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Selective inhibition of human cytomegalovirus replication by naphthalenedisulfonic acid derivatives

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Summary

Several naphthalenedisulfonic acid derivatives were found to be selective inhibitors of human cytomegalovirus (CMV) replication in MRC-5 cells. Among the test compounds, the bis-naphthalenedisulfonic acid derivative having an hexamethylene spacer emerged as the most potent inhibitor of CMV replication. Its 50% antivirally effective concentration (EC_{50}) for AD-169 strain was 12 μM, whereas the compound did not affect the growth of mock-infected MRC-5 cells at concentrations up to 500 μM. The naphthalenedisulfonic acid derivatives were also inhibitory to CMV clinical isolates. Virus yield reduction assay revealed that the compounds significantly reduced virus growth in CMV-infected MRC-5 cells. The bis-naphthalenedisulfonic acid derivatives with an hexamethylene spacer suppressed the expression of CMV-induced immediate early, and early antigens at a concentration of 20 μM, whereas the anti-CMV nucleoside ganciclovir did not do so even at the concentration that was 10-fold higher than its EC_{50} for CMV-induced plaque formation. Furthermore, naphthalenedisulfonic acid derivatives had to be present at the time of virus infection to exert their anti-CMV activity. These results suggest that the compounds are targeted at an early event in the virus replicative cycle, presumably, virus adsorption.

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Introduction

Human cytomegalovirus (CMV) is one of the most important pathogens in immunocompromized hosts such as organ transplant recipients and patients with the acquired immune deficiency syndrome (AIDS) (Macher et al., 1983). Antiviral agents acyclovir (ACV), bromovinyldeoxyuridine (BVDU), and bromovinylarabinofuranosyluracil (BVaraU) have already been used in the treatment of patients with herpes simplex virus (HSV) and varicella-zoster virus (VZV) infections (Straus et al., 1984; Tricot et al., 1986; Hiraoka et al., 1991). These compounds need to be phosphorylated by the virus-encoded thymidine kinase to exert their antiviral activity (De Clercq, 1984). Since human CMV does not encode such a virus-specific enzyme, ACV, BVDU and BVaraU show little, if any, inhibition of the replication of CMV (Shigeta et al., 1991). The only agents that have currently been approved for the treatment of CMV infections in vivo are 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG, ganciclovir) and phosphonoformic acid (PFA, foscarnet). However, prolonged use of ganciclovir is often associated with serious side effects such as anemia and neutropenia (Collaborative DHPG Treatment Study Group, 1986; Laskin et al., 1987; Hecht et al., 1988). It seems, therefore, still imperative to find novel chemotherapeutic agents active against CMV replication, preferably, through a different mechanism of action.

We have recently reported that several naphthalenedisulfonic acids derivatives are potent and selective inhibitors of HIV-1 replication in vitro (Mohan et al., 1991a,b,c). In this study, we have examined these compounds for their inhibitory effects on CMV replication in cell culture and found that they are also selective inhibitors of CMV.

Materials and Methods

Compounds

4-Acetylamino-5-hydroxy-2,7-naphthalenedisulfonic acid (**1**), 4-palmitoylamino-5-hydroxy-2,7-naphthalenedisulfonic acid (**2**), 4,4′-[1,6-hexanediylbis(carbonylamino)]bis(5-hydroxy-2,7-naphthalenedisulfonic acid) (**3**), 4,4′-[1,8-octanediylbis(carbonylamino)]bis(5-hydroxy-2,7-naphthalenedisulfonic acid) (**4**), 4,4′-[1,10-decanediylbis(carbonylamino)]bis(5-hydroxy-2,7-naphthalenedisulfonic acid) (**5**), 3,3′-[1,10-decanediylbis(carbonylamino)]bis(1,5-naphthalenedisulfonic acid) (**6**), 4,4′-[4,4′-biphenyldiylbis(sulfonylamino)]bis(5-hydroxy-2,7-naphthalenedisulfonic acid) (**7**), and 3,3′-[4,4′-biphenyldiylbis(sulfonylamino)]bis(1,5-naphthalenedisulfonic acid) (**8**) (Fig. 1) were

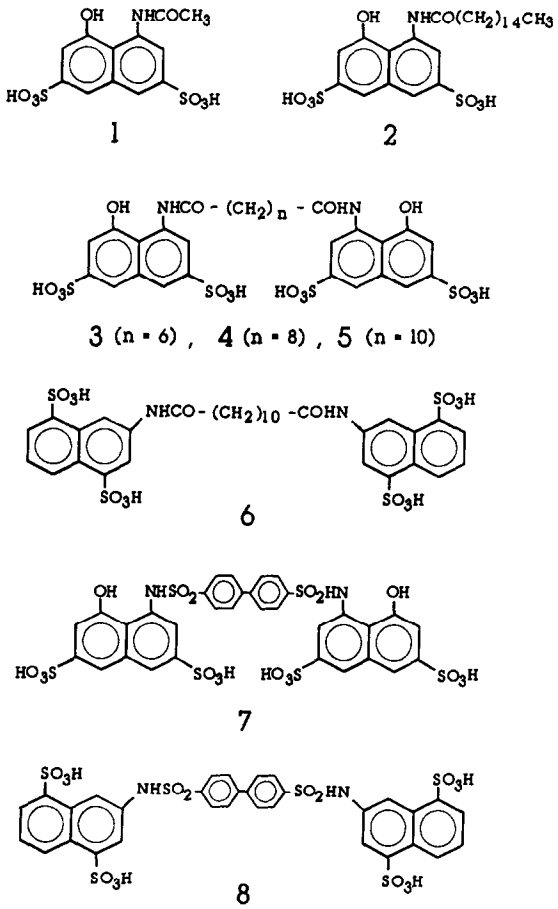

Fig. 1. Structures of naphthalenedisulfonic acid derivatives.

prepared according to the procedures, as previously described (Mohan et al., 1991a,c). Suramin was obtained from Bayer AG (Wuppertal, Germany). Ganciclovir was provided by Nippon Synthex (Tokyo, Japan).

Cells

Human diploid embryonic lung fibroblast (MRC-5) cells were used in anti-CMV assays. The cells were grown in Eagle's minimal essential medium supplemented with 10% fetal calf serum (FCS) (Cell Culture Laboratories, Cleveland, OH), 100 units/ml penicillin G, ad 100 μg/ml streptomycin. The cells were maintained in the medium containing 2% FCS and antibiotics (maintenance medium).

Viruses

All CMV strains, except for AD-169, were isolated in Fukushima Medical

College from either urine, blood, or throat swabs of bone marrow transplant recipients. AD-169, a laboratory strain of CMV, was provided by Syva (Palo Alto, CA). To obtain cell-free virus, virus-infected cells were dislodged by glass beads in 35% sorbitol and disrupted by sonication for 60 s. Cells were then centrifuged at 500 × *g* for 10 min. The supernatant was collected and stored at −80°C until use.

Antiviral assay

Inhibitory effects of the test compounds on CMV replication were monitored by the inhibition of virus-induced plaque formation in MRC-5 cells, as previously described (Shigeta et al., 1991). In all experiments, a centrifugal culture technique was used for infection of MRC-5 cells with CMV (Gleaves et al., 1985). Briefly, MRC-5 cells were seeded in 24-well tissue culture plates at 10^5 cells per well and incubated at 37°C. When confluent, the cells were infected with 50 plaque forming units (PFU) of virus per well. The test compounds were added at the time of virus inoculation. The plates were then centrifuged at 700 × *g* for 40 min at room temperature and washed once with maintenance medium. The cells were overlaid with maintenance medium containing appropriate concentrations of the test compounds and 0.6% methylcellulose. After a 7-day incubation at 37°C, the cells were fixed with 5% formalin and stained with 0.02% crystal violet. The number of CMV plaques was determined microscopically.

Cytotoxicity assay

Cytotoxicity measurements were based on the inhibition of cell growth. MRC-5 cells were seeded at 5 × 10^3 cells per well into 96-well microtiter plates and allowed to proliferate at 37°C for 24 h. The medium was then replaced by maintenance medium containing various concentrations of the test compounds. After a 5-day incubation at 37°C, the number of viable cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method, as previously described (Pauwels et al., 1988).

Virus yield assay

MRC-5 cells in 24-well tissue culture plates were infected with 50 PFU of virus per well in the presence of test compounds. The concentration of compounds used in this assay was twice the EC_{50} of each compound for CMV-induced plaque formation. The plates were then centrifuged at 700 × *g* for 40 min at room temperature and washed once with maintenance medium. The cells were incubated at 37°C in the presence of test compounds. On day 5, after virus infection, culture medium was replaced with fresh medium containing the same concentration of test compounds. On day 3, 6 and 9, the cells were harvested, washed three times with maintenance medium, and disrupted by sonication for 60 s. The disrupted cells were centrifuged at 500 × *g* for 10 min, and the supernatant was assayed for its infectivity in MRC-5 cells.

Assay for CMV antigen expression

Inhibitory effects of the compounds on the expression of CMV-induced immediate early antigen (IEA) and early antigen (EA) were evaluated by indirect immunofluorescence using the MicroTrak® CMV culture identification test kit (Syva, Palo Alto, CA). Briefly, MRC-5 cells were seeded in shell vials with 12-mm coverslips and infected with CMV in the presence of test compounds. Except for ganciclovir, the concentration of compounds used in this assay was twice the EC_{50} of each compound for CMV-induced plaque formation. The concentration of ganciclovir was 10-fold higher than its EC_{50}. The shell vials were then centrifuged at 700 × *g* for 40 min at room temperature and incubated at 37°C in the presence of test compounds. At 24 and 48 h after virus infection, the cells were washed twice with phosphate-buffered saline and fixed with acetone. The samples were then subjected to indirect immunofluorescent staining with monoclonal antibodies specific for CMV-induced IEA and EA. The number of antigen-positive cells was determined microscopically.

Results

When the naphthalenedisulfonic acid derivatives were evaluated for their inhibitory effects on the plaque formation of CMV (AD-169 strain) in MRC-5 cells, compounds **2**, **3**, **4**, **7**, and **8** showed selective inhibition of CMV replication with 50% antivirally effective concentrations (EC_{50}) ranging from 12 to 43 μM (Table 1). These compounds, except for compound **2**, did not affect the growth of mock-infected MRC-5 cells at concentrations up to 500 μM, thus, their 50% cytotoxic concentrations (CC_{50}) were > 500 μM (Table 1). Among the compounds, the bis-naphthalenedisulfonic acid having a

TABLE 1

Inhibitory effects of naphthalenedisulfonic acid derivatives on the replication of CMV (AD-169 strain) in MRC-5 cells

Compound	EC_{50}[a] (μM)	CC_{50}[b] (μM)	SI[c]
1	> 100	> 500	–
2	33 ± 9	98 ± 2	3
3	12 ± 2	> 500	> 41
4	43 ± 4	> 500	> 11
5	> 100	> 500	–
6	> 100	191 ± 5	–
7	33 ± 3	> 500	> 15
8	42 ± 3	> 500	> 11
Suramin	21 ± 1	> 100	> 4

[a]50% Effective concentration, required to reduce the number of plaques by 50% of the virus-infected control.

[b]50% Cytotoxic concentration, required to reduce cell growth by 50% of the mock-infected control.

[c]Selectivity index (ratio of CC_{50} to EC_{50}).

All data represent mean values (± S.D. or range) for at least two separate experiments.

TABLE 2

Inhibitory effects of naphthalenedisulfonic acid derivatives on the replication of clinical isolates of CMV in MRC-5 cells

Compound	EC_{50}[a] (μM)		
	T-156	T-1082	T-1095
2	N.D.[b]	12	N.D.
3	16	10	25
4	48	18	65
7	N.D.	25	N.D.
8	N.D.	40	N.D.
Suramin	18	25	25

[a]50% Effective concentration, required to reduce the number of plaques by 50% of the virus-infected control.

[b]Not determined.

Data from a representative experiment are shown.

hexamethylene spacer (compound **3**) was the most potent inhibitor (EC_{50}: 12 μM). Its selectivity index (SI) based on the ratio of CC_{50} to EC_{50} was > 41 (Table 1). The reference compound suramin proved also inhibitory to CMV replication. Interestingly, the palmitoylamino derivative (compound **2**) but not the acetylamino derivative (compound **1**) of naphthalenedisulfonic acids was found to be a selective inhibitor of CMV replication (Table 1).

In the next experiment, the active compounds were further evaluated for their inhibitory effects on the replication of clinical isolates of CMV (T-156, T-1082, and T-1095). As shown in Table 2, these compounds were also effective in inhibiting the replication of these strains. Again, compound **3** was found to be the most potent inhibitor. Its EC_{50} for the clinical isolates ranged from 10 to 25 μM (Table 2).

When compounds **3** and **4** were examined for their inhibitory effects on the growth of CMV (AD-169 strain) in MRC-5 cells, virus titer of the control culture reached 8.3×10^6 PFU per ml on day 9 after virus infection, whereas compounds **3** and **4** achieved 10 and 69-fold reduction of the virus titer at a concentration of 20 and 80 μM, respectively, (Table 3). These concentrations were only twice as much as the EC_{50} of each compound for CMV-induced plaque formation (Table 1). Suramin was more inhibitory to the virus growth. It reduced the virus titer 2500-fold at a concentration of 40 μM. The inhibitory effects of the compounds on the virus growth were also observed for a clinical isolate T-1082, yet the inhibition was less pronounced. On day 9, both compounds **3** and **4** achieved approx. 5-fold reduction of the virus titer at a concentration of 20 and 36 μM, respectively, (Table 3). In the presence of suramin (50 μM), the titer of T-1082 strain was 25-fold lower than that in the control culture.

When compound **3** and suramin were evaluated for their inhibitory effects on the expression of CMV-specific IEA and EA by indirect immunofluorescence at 24 and 48 h post-infection, both compounds suppressed the expression

TABLE 3

Inhibitory effects of naphthalenedisulfonic acid derivatives on the growth of CMV in MRC-5 cells

Strain	Compound[a]	Virus titer[b] (PFU/ml)			
		day 0	day 3	day 6	day 9
AD-169	Control	< 10	2.2×10^2	5.3×10^4	8.3×10^6
	3		1.2×10	7.8×10^3	8.1×10^5
	4		1.2×10	2.0×10^3	1.2×10^5
	Suramin		< 10	1.4×10^3	3.3×10^3
T-1082	Control	< 10	1.5×10^2	1.2×10^3	9.5×10^3
	3		1.1×10^2	1.1×10^3	2.0×10^3
	4		6.2×10	7.5×10^2	1.8×10^3
	Suramin		1.2×10	4.3×10^2	3.8×10^2

[a]The concentration of compounds used in this assay was twice the EC_{50} for CMV-induced plaque formation (see Tables 1 and 2).

[b]Supernatant of the cell suspension was assayed for their infectivity in MRC-5 cells.

Data from a representative experiment are shown.

of IEA and EA at a concentration of 20 and 40 μM ($2 \times EC_{50}$), respectively, (Fig. 2). In contrast, ganciclovir did not inhibit the antigen expression even at a concentration of 20 μM ($10 \times EC_{50}$) (Fig. 2).

To gain further insight into the mechanism of action, we examined the anti-CMV activity of the compounds by different treatment regimens. As shown in Table 4, compounds **3**, **4** and suramin had to be present at the time of virus infection (treatment A) to exert their inhibitory effect on CMV-induced plaque

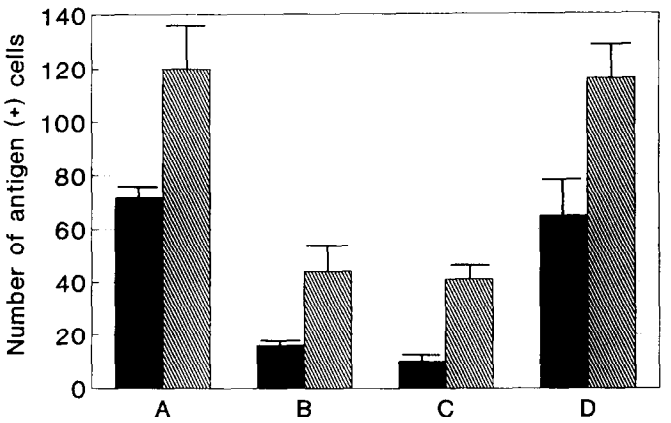

Fig. 2. Inhibitory effects of compound **3** and suramin on the expression of CMV-specific IEA and EA in MRC-5 cells. MRC-5 cells were infected with CMV (AD-169 strain) in the absence (A) or presence of compound **3** (B), suramin (C), and ganciclovir (D). Except for ganciclovir, the concentration of compounds used in this assay was twice the EC_{50} for CMV-induced plaque formation (see Table 1). For ganciclovir, the concentration was 10-fold higher than its EC_{50} (20 μM). At 24 h (■) and 48 h (▨) after virus infection, the cells were subjected to indirect immunofluorescent staining with monoclonal antibodies specific for CMV-induced IEA and EA.

TABLE 4

Inhibitory effects of naphthalenedisulfonic acid derivatives on CMV-induced plaque formation following different treatment regimens

Compound[a]	Number of plaques	
	Treatment A[b]	Treatment B[b]
Control	80 ± 3	–
3	23 ± 6	78 ± 8
4	26 ± 7	75 ± 3
Suramin	43 ± 7	80 ± 2
Ganciclovir	34 ± 4	38 ± 1

[a]AD-169 strain was used in this experiment. The concentration of compounds used in this assay was twice the EC_{50} for CMV-induced plaque formation (see Table 1).
[b]The compounds were present during virus adsorption and thereafter (treatment A) or only after virus adsorption (treatment B).
All data represent mean values (± S.D.) for quadruplicated experiments.

formation. However, ganciclovir was still effective in inhibiting CMV replication, when added after virus adsorption (treatment B). There results suggest that the naphthalenedisulfonic acid derivatives act at the stage of virus adsorption.

Discussion

In the search for effective chemotherapeutic agents against CMV infections, several nucleoside analogues have been shown to selectively inhibit the replication of CMV in cell culture, which include ganciclovir, carbocyclic OXT-G, (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine (HPMPA) 9-(2-deoxy-2-hydroxymethyl-β-D-erythro-oxetanosyl)guanine (OXT-G), and (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (HPMPC) (De Clercq et al., 1987; Nishiyama et al., 1988; Snoeck et al., 1988; De Clercq et al., 1991). These nucleoside analogues do not prevent IEA expression in CMV-infected cells, and their mode of action can be attributed to selective inhibition of viral DNA synthesis (Nishiyama et al., 1988; Neyts et al., 1990).

Sulfated polymers such as dextran sulfate, pentosan polysulfate, carrageenans, and polyvinylalcohol sulfate have proved to be highly potent and selective inhibitors of several enveloped viruses, including CMV, HSV and human immunodeficiency virus type 1 (HIV-1) (Baba et al., 1988a, 1990; Schols, et al., 1990). They are targeted at the virus adsorption to cell membrane (Baba et al., 1988b; Neyts et al., 1992b). Furthermore, anti-CMV activities of poly(hydroxy)carboxylate and mannose-specific plant lectins have recently been reported (Neyts et al., 1992a; Balzarini et al., 1991). In this study, we have demonstrated that naphthalenedisulfonic acids, another class of anionic substances, are also inhibitory to the replication of CMV at their non-toxic concentrations to the host cells.

Studies on the structure-activity relationship of the compounds revealed that replacement of the hexamethylene spacer of compound **3** by octamethylene spacer (compound **4**) or decamethylene spacer (compound **5**) reduced or even annihilated the potency of compound **3** against CMV (Table 1). These results suggest that an appropriate distance between two naphthalenedisulfonic moieties is necessary to retain anti-CMV activity. On the other hand, the 'bis' structure of naphthalenedisulfonic acids is not always required for the antiviral activity, since compound **2** was also inhibitory to CMV replication.

Previous studies using a flow cytometric method have proved that naphthalenedisulfonic acids and suramin block the binding of HIV-1 to the host cells (Schols et al., 1989; Baba et al., 1992). In fact, all naphthalenedisulfonic acids examined in this study have been shown to inhibit the replication of HIV-1 in cell cultures (Mohan et al., 1991a,b,c). Neyts and his coworkers have recently demonstrated that sulfated polymers block the binding of CMV to the host cells and suppress the expression of CMV-induced IEA (Neyts et al., 1992b). They have postulated that the sulfated polymers interact with the viral envelope site involved in virion attachment to heparan sulfate on the cell surface. Like sulfated polymers, the naphthalenedisulfonic acid derivatives were also inhibitory to the expression of IEA and EA (Fig. 2), suggesting that their mode of action is similar to that of sulfated polymers. In fact, the naphthalenedisulfonic acid derivatives have to be present at the time of virus infection to exert their anti-CMV activity (Table 4). Further studies are needed to elucidate their precise mechanism of action.

In conclusion, certain naphthalenedisulfonic acid derivatives are selective inhibitors of CMV replication in vitro. They may block an early event in the virus replicative cycle. Since they are also effective in inhibiting HIV-1 replication in cell culture, this class of compounds may have potential as candidate drugs for the treatment of AIDS.

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