhibitory to SSPE virus only at a concentration of 810 and 100 μg/ml, respectively. These concentrations are clearly higher than the drug levels that could ever be achieved in the plasma or CNS upon systemic administration of these drugs. It may be inferred therefore that if any efficacy were to be shown with these drugs against SSPE, such benefit must result from an indirect action of the drugs.

Glycyrrhizin, which has been shown to inhibit the replication of varicella-zoster virus (Baba et al., 1987) and human immunodeficiency virus (Ito et al., 1987), proved active against SSPE virus only at a concentration (720μg/ml) that may not be readily attainable in vivo.

In conclusion, our present findings indicate that of all the compounds that were evaluated for their anti-SSPE virus activity, pyrazofurin, 3-deazaguanine, 6-azauridine and ribavirin show the greatest promise as candidate drugs for the treatment of SSPE and should be further pursued for this purpose.

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