**L-1**

# A Strategy for Discovery of Novel Antivirally Active Substances from Algae

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## Introduction

Vaccines have been very succesful in prevention of viral diseases. However, some serious virus infectious diseases caused by human immunodeficiency virus (HIV) and herpes viruses such as herpes simplex virus type 1 (HSV-1) and human cytomegalovirus (HCMV) are likely to be controlled only by antiviral chemotherapy. So far, synthetic nucleoside analogs including 3'-azido-3'-deoxythymidine (AZT), 2',3'-dideoxyinosine (ddI), 2',3'-deoxycytidine (ddC), acyclovir (ACV) and ganciclovir (GCV) have been clinically used. In recent years, some peptide inhibitors of viral protease were found to be clinically efficient against HIV. Although these drugs have a potent inhibitory activity on the viral enzymes, serious side effects of the drugs and the emergence of drug-resistant strains of viruses during long-term treatment with monotherapy have been frequently reported. To optimize chemotherapeutic schedules for viral infection, it is desired to discover new agents that are inhibitory to different stages of the virus replication cycle (e.g. viral adsorption to host cell, virus-cell fusion, penetration, uncoating, viral protein and DNA synthesis, assembly, budding, and release).

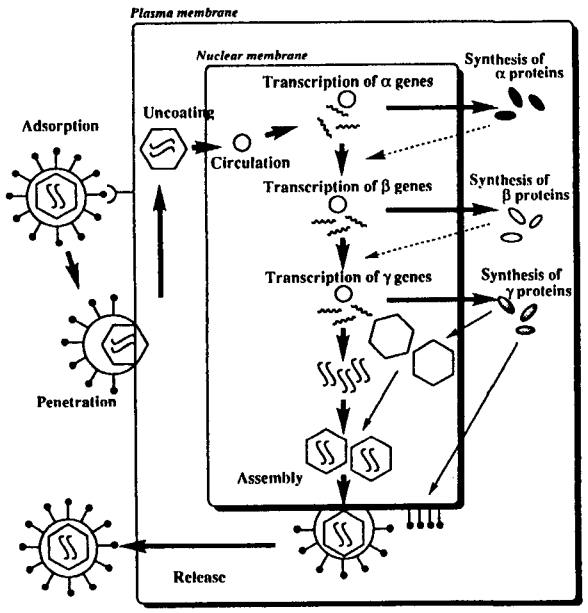
In an attempt to find new sources of antiviral compounds with different mechanisms of action, we have evaluated the antiviral activity of extracts of algae in anti-HSV and anti-HIV screening assay systems. In screening assays, we used assay systems which include all steps of the viral replication cycle.

## Materials for screening

Materials for screening were obtained by collection at seashores and lakes in Toyama and Ishikawa prefectures, Japan, or by cultivation. Freeze-dried algae were used for extraction.

## Bioassay systems

***Cells and viruses*** : HeLa cells were grown in Eagle's minimum essential medium (MEM) supplemented with 5% fetal calf serum (FCS) and 60 μg/ml




**Replication cycle of HSV-1**

kanamycin. HSV-1 (HF strain) was propagated and plaque-assayed in HeLa cells. MT-4, Molt-4 clone No. 8 and Molt-4/HTLV-IIIB, a HIV-producing cell line, were cultured in RPMI-1640 medium containing 10% FCS.

***Cytotoxicity*** : For cell growth inhibition studies, $5x10^4$ HeLa or MT-4 cells were inoculated in 24-well plates and incubated for 72 h at 37°C in the presence of increasing amounts of sample. After the medium was removed, viable cells were counted by trypan blue dye exclusion test. The inhibition data were plotted as dose-response curves, from which the 50% inhibitory dose ($ID_{50}$) was obtained.

***Anti-HSV-1 activity*** : HeLa cell monolayers in 24-well plates were infected with HSV-1 and incubated in maintenance medium (MEM plus 2% FCS) containing various dilutions of sample. The cultures were incubated for 24 h at 37°C, harvested, and disrupted by three cycles of freezing and thawing. Virus yields were determined by plaque assay. The antiviral activity was expressed as the 50% effective dose ($ED_{50}$) for viral replication, which was the lowest drug concentration that reduced plaque numbers by 50% in the treated cultures compared with untreated ones.

***Assay for HIV-induced cytopathic effect*** : Exponentially growing MT-4 cells were pelleted from the medium, infected with HIV-1 at a multiplicity of infection (MOI) of 0.0007 at room temperature for 1 h with agitation, and then diluted in growth medium to yield $3x10^4$ cells/100 μl/well after inoculation. Equal aliquots (100 μl) of the test solutions were added to each well. After 5 days of incubation at 37°C, viable cells were counted by the trypan blue dye exclusion test. From the data, the $IC_{50}$ values for a cytopathology assay were calculated.

***HIV-induced syncytium formation*** : Molt-4 ($2.5 \times 10^5$/250 μl) and Molt-4/HTLV-IIIB ($2.5 \times 10^5$/250 μl) cells were mixed with 500 μl of medium containing test sample or control medium. Syncytium formation was evaluated at 20 h after co-cultivation at 37°C. The results were expressed as the percent inhibition of syncytium formation, which was calculated as follows:

Percent inhibition = (1-syncytia formed in the presence of sample/syncytia formed in the absence of sample) x 100

***Criteria for selection of antivirally active samples*** : Samples were regarded as being effective for each virus using the following criteria.

(a) for HSV-1, the selectivity index ($ID_{50}/ED_{50}$) is more than 10 when sample is added immediately after virus infection, and is more than 20 when the sample is added at just before infection and throughout the incubation.

(b) for HIV, the selectivity index is more than 10 in the acute infection system, and more than 50% inhibition of syncytium formation is observed at a concentration of less than 20 μg/ml of sample when compared with drug-free control cultures.

## Bioassay guided isolation of antivirally active substances from *Spirulina platensis* and *Sargassum horneri*

1. Separation by trichloroacetic acid (TCA) treatment. 2. Dialysis of TCA-soluble fraction.
3. Gel filtration of nondialyzate (Sepharose 6B) eluting with 0.01 M citrate buffer containing 0.1 M NaCl
4. Ion exchange column chromatography on DEAE Toyopearl 650M eluting with a linear gradient solvent system (0 - 2 M NaCl). 5. Gel filtration on Toyopearl HW65S eluting with 0.1 M NaCl

## Structural analysis of antivirally active substances

1. Analysis of the component sugars by GC and GC-MS

   Hydrolysis and conversion of the hydrolysates to their alditol acetates
2. Elemental analysis (sulfur content, metal ion)
3. Determination of the glycosidic linkage and the position of sulfate group

   Methylation analysis of the triethylamine salt and the desulfated polysaccharide
4. Estimation of apparent molecular weight

   HPLC method (TSK GMPW$_{XL}$ gel filtration column) eluting with 0.2 M NaCl

   Pullulans (Shodex P-52, Showa Denko) were used as standard molecular markers.

Table 1. Results of methylation analysis of antivirally active substances

| partially methylated alditol acetates | deduced linkage | composition (mol %) TEA-SP | DS-SP |
|---|---|---|---|
| 2,3,4-Rha* | Rha(1- | 0.2 | 4.2 |
| 2,4-Rha | -3)Rha(1- | 6.7 | 44.6 |
| 3,4-Rha | -2)Rha(1- | 24.0 | 32.6 |
| 3-Rha | -4,2)Rha(1- | 17.5 | |
| 4-Rha | -3,2)Rha(-1 | 27.0 | 2.9 |
| Rha | -4,3,2)Rha(1- | 19.8 | 2.9 |
| 2,3-Fuc | -4)Fuc(1- | 0.4 | 2.4 |
| 3-Fuc | -4,2)Fuc(1- | | 0.3 |
| 2,3,4-Xyl | Xyl(1- | 0.2 | 5.6 |
| 2,3-Xyl | -4)Xyl(1- | 4.1 | 2.5 |
| 2,4-Xyl | -3)Xyl(1- | | 0.4 |
| 2,3-Ara | -4)Ara(1- | 0.1 | 0.4 |
| 3,4-Glc | -6,2)Glc(1- | | 1.2 |

*2,3,4-Rha =1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylrhamnitol.
TEA-SP: triethylamine salt of calcium spirulan (Ca-SP).
DS-SP: desulfated calcium spirulan.

| partially methylated alditol acetates | deduced linkage | composition (mol %) TEA-HOR | DS-HOR |
|---|---|---|---|
| 2,3,4-Fuc* | Fuc(1- | 12.9 | 20.8 |
| 2,3-Fuc | -4)Fuc(1- | 13.6 | 27.1 |
| 2,4-Fuc | -3)Fuc(1- | 9.2 | 37.5 |
| 3,4-Fuc | -2)Fuc(1- | 10.0 | |
| 2-Fuc | -4,3)Fuc(1- | 27.5 | 14.6 |
| 4-Fuc | -3,2)Fuc(1- | 15.0 | |
| Fuc | -4,3,2)Fuc(1- | 11.8 | |

*2,3,4-Fuc =1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl fucitol
TEA-HOR: triethylamine salt of sodium hornan (Na-HOR).
DS-HOR: desulfated sodium hornan.

## Antiviral activity

In order to elucidate the mode of action of the inhibition of virus replication, the effects of Ca-SP and Na-HOR were studied under various conditions. Ca-Sp was found to exert its antiviral activity

on enveloped viruses including HSV-1, HCMV, and HIV-1, but not on unenveloped viruses including poliovirus and coxackievirus (Table 2). Na-HOR showed similar antiviral spectrum to that of Ca-SP.

Table 2. Inhibitory effect of Ca-SP on the replication of different viruses

| virus | host cell | cytotoxicity ($ID_{50}$, μg/ml)[a] | antiviral activity ($ED_{50}$, μg/ml)[b] | | selectivity index ($ID_{50}/ED_{50}$) | |
|---|---|---|---|---|---|---|
| | | | A | B | A | B |
| HSV-1 | HeLa | 7900 | 16.5 | 0.92 | 479 | 8587 |
| HCMV | HEL | 4800 | 41 | 8.3 | 117 | 578 |
| Measles virus | Vero | 6300 | 39 | 17 | 162 | 371 |
| Mumpsvirus | Vero | 6300 | 92 | 23 | 68 | 274 |
| Influenza A virus | MDCK | 5400 | 230 | 9.4 | 23 | 574 |
| Poliovirus | Vero | 6300 | 2300 | 2200 | 2.7 | 2.9 |
| Coxackievirus | Vero | 6300 | 2600 | 1850 | 2.4 | 3.4 |
| HIV-1 | MT-4 | 2900 | 11.4 | 2.3 | 254 | 1261 |

[a] Concentration required to reduce cell growth by 50%; mean value for two experiments.
[b] Concentration required to reduce virus replication by 50%; mean value for two experiments.
A: Ca-SP was added to the medium immediately after viral infection.
B: Ca-SP was added to the medium 3 h before viral infection.

Table 3. Effect of Ca-SP and its related polysaccharides on syncytium formation

| sample | percent inhibition of syncytium formation | | | | 50% inhibitory concentration (μg/ml) |
|---|---|---|---|---|---|
| | 0.2 μg/ml | 1 μg/ml | 5 μg/ml | 25 μg/ml | |
| Ca-SP | 6.3 ± 1.2 | 14 ± 3.9 | 27 ± 6.7 | 99 ± 1.27 | 7.3 |
| Na-SP | -8 ± 5.0 | -7.3 ± 2.9 | 10 ± 2.2 | 95 ± 2.9 | 10.5 |
| H-SP | -8.7 ± 1.7 | -9.7 ± 6.0 | -1.3 ± 0.45 | 13 ± 3.4 | >100 |
| DS-SP | -12 ± 4.9 | -13 ± 5.4 | -12 ± 3.5 | 9.3 ± 4.0 | >25 |
| Dextran sulfate | -13 ± 5.4 | -8.6 ± 1.2 | 3.8 ± 0.64 | 74 ± 9.9 | 14.2 |

Na-SP: $Ca^{+2}$ was exchanged with $Na^{+}$, H-SP:$Ca^{+2}$ was exchanged with $H^{+}$, DS-SP: desulfated calcium spirulan.

Table 3 shows that Ca-SP exhibited dose-dependent inhibition against syncytium formation, while other related polysaccharides showed stimulating effect on cell fusion at lower concentration. H-SP and DS-SP did not exert marked inhibition against sycytium formation up to 25 μg/ml. When Na-HOR and Ca-HOR were evaluated for their effect against syncytium formation, dose-dependent inhibition was observed in Ca-HOR, and the stimulation of cell fusion was seen in Na-HOR at less than 2 μg/ml.

So far, the mechanism by which sulfated polysaccharides inhibit virus replication has been

generally explained by the inhibition of virus attachment to host cells and by the subsequent virus-cell fusion, or virus penetration. In the time-of-addition experiments, the anti-HSV-1 activites of Ca-SP and Na-HOR were compared with that of DS (Table 4). Treatment of host cells with the

Table 4. Effect of time of addition on HSV-1 replication

| Time of addition | | | | | antiviral activity ($IC_{50}$, μg/ml) | | |
|---|---|---|---|---|---|---|---|
| 3h before infection | during infection | 0-1h p.i[a] | 1-2 h p.i | 2-24 h p.i | Ca-SP | Na-HOR | DS |
| +[b] | - | - | - | - | > 200[c] | >200 | >200 |
| - | + | - | - | - | 0.97 | 1.6 | 0.72 |
| - | - | + | - | - | > 200 | >200 | >200 |
| - | - | - | + | - | > 200 | >200 | >200 |
| - | - | - | - | + | 24.5 | 3.8 | >200 |
| + | + | - | - | - | 0.92 | 1.2 | 2.5 |
| - | + | + | - | - | 1.2 | 3.4 | 2.9 |
| - | - | + | + | - | > 200 | >200 | >200 |
| + | + | + | + | + | 0.95 | 3.0 | 0.91 |
| - | + | + | + | + | 0.83 | 2.4 | 19 |
| - | - | + | + | + | 13.2 | 7.6 | 146 |

[a] post infection. [b] HeLa cells were treated in the absence (-) or presence (+) of different concentration of sample during the period indicated. [c] Each value is the mean ± SD of triplicate assays. Ca-SP: calcium spirulan, NaHOR: sodium hornan, DS: dextran sulfate.

sample for 3 h before infection showed no inhibitory effect against HSV-1 replication. When they were present during infection, all polysaccharides suppressed virus replication efficiently, and there was no striking difference in antiviral effect among three compounds. When antiviral activity was evaluated in the cultures treated with the compound immediately after or 2 h after infection and throughout the incubation thereafter, however, the $IC_{50}$ values for Ca-SP and Na-HOR were 10- or 50-fold lower than that of DS, respectively. These findings suggested that Ca-SP and Na-HOR inhibited not only the initial stages of viral infection but also later stages after virus penetration.

Thus, Ca-SP and Na-HOR could be good candidates for antiviral agents with novel action mechanism. Further detailed action mechanisms of these sulfated polysaccharides are currently under investigation.

## References

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