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Application of a gastric cancer cell line (MKN-28) for anti-adenovirus screening using the MTT method

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Abstract

We established a sensitive and accurate method for screening of anti-adenovirus agents using the 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. MKN-28 cells, which are well-differentiated stomach adenocarcinoma cells, were used for adenovirus (ADV) infection and examined for the anti-ADV activities of several established anti-herpes virus agents. ADV-11 is the causative agent of respiratory and urinary infections. It frequently causes hemorrhagic cystitis in immunocompromised hosts. One laboratory strain and 4 clinical isolates of ADV-11 were examined, and found susceptible (in order of decreasing activity) to 2-amino-7-[(1,3-dihydroxy-2-propoxy)methyl]purine (S-2242), (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine[(*S*)-HPMPA], and (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine[(*S*)-HPMPC]. On the other hand, ganciclovir and iododeoxyuridine were only weakly effective and dextran sulfate was ineffective. Our findings indicate that the MTT assay using MKN-28 cells is applicable to anti-ADV screening. The anti-ADV activity of (*S*)-HPMPA and (*S*)-HPMPC was confirmed, and, furthermore, S-2242 emerged as a highly potent and selective inhibitor of ADV-11.

Keywords: Adenovirus; Antiviral screening; MTT method

1. Introduction

Adenoviruses (ADV) infect epithelial cells or mucus membrane cells of conjunctiva, cornea, respiratory tract, intestinal tract, and other organs. The clinical manifestations caused by ADV infections vary considerably; most of them are mild and heal without special chemotherapy. However, it has been reported that severe ADV infections occur in immunocompromised patients, i.e. patients with leukemia (Zahradnik et al., 1980), or the acquired immune deficiency syndrome (AIDS) (De Jong et al., 1983), and recipients of kidney (Stalder et al., 1977) or bone marrow (Shields et al., 1985) allografts. Several reports of especially severe ADV-11 urinary infec-

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tion in immunocompromised patients (Ambinder et al., 1986; Shindo et al., 1986; Thomas and Myron, 1994) prompted us to search for antiviral compounds which are effective against ADV replication.

Recently several investigators reported that (*S*)-HPMPA, (*S*)-HPMPC, 2′-nor-cyclic GMP, and Schiff bases of aminohydroxyguanidine were effective in inhibiting ADV replication (Baba et al., 1987; Gordon et al., 1991; Hui et al., 1994). 5-iodo-2′-deoxyuridine (IDU) and ganciclovir (GCV), two well-known antiherpetic compounds, have been used in the chemotherapy of ADV infection (Dudgeon et al., 1969; Gordon et al., 1991). In vitro, these compounds have generally been examined by the plaque reduction method which, while being sensitive and reproducible, is rather tedious. 3-(4,5-Dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is a water-soluble, yellow dye and is easily taken up into viable cells. MTT is converted to water-insoluble, blue crystal formazan in viable cells by mitochondrial enzymes. The amount of formazan formation correlates well to the number of viable cells. The MTT assay has been widely applied to the screening of anti-cancer substances (Carmichael et al., 1987). Pauwels et al. (1988) recently described a rapid and sensitive MTT assay system for detecting agents with anti-HIV activity in vitro. This colorimetric assay system has been used broadly for screening of various antiviral agents (Hansen et al., 1990; Takeuchi et al., 1991; Hosoya et al., 1992; Sudo et al., 1994), but it has not been applied to the evaluation of anti-ADV activity. We have now established an anti-ADV screening method with gastric cancer cells (i.e. MKN-28 cells) using the MTT assay.

2. Materials and methods

2.1. Cells

The human gastric cancer cell line (MKN-28) (Hojo, 1991; Ura et al., 1991), was used in the MTT assay. The MKN-28 cells were derived from well-differentiated adenocarcinoma cells and grown as adherent cells in RPMI 1640 medium supplemented with 10% newborn calf serum (NCS) (Biocell laboratories, CA), 100 units/ml penicillin G, and 50 μg/ml streptomycin. In the antiviral assay, the medium was supplemented with 5% NCS and the antibiotics.

2.2. Viruses

ADV-type 11 was used throughout the experiments. One laboratory strain (NS-3) and four clinical isolates (AD-0172, AD-0763, AD-0820 and AD-1772) were provided by Y. Numazaki, National Sendai Hospital and H. Gondo, Kyushu University, respectively. These strains were propagated in the MKN-28 cells and stored at −80°C until use.

2.3. Compounds

(*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl) adenine [(*S*)-HPMPA] and (*S*)-1-(3-hydroxy-2 phosphonylmethoxypropyl)cytosine[(*S*)-HPMPC], were obtained from Dr. A Holy (Institute of Organic Chemistry and Biochemistry, Czech Republic Academy of Science, Prague, Czech Republic) and 2-amino-7-[(1,3-dihydroxy-2-propoxy)-methyl]purine (S-2242) was obtained from Hoechst A9, Anti-infectives Research, Frankfurt am Main, Germany. 5-Iodo-deoxyuridine (IDU), dextran sulfate (DS; molecular weight 5000), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 9-(1,3-Dihydroxy-2-propoxy-methyl)-guan-osine (ganciclovir, GCV) was obtained from Nippon Syntex (Tokyo, Japan).

2.4. Antiviral assay using MTT method

An aliquot of 125 μl of culture medium containing 400 μg/ml of the test compound was added to two series of triplicate wells (the first row) of flat-bottomed 96-well microtiter trays, and 100 μl of culture medium was added to the rest of the wells. Serial 5-fold dilutions were made directly in the microtiter trays using an eight-channel pipette. The last row of the wells was used as the untreated control. After 3 days,

MKN-28 cells were harvested with trypsin and suspended in culture medium at a concentration of 2×10^5 cells/ml and mixed with 50 $CCID_{50}$ (50% cell culture infective dose) of ADV. Immediately after the cells and virus had been mixed, 100 μl of mock- and virus-exposed cell suspensions were transferred to each well containing the diluted compound solution. The tray was centrifuged at 800 *g* for 10 min and incubated at 37°C for 5 days. At the end of incubation period, 20 μl of the MTT solution (7.5 mg/ml) in phosphate-buffered saline (PBS) was added to each well of the tray. The tray was then incubated at 37°C for 2 h. After incubation, 150 μl of medium was removed from each well. To solubilize the formazan crystals, 100 μl of acidified isopropanol (4 ml concentrated HCl per 1 l isopropanol) containing 10% (v/v) Triton X-100 was added to each well, and the tray was shaken by a plate shaker for at least 10 min. After confirming microscopically that formazan crystals were completely solubilized, the absorbance of the wells was read in a computer-controlled microplate reader (Model 3550, Biorad) at two different wavelengths (540 nm and 690 nm).

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% antivirally effective concentration (EC_{50}) was expressed as the concentration that achieved 50% protection of virus-infected cells against virus-induced destruction. The percent protection was calculated by the following formula:

$$\frac{(OD_T)_{ADV} - (OD_C)_{ADV}}{(OD_C)_{MOCK} - (OD_C)_{ADV}} \times 100(\%)$$

$(OD_T)_{ADV}$, $(OD_C)_{ADV}$, and $(OD_C)_{MOCK}$ indicate the absorbances of the test sample, the virus-infected control (no compound), and the mock-infected control, respectively.

Infectivity of virus stocks was also determined by the MTT method. In brief, the reciprocals of dilution of stock virus which resulted in 50% reduction of absorbance of formazan in the infected cells at 5 days was determined as infectivity of virus by MTT ID_{50} (50% infective dose).

2.5. Statistical analysis

The effects of compounds on inhibition of ADV-11 replication were compared statistically using the Student's *t*-test.

3. Results

3.1. Formazan formation in MKN-28 cells

In MKN-28 cells, MTT was rapidly converted to formazan and after 2 and 6 h incubation periods the OD value reached 0.7 and 1.5 respectively (Fig. 1). In a previous report it was recommended that for the MTT assay, the OD (optical density) value of formazan converted from MTT in mock-infected cells should be more than 0.4 (Sudo et al., 1994). From the results in this and Sudo's studies we decided that MTT incubation for 2 h is suitable for the assay using MKN-28 cells.

3.2. Inhibition of formazan formation in virus-infected cells

The inhibition of formazan formation was examined in ADV-11- infected MKN-28 cells. From the results reported previously (Takeuchi et al.,

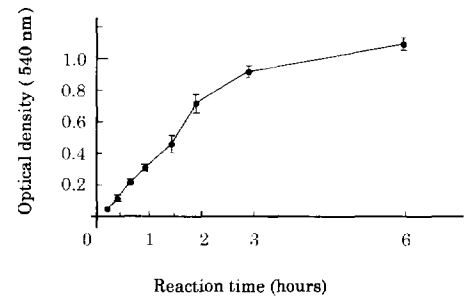

Fig. 1. Formazan formation in MKN-28 cells. After addition of 20 μl of MTT solution (7.5 mg/ml) to each well, the cell cultures were incubated at 37°C in 5% CO_2. At the indicated time, the formazan crystals were dissolved in acidified isopropanol containing 10% Triton X-100 and the optical density (OD) was read by a microplate reader at two wavelengths (540 and 690 nm). Each point is the mean ($\pm$ S.D.) of six separate wells.

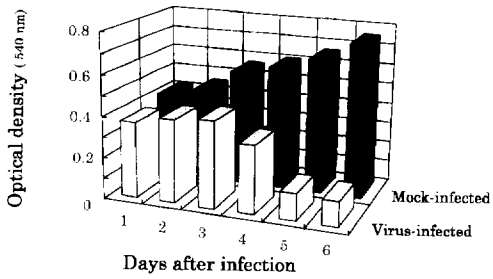

Fig. 2. Inhibition of formazan formation in virus-infected and mock-infected MKN-28 cells. Formazan formation by mock-infected MKN-28 cells and ADV-11 infected cells (50 $CCID_{50}$ per well) was monitored. Each value is the mean of 10 separate wells.

1991; Sudo et al., 1994), at least 80% inhibition of formazan formation following virus infection is necessary for determing the antiviral activity. Upon infection with ADV-11 at 50 $CCID_{50}$ per well, the OD value decreased to less than 20% of the control at 5 days (Fig. 2). Therefore we used 50 $CCID_{50}$ of ADV and incubated the cells for 5 days after infection in the MTT antiviral assay.

3.3. Activity of antiviral compounds as determined by the MTT method

To determine whether the MTT assay using MKN-28 cells is practically useful and reliable for the screening of anti-ADV compounds, a selected number of compounds were evaluated for their inhibitory effect on the cytopathogenicity of ADV-11, NS-3 strain. S-2242 showed the most potent and selective anti-ADV activity (Table 1). Also (*S*)-HPMPA and (*S*)-HPMPC proved to be potent and selective ADV inhibitors, whereas GCV and IDU showed little activity against ADV. Dextran sulfate was absolutely ineffective against ADV-11 replication. In previous reports, (*S*)-HPMPA and (*S*)-HPMPC were shown to inhibit replication in the conventional plaque reduction assay (Baba et al., 1987; Gordon et al., 1991). Their anti-ADV activity was confirmed by the MTT method using MKN-28 cells (Table 1).

3.4. Evaluation of antiviral activity against clinical isolates.

S-2242 and (*S*)-HPMPA were also examined for their activity against 4 clinical isolates of ADV-11. These strains had been freshly isolated from the urine of patients with acute hemorrhagic cystitis. S-2242 and (*S*)-HPMPA were effective against the clinical isolates at an average EC_{50} of 1.36 and 7.40 μg/ml respectively, that is about 2.65-fold higher than their EC_{50} for the laboratory strain (Table 2).

4. Discussion

The MTT assay as a method for the evaluation of antiviral activity was first established by Pauwels et al. (1988) for the detection of inhibitors of the human immunodeficiency virus (HIV). This method is rapid and can be readily automated, and thus, large numbers of compounds could be evaluated in a short time without tedious experimental procedures. Although activity against ADV has been determined in some studies, at present only a few compounds are known be effective (Baba et al., 1987; Gordon et al., 1991; Hui et al., 1994).

Table 1
Anti-ADV activity of various compounds against the laboratory NS-3 strain of ADV-11

Compound	EC_{50} [a] (μg/ml)	CC_{50} [b] (μg/ml)	SI [d]
(*S*)-HPMPA	2.81 ± 0.47 [c]	197.3 ± 26.8	70.2
(*S*)-HPMPC	15.8 ± 3.97	173.2 ± 15.9	11.0
S-2242	0.51 ± 0.10	149.0 ± 10.0	292.2
GCV	57.9 ± 22.1	123.1 ± 5.15	2.1
IDU	39.7 ± 11.9	136.1 ± 4.5	3.4
DS5000	>200	>200	1.0

[a] EC_{50}: 50% antivirally effective concentration
[b] CC_{50}: 50% cytotoxic concentration
[c] Data represent mean values for at least three independent experiments (mean value ± S.D.).
[d] Selectivity index
Differences between antiviral activity of S-2242 and that of (*S*)-HPMPA or (*S*)-HPMPC were statistically analyzed and are significant (both P values <0.05).

Table 2
Comparison of EC_{50} values of (*S*)-HPMPA and S-2242 for several clinical ADV-11 isolates

Compound	EC_{50} (μg/ml) [a,b]				
	ADV-0196	ADV-0736	ADV-0820	ADV-1772	Average
(*S*)-HPMPA	10.3 ± 9.6	2.19 ± 1.54	5.03 ± 3.40	12.1 ± 7.48	7.40 ± 3.81
S-2242	1.26 ± 0.27	1.60 ± 0.76	0.88 ± 0.17	1.68 ± 0.18	1.36 ± 0.29

[a] EC_{50}: 50% antivirally effective concentration
[b] Data represent mean values for at least three independent experiments (mean value ± S.D.).
Difference between antiviral activity of S-2242 and that of (*S*)-HPMPA was statistically analyzed and is significant (P value <0.05).

The MTT method for antiviral assay was applied to HIV at first and later to herpes simplex virus (HSV) (Pauwels et al., 1988; Takeuchi et al., 1991; Sudo et al., 1994). HIV shows stronger cytopathic effects in tissue culture cells (human T-lymphocytes) than ADV. HSV has a broad host cell spectrum compared with ADV, and both HIV and HSV were easily applicable to the MTT method. However, there has been no report on anti-ADV assay by the MTT method. Therefore, a new anti-ADV assay which is simple, sensitive and automated for evaluation of antiviral activity is needed to screen a large number of compounds.

We examined several human and monkey cell lines for antiviral MTT method against ADV-11 and MKN-28 was the only cell line which was applicable to the MTT method for anti-ADV assay.

Here we report a convenient screening method for anti-ADV agents. Using this method, we determined the efficacy against ADV of a number of compounds known to be effective against herpes viruses. IDU has been reported to offer little benefit in the treatment of kerato-conjunctivitis due to ADV (Dudgeon et al., 1969). Also GCV has little, if any, activity against ADV (Gordon et al., 1991). As could be anticipated, IDU and GCV were only slightly active in our anti-ADV assay system. (*S*)-HPMPA, (*S*)-HPMPC, and S-2242 were reported previously to be inhibitors of HSV replication (Neyts et al., 1994). (*S*)-HPMPA and (*S*)-HPMPC were also shown to be inhibitory to ADV (Baba et al., 1987). In the present study, we found that S-2242 to be a highly potent and selective inhibitor of the replication of 5 ADV-11 strains including 1 laboratory strain and 4 clinical isolates.

ADV-11 is assumed to be the causative agent of acute hemorrhagic cystitis (Numazaki et al., 1968). ADV-11 was isolated from urine samples of immunocompromised patients (such as bone marrow recipients, AIDS patients) presenting with severe hemorrhagic cystitis (Ambinder et al., 1986; Heirholzer et al., 1988; Thomas and Myron, 1994). Therefore, urgent chemotherapy seems to be needed for ADV-11 infections. In a previous paper, we reported (*S*)-HPMPA was the most potent and selective compound of a number of compounds tested for anti-ADV activity (Baba et al., 1987). Now we found that S-2242 which has broad spectrum anti-herpes virus activity, i.e. against thymidine-kinase-deficient herpes simplex and varicella-zoster viruses, and cytomegalovirus (Neyts et al., 1994), is even more potent and selective as inhibitor of ADV-11 than (*S*)-HPMPA.

In conclusion, we have developed the MTT assay for the screening of anti-ADV agents. This method is simple and reproducible to evaluate anti-ADV activity. (*S*)-HPMPC, (*S*)-HPMPA and, in particular, S-2242 emerged as potent and selective inhibitors of ADV-11 replication.

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