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Antiviral activity of NMSO3 against adenovirus in vitro

Hisatoshi Kaneko [a,b,*], Keiichiro Kato [a], Shuichi Mori [b], Shiro Shigeta [b]

[a] *Department of Ophthalmology, Fukushima Medical University School of Medicine, 1, Hikarigaoka, Fukushima 960-1295, Japan*
[b] *Department of Microbiology, Fukushima Medical University School of Medicine, 1, Hikarigaoka, Fukushima 960-1295, Japan*

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Abstract

NMSO3, a sulfated sialyl lipid, was evaluated for its efficacy against adenovirus (AdV) in vitro. The median effective concentration (50% effective concentration, EC_{50}) of NMSO3 against replication of AdV type 2 (AdV2), type 4 (AdV4), type 8 (AdV8) and type 37 (AdV37) was 0.21–0.71 μg/ml in HEp-2 cells and 1.01–1.41 μg/ml in MKN-28 cells. The EC_{50} values of NMSO3 were lower than those of HPMPC and ddC, which were also evaluated. NMSO3 exhibited minimal cytotoxicity against HEp-2 cells and MKN-28 cells, both for which the median cytotoxic concentration (50% cytotoxic concentration, CC_{50}) was more than 1000 μg/ml. NMSO3 was the most potent and selective anti-AdV compound of those examined. NMSO3 inhibited AdV infection of HEp-2 cells only when present during the virus adsorption period. A virus binding assay using radiolabeled AdV4 revealed that NMSO3 inhibited viral binding to the HEp-2 cells. NMSO3 itself bound to the virus particles, but not to the HEp-2 cell membrane. Thus, the mechanism of anti-AdV activity by NMSO3 involves inhibition of virus adsorption to cells by NMSO3 binding to viral particles.

Keywords: Adenovirus; Anti-adenovirus; NMSO3; Adsorption inhibitor; MTT method

1. Introduction

The adenovirus (AdV) family consists of 51 known serotypes, which fall into six subgenera, A to F (Wadell et al., 1980; De Jong et al., 1999). Many AdV serotypes cause the most common external ocular viral infections worldwide (Ford et al., 1987; Gordon et al., 1996a). In particular, adenoviral conjunctivitis is known to be the major cause of acute contagious infections associated with community and nosocomial epidemics. Although adenoviral conjunctivitis is usually a mild contagious disease, the economic and social impact of community epidemics is enormous, involving not only the loss of valuable time and resources in the commercial, governmental and academic worlds, but also a decrease in the quality of enjoyment derived from everyday activities. As a systemic infection, AdV infects the respiratory tract, intestinal tract, and in rare cases the liver and kidneys, and has a variety of clinical manifestations. Moreover, severe systemic AdV infection can occur in immunocompromised patients, such as those with leukemia, acquired im-

* Corresponding author. Tel.: +81-24-548-2111; fax: +81-24-548-5072.
E-mail address: h-kane@chive.ocn.ne.jp (H. Kaneko).

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mune deficiency syndrome (AIDS) and kidney or bone marrow allograft transplantation (Hierholzer, 1992; Zarraga et al., 1992). Thus, the development of anti-AdV drugs effective in the clinical treatment of adenoviral conjunctivitis or systemic AdV infectious diseases is of paramount importance.

Recently, several investigators reported that cidofovir (HPMPC) (Gordon et al., 1991, 1994; De Oliveira et al., 1996; Kodama et al., 1996; Romanowski and Gordon, 2000) and zalcitabine (ddC) (Mentel et al., 1997; Mentel and Wegner, 2000) are effective in inhibiting AdV replication. HPMPC and ddC have been used in the clinical treatment of other viral diseases, that is, cytomegalovirus (CMV) infection and AIDS, respectively.

NMSO3 is a chemically synthesized sulfated sialyl lipid that has been shown to exhibit antiviral activity against respiratory syncytial virus (RSV). The mechanism of this anti-RSV activity is inhibition of virus adsorption and penetration (Kimura et al., 2000; Shigeta, 2000). NMSO3 is a nontoxic, broad-spectrum compound, exhibiting antiviral activity against human immunodeficiency virus (HIV) (M. Terada, unpublished data), influenza virus type A (FluV-A) (Kimura et al., 2000), and rota virus (K. Takahashi, unpublished data). The antiviral activity of NMSO3 against AdV has not been previously examined.

In the present study, we examined the antiviral effects of HPMPC, ddC and NMSO3 against several AdV serotypes using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method in vitro. We also analyzed the mechanism of the anti-AdV activity of NMSO3.

2. Materials and methods

2.1. Cells

The human larynx epidermoid cancer cell line (HEp-2 cells) and the human gastric cancer cell line (MKN-28 cells) were used. The usefulness of MKN-28 cells for evaluation of the anti-AdV activity of compounds has been previously established (Kodama et al., 1996). Cells were grown and maintained in Eagle's minimum essential medium (MEM) supplemented with 2–5% fetal calf serum (FCS), 100 units/ml penicillin G, 50 μg/ml streptomycin and 2 mM L-glutamine.

2.2. Viruses

The viruses used were AdV type 2 (AdV2), type 4 (AdV4), type 8 (AdV8) and type 37 (AdV37). All four types were prototype strains, and were provided by the National Institute of Infectious Diseases, Tokyo, Japan. These strains were propagated in HEp-2 cells and stored at −80 °C until use. ^{35}S-methionine labeled AdV was prepared in HEp-2 cells. Briefly, AdV-infected HEp-2 cells were radiolabeled with 100 μCi ^{35}S-methionine for 12 h until massive CPE was observed. The radiolabeled virus particles were purified on a CsCl gradient by ultracentrifugation (Green and Pina, 1964) and 100-μl aliquots were then tested to determine the virus infectivity titer and radioactivity.

2.3. Compounds

NMSO3 was synthesized at the Central Research Institute of Nissin Food Products Co. Ltd., Kusatsu, Shiga, Japan. The chemical name of NMSO3 is sodium [2,2-bis(docosyloxymethyl)propyl-5-acetoamido-3,5-dideoxyl-4,7,8,9-tetra-*O*-(sodium-oxysulfonyl)-D-glycero-a-D-galacto-2-nonulopyranosid] onate. The structural formula of NMSO3 is shown in Fig. 1. (*S*)-1-[3-hydroxy-(2-phosphonylmethoxypropyl)cytosine] (cidofovir,

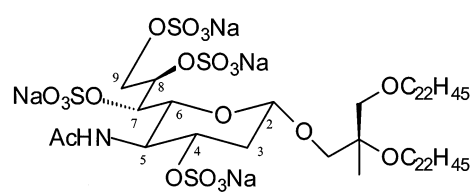

Fig. 1. Chemical structure of NMSO3. The chemical name of NMSO3 is sodium[2,2-bis(docosyloxymethyl)propyl-5-acetoamido-3,5-dideoxy-4,7,8,9-tetra-*O*-(sodiumoxysulfonyl)-D-glycero-α-D-galacto-2-nonulo-pyranosid]onate. Molecular weight is 1478.7.

HPMPC) was purchased from Gilead Sciences (Foster City, CA). 2′,3′-Dideoxycytidine (zalcitabine, ddC) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Company (St. Louis, MO).

2.4. Virus titration

The infectivity of each virus serotype was determined by the MTT method. Briefly, the reciprocal of the dilution of virus which resulted in a 50% reduction in absorbance of formazan by the infected cells at 7 days after infection was defined as the ID_{50} (50% infective dose) (Kodama et al., 1996).

2.5. Antiviral assay against AdV using the MTT method

The antiviral activity against the four AdV serotypes and the cytotoxicity of the three compounds was assessed using the MTT method. The MTT antiviral assay for AdV was performed according to Kodama et al., (1996). The concentration that reduced the absorbance of mock-infected cells by 50% of that of the control was defined as the 50% cytotoxic concentration (CC_{50}). The 50% antiviral effective concentration (EC_{50}) was defined as the concentration that achieved 50% protection of virus-infected cells against virus-induced destruction.

2.6. Inhibition assay for virus adsorption

At low temperature (4 °C), AdV binds to cells but does not penetrate the cell membrane. However, when virus-bound cells are exposed to a temperature of 35 °C, the virus begins to penetrate the cell membrane (Defer et al., 1990; Greber et al., 1997; Hong et al., 1997). The inhibition assay of virus binding and penetration used was that performed for myxoviruses (Hosoya et al., 1991; Kimura et al., 2000) with a few minor modifications. First, HEp-2 monolayers were prepared in 96-well microplates (Falcon 3072, Becton Dickinson, Lincoln Park, NJ). Approximately 100 ID_{50} of AdV 2, AdV 4 or AdV 37 was then added to the cells, after which they were incubated at 4 °C for 1.5 h in the presence or absence of NMSO3 at various concentrations. After 1.5 h of virus adsorption at 4 °C, the cells were washed with maintenance medium, and then pre-warmed maintenance medium without NMSO3 was added to each well. The plates were incubated for 7 days at 35 °C. At the end of the incubation period, the antiviral activity against AdV was assessed by determining the formation of MTT by viable cells.

2.7. Virus binding assay with radiolabeled virus

The virus binding assay was performed with ^{35}S-methionine-labeled purified AdV4 (von Oostrum and Burnett, 1985; Greber et al., 1993). Radiolabeled AdV 4 (100 $ID_{50} = 10^4$ DPM/100 μl) was mixed with NMSO3 at various concentrations and incubated at 4 °C for 1.5 h. After incubation, the viruses were collected by ultracentrifugation and unbound NMSO3 removed. The viruses were then added to a confluent monolayer of HEp-2 cells in a 96-well microplate and incubated at 4 °C for 1.5 h. After adsorption, the cells were washed with maintenance medium, lysed with lysing solution (1% Triton-X, 0.15 M NaCl, 10 mM Tris–HCl), and radioactivity quantified using a liquid scintillation counter (Aloka, Tokyo Japan). The percentage of bound viruses was calculated as (DPM of membrane-bound virus/DPM of total input AdV4 in medium) × 100%. In a separate experiment, NMSO3 at various concentrations was added to HEp-2 cells, which were incubated at room temperature for 1.5 h and unbound NMSO3 then removed. Radiolabeled virus was added to the cells and incubated at 4 °C for 1.5 h. The percentage of bound virus was calculated using the method described above.

3. Results

3.1. Activity of antiviral compounds as determined using the MTT method

The inhibitory effect of HPMPC, ddC and

Table 1
Antiviral activities of HPMPC, ddC and NMSO3 for AdV

Compound	EC_{50}[a] (μg/ml)				CC_{50}[b] (μg/ml)
	AdV2	AdV4	AdV8	AdV37	
HEp-2 cells					
HPMPC	7.11 ± 1.99[c]	8.86 ± 1.87	7.50 ± 3.51	4.61 ± 1.92	184.8 ± 10.5
ddC	3.95 ± 2.15	4.29 ± 1.13	4.00 ± 1.62	3.82 ± 1.49	381.9 ± 15.6
NMSO3	0.40 ± 0.55	0.71 ± 0.07	0.47 ± 0.17	0.21 ± 0.06	>1000
MKN-28 cells					
HPMPC	6.78 ± 2.21	8.74 ± 1.56	6.67 ± 0.94	5.51 ± 1.00	194.8 ± 10.2
ddC	3.56 ± 2.01	3.23 ± 0.76	3.85 ± 2.29	2.82 ± 0.91	451.6 ± 19.8
NMSO3	1.35 ± 0.89	1.41 ± 1.06	1.31 ± 1.17	1.01 ± 0.95	>1000

[a] 50% effective concentration.
[b] 50% cytotoxic concentration.
[c] Data represent mean values for five independent experiments (mean value ± S.D.)

NMSO3 on AdV replication was examined using the MTT method. The amount of formazan formation in uninfected HEp-2 cells and MKN-28 cells was determined by a colorometric assay, and confirmed that formazan formation correlates well with the number of viable cells.

HPMPC, ddC and NMSO3 inhibited all four AdV serotypes tested. The EC_{50} of NMSO3 was 0.21–0.71 μg/ml in HEp-2 cells and 1.01–1.41 μg/ml in MKN-28 cells. The EC_{50} values of NMSO3 were lower than those of HPMPC and ddC in both HEp-2 and MKN-28 cells (Table 1). On the other hand, the CC_{50} of NMSO3 was greater than 1000 μg/ml in both cell types, which was higher than those of HPMPC and ddC (Table 1). The selectivity index [$SI = (CC_{50}/EC_{50})$] of NMSO3 was the highest of the compounds examined, with that against ADV 37 being greater than 4761 in HEp-2 cells and greater than 990 in MKN-28 cells.

3.2. Inhibitory activity of NMSO3 against AdV adsorption

The inhibitory activity of NMSO3 against adsorption of AdV to the cells was examined during the contact period of the virus with the cells. NMSO3 at various concentration was added to HEp-2 cells at the start of 1.5 h of AdV adsorption to the cells and then removed from the cell cultures. After 7 days of incubation of the culture at 37 °C, NMSO3 was found

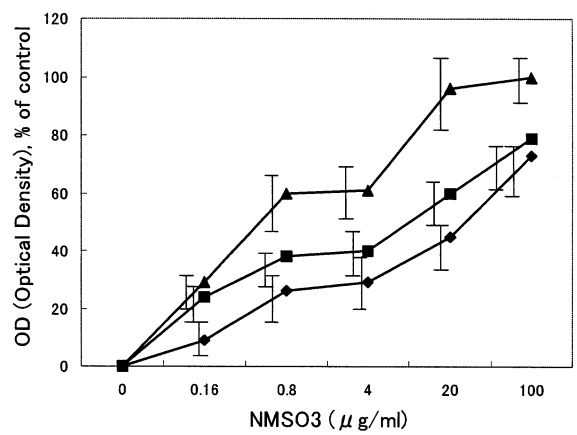

Fig. 2. Inhibitory effects of NMSO3 on adsorption of AdV2 (●), AdV4 (■) and AdV37 (▲). A five-fold dilution of NMSO3 in cold (4 °C) maintenance medium was added to HEp-2 monolayer cells in each of four wells. An AdV of 100 ID_{50} was added to the cells, which were then incubated with or without NMSO3 at 4 °C for 1.5 h. The medium was removed, the cells washed, maintenance medium without NMSO3 added and the cells then incubated at 37 °C for 7 days. The abscissa indicates optical density of formazan (% of control) and the ordinate indicates NMSO3 concentration. The data represents the average of three independent experiments. The bars indicate S.D.

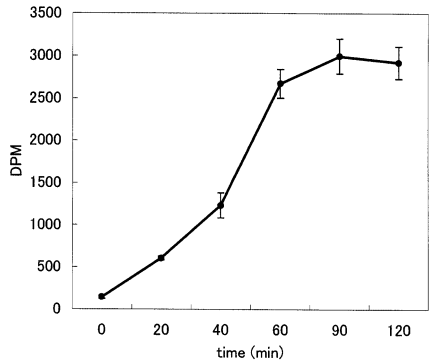

Fig. 3. Radioactivity of bound AdV 4 at different times (min) after virus inoculation. ^{35}S-labeled AdV 4 was added to HEp-2 cells and incubated at 4 °C. After incubation, the cells were washed and unbound viruses were removed. The radioactivity of adsorbed virus to HEp-2 cells was calculated in a liquid scintillation counter. Data represent radioactivity of membrane-bound virus (DPM) as a function of incubation time, and correspond to average values for three independent experiments. The bars indicate S.D.

to inhibit AdV replication, that is, 100 μg/ml of NMSO3 protected 75–100% of infected cells, and 20 μg/ml of NMSO3 protected 50–96% of cells, from death (Fig. 2).

3.3. Effect of NMSO3 on the binding of radiolabeled AdV to cells

Since NMSO3 inhibited AdV adsorption, we examined whether NMSO3 inhibited the binding of radiolabeled purified AdV 4 to HEp-2 cells. First, we examined the adsorption of radiolabeled AdV 4 to HEp-2 cells. Labeled virus was added to HEp-2 cells and incubated at 4 °C. At the times (min) indicated in Fig. 3 after virus inoculation, the cells were removed and washed with maintenance medium. As shown in Fig. 3, the radioactivity (DPM) of adsorbed virus increased, and then reached a plateau at 1.5 h.

When NMSO3 was added to the virus particles prior to virus adsorption, NMSO3 blocked the binding of AdV4 to the cells in a dose-dependent manner (Fig. 4A). However, when NMSO3 was added to the cells before virus adsorption, the percentage of the bound virus did not decrease at 100 μg/ml NMSO3 (Fig. 4B), suggesting that NMSO3 bound to the virus particles and thereby prevented viral binding to the cell membrane, but did not bind directly to the cell membrane.

4. Discussion

At present, there are no antiviral agents available to treat AdV infections. Several investiga-

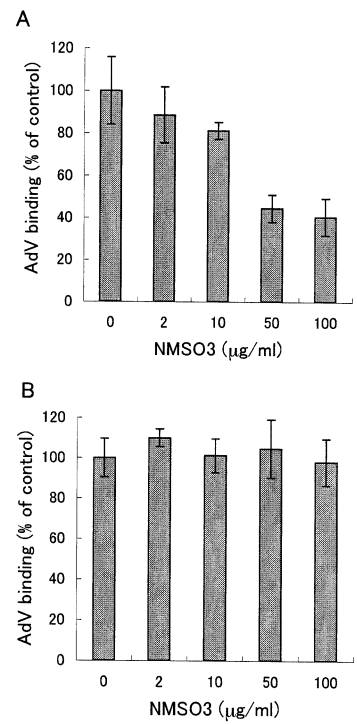

Fig. 4. The percentage of bound AdV 4 to HEp-2 cells. (A) ^{35}S-labeled AdV 4 was mixed with NMSO3 at various concentrations and incubated at 4 °C for 1.5 h. After incubation, virus was collected by ultracentrifugation and the unbound NMSO3 removed. The viruses were then added to HEp-2 cells and incubated at 4 °C for 1.5 h. The percentage of bound virus was calculated. (B) NMSO3 was added at various concentrations to HEp-2 cells, incubated at room temperature for 1.5 h and the unbound NMSO3 removed. ^{35}S-labeled AdV 4 was then added to HEp-2 cells and incubated at 4 °C for 1.5 h. The percentage of bound virus was calculated. Data represents percentages of membrane-bound virus and correspond to average values for three independent experiments. The bars indicate S.D.

tors have reported that HPMPC and ddC do inhibit AdV replication. HPMPC exhibits antiviral activity against several DNA viruses and has been clinically used as a drug for the treatment of CMV infections (Neyts et al., 1990; De Clercq, 1993). Furthermore, studies have shown that HPMPC exhibits antiviral activity against several AdV serotypes in vitro (Gordon et al., 1991; Kodama et al., 1996) as well as in vivo (Gordon et al., 1994; De Oliveira et al., 1996; Romanowski and Gordon, 2000). Gordon et al. reported that topical HPMPC application inhibits ocular AdV infections in rabbits. ddC is a reverse transcriptase inhibitor of HIV (Mitsuya et al., 1987), and is also known to exhibit antiviral activity against AdV both in vitro (Mentel et al., 1997) and in vivo (Mentel and Wegner, 2000). However, neither HPMPC nor ddC have been used clinically as an anti-AdV drug, except for cases of HPMPC as eyedrops in the treatment of adenovirus keratoconjunctivitis (Gordon et al., 1996b; Hillenkamp et al., 2001).

In the present study, NMSO3 inhibited the replication of all four AdV prototype strains at significantly lower concentrations than those of HPMPC and ddC. The CC_{50} of NMSO3 was greater than 1000 μg/ml, while the corresponding values for HPMPC and ddC were much lower. Thus, NMSO3 was the most potent and selective inhibitor of AdV of the three compounds examined.

NMSO3 has been shown in previous studies to inhibit adsorption of RSV (Kimura et al., 2000; Shigeta, 2000). Furthermore, unpublished data from our experiments has indicated that NMSO3 inhibits adsorption of rota virus. In the present study, NMSO3 inhibited AdV replication only when added during virus adsorption (0–1.5 h). Furthermore, experiments on radiolabeled virus binding showed that NMSO3 blocked virus binding to HEp-2 cells. These findings suggest that the mechanism of the anti-AdV activity of NMSO3 is based upon inhibition of virus adsorption to cells.

In general, negatively charged polysaccharides inhibit the adsorption of viruses to the cell membrane by interference of static electric binding between the virus and cell membrane. As NMSO3 contains four negatively charged residues in its molecule, it may inhibit the binding of AdV to the cell membrane in this way. The virus binding assay using radiolabeled virus showed that NMSO3 bound to the virus particle, and not to the cell membrane. NMSO3 possesses a sialic acid moiety, which is used by several viruses as a cellular receptor. Conceivably, NMSO3 interferes with the binding of AdV to the cell membrane by competing with the cellular receptors for virus particles. Three cellular receptors for AdV, namely Coxsackievirus-adenovirus receptor (CAR), major histocompatibility complex class I (MHC-I) α2 and sialic acid, have recently been identified. Of the AdV serotypes used in the present study, AdV 8 and AdV 37 of subgenus D use sialic acid as their cellular receptor (Arnberg et al., 2000a,b), AdV 2 of subgenus C uses CAR (Bergelson et al., 1997), and that of AdV 4 is unclear. NMSO3 inhibited the adsorption of all four AdV serotypes, regardless of preferred cellular receptor. Given that NMSO3 bound to the AdV particle, but not the cellular receptor, these findings suggest that the bound NMSO3 hinders the binding of the virus particle and the receptor. The detailed mechanism of inhibition of AdV adsorption by NMSO3 requires further clarification.

In the present study, NMSO3 inhibited adsorption of AdV and thus exhibited antiviral activity shortly before or immediately after AdV. It is also conceivable that NMSO3 inhibits adsorption of released viruses during secondary infection. Thus, NMSO3 is a candidate for the prophylactic and therapeutic application in AdV infection.

In conclusion, NMSO3 was shown to be a potent and selective anti-AdV compound. Furthermore, NMSO3 was nontoxic to the cell cultures. The mechanism of antiviral activity of NMSO3 involves inhibition of virus adsorption, although further clarification is required. These findings suggest that NMSO3 is worth investigating as an antiviral agent in the therapy and prophylaxis of AdV infections.

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