New Substances against Human Immunodeficiency Virus: Sulfated 5′-Nucleotidase Inhibitory Polyphenols

Toshikatsu TOUKAIRIN,[a] Keijiro UCHINO,*[,a] Takashi MIZUNO,[a] Hideki NAKASHIMA,[b] Naoki YAMAMOTO,[b] and Hiroshi OGAWARA[c]

Central Laboratory, Nippon Flour Mills Co., Ltd.,[a] 2114-2, Nurumizu, Atsugi, Kanagawa 243, Japan, Department of Virology and Parasitology, Yamaguchi University, School of Medicine,[b] 1114, Kogushi, Ube, Yamaguchi 755, Japan, and Department of Biochemistry, Meiji College of Pharmacy,[c] Nozawa-1, Setagaya-ku, Tokyo 154, Japan. *Received September 7, 1991*

Two sulfated polyphenols, NF-86II-S-13.3 and NF-86II-S-6.2, were synthesized from NF-86II without sulfur their inhibitory effect on human immunodeficiency virus (HIV) infection *in vitro* was examined, using cytopathic effect and an antigen expression assay system in MT-4 cells. NF-86II-S-13.3 (sulfur content, 13.3%) completely inhibited the cytopathic effect of HIV and the HIV-specific antigen expression in MT-4 cells at concentrations of more than 6.3 μg/ml. The effect of NF-86II-S-6.2 (sulfur content, 6.2%) was much weaker than that of NF-86IIS-13.3. On the other hand, NF-86II without sulfur did not show any activity at all.

Keywords AIDS; HIV; sulfated polyphenol; 5′-nucleotidase; inhibitor; cytopathic effect; *Areca catechu*

Recently, it has been discovered that several retroviruses can cause severe human diseases.[1-4] Human immunodeficiency virus [HIV (HTLV-III/LAV)] is a newly recognized retrovirus which is cytopathic for helper-induced T cells. This virus is an etiological agent associated with the acquired immune deficiency syndrome (AIDS) and its related disorders.[5-10] Therefore, development of anti-HIV drug is urgent. Several compounds such as azidothymidine (AZT),[11,12] glycyrrhizin,[13] interferon-α,[14] and interferon-γ[15] have already been shown to have an inhibitory effect on HIV infection. Furthermore, we reported that sulfated polysaccharides[16] and sulfated glycyrrhizin[17] efficiently inhibited the HIV infection *in vitro*. However, the anti-HIV effect of sulfated polyphenols has not been described.

We have isolated a new 5′-nucleotidase inhibitor, named NF-86II, from the seed of *Areca catechu* L.[18] This compound was a polyphenolic substance and showed antitumor activity *in vitro*.[19] In the present study, we synthesized sulfated derivatives of the 5′-nucleotidase inhibitory polyphenol and examined their inhibitory effect on HIV infection using cytopathic effect (CPE) and an antigen expression assay system in MT-4 cells.

Materials and Methods

Sulfation of NF-86II 5′-Nucleotidase inhibitor, NF-86II was prepared from the seeds of *Areca catechu* L. as described in the previous paper.[18] NF-86II is a one-to-one mixture of two compounds, NPF-86IIA and NPF-86IIB.

NF-86II (200 mg) was suspended in 30 ml of anhydrous pyridine and treated with chlorosulfonic acid (7.6 g) at 0 °C. The reaction mixture was kept at room temperature for 2 d. The pasty reaction product was dissolved in 80 ml of ice water, neutralized with a saturated solution of Na_2CO_3, and dialyzed in cellulose tubing (spectra/pore 6: molecular mass cut: 1000) against distilled water for 7 d. The non-dialyzable solution was lyophilized to yield NF-86II sulfate (NF-86II-S, 254 mg). We synthesized two derivatives depending on the sulfur content (S content, 13.3% = NF-86II-S-13.3; S content, 6.2% = NF-86-II-S-6.2).

Cells and Virus MT-4 cells, which are an HTLV-I carrying cell line, were cultured in an RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin (100 IU/ml) and streptomycin (100 μg/ml) at 37 °C in a humidified atmosphere of 5% CO_2. The virus strain used was $HIV_{HTLV-IIIB}$.

Cell Growth and HIV-Induced Cytopathic Effect MT-4 cells and HIV-infected MT-4 cells were adjusted to 3×10^5 cells/ml and cultured in the presence of various concentrations of the drug. On the 3rd d after infection, one-third of the medium in each culture was changed. At 3 and 6 d after infection, the viable cells were counted by a trypan blue dye exclusion method.[12]

Immunofluorescence Assay Inhibitory effect of NF-86II-S on the expression of HIV-specific antigen was determined by an indirect immunofluorescence (IF) method using HIV antibody-positive sera.[12] IF-positive cells were counted under a fluorescence microscope, and the percentage of HIV antigen-positive cells was calculated.

Results

Effect on the Cell Growth and Virus-Induced Cytophathic Effect The effects of NF-86II-S-13.3 and NF-86II-S-6.2 on the growth of MT-4 cells, and their inhibitory effect on the virus-induced cytophathic effect in HIV-infected MT-4 cells were tested after 3 and 6 d in culture (Fig. 1). HIV-induced CPE was completely prevented by NF-86II-S-13.3 at concentrations of more than 6.3 μg/ml. In the absence of virus, NF-86II-S-13.3 had no significant cytotoxicity up to 100 μg/ml. However, NF-86II-S-6.2 was more toxic than NF-86II-S-13.3. Also, the inhibitory effect on the HIV-induced cytophathic effect of NF-86II-S-6.2 was much weaker than that of NF-86II-S-13.3. NF-86II without sulfur did not show this activity (data not shown).

Inhibitory Effect on the Expression of HIV-Specific Antigen When MT-4 cells were infected with HTLV-III, 73% of the cells became positive for virus antigens 3 d after infection, and more than 90% were positive 6 d after infection. At concentrations of more than 6.3 μg/ml, positive cells were only less than 1% (Table I). The frequency of antigen-positive cells was 1% and 30% in HIV-infected MT-4 cells at 6 d after infection treated with 100 μg/ml and 50 μg/ml of NF-86II-S-6.2, respectively.

Discussion

AZT, a nucleoside analog, was reported to block the *in vitro* infectivity and cytopathic effect of HTLV-III.[11] AZT was further examined clinically, but many issues still remained to be solved concerning the clinical use of this drug, especially for long-term administration.[20] Therefore, it seems important to explore other effective drugs.

In the course of screening work for new types of 5′-nucleotidase inhibitors, novel polyphenolic substances were isolated from the seeds of *Areca catechu* L. (Palmae).[18] These inhibitors showed antitumor activity, prolonging the life span of mice inoculated with Ehrlich ascites carci-

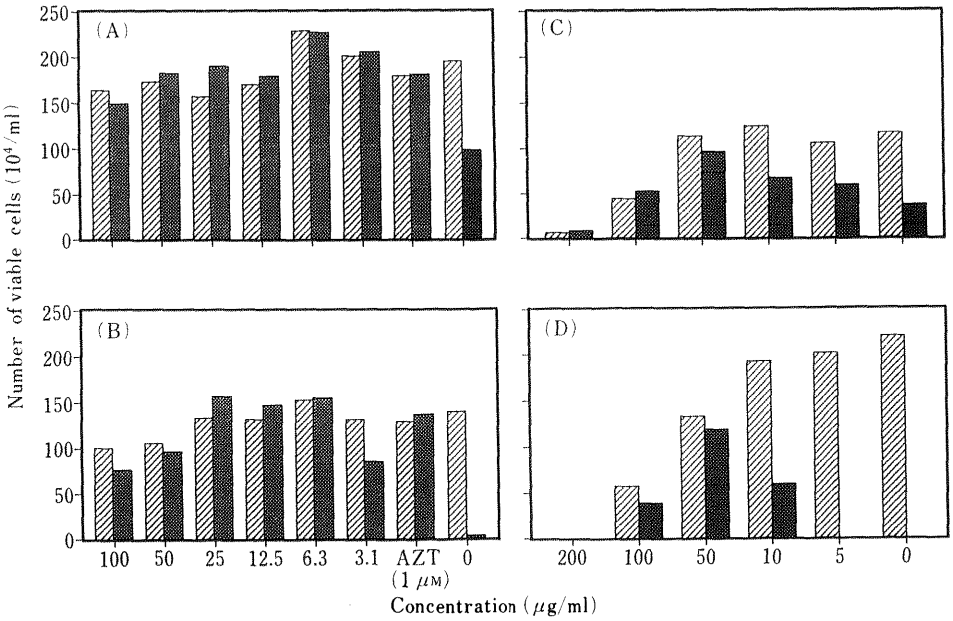

Fig. 1. Effects of NF-86II-S-13.3 (A: 3d, B: 6d) and NF-86II-S-6.2 (C: 3d, D: 6d) on the Cell Growth of MT-4 Cells (▨), and Inhibitory Effect on the Virus-Induced Cytopathic Effects in HIV-Infected MT-4 Cells (▩).

TABLE I. Inhibitory Effects of NF-86II-S-13.3 and NF-86IIS-6.2 on the Expression of HIV-Specific Antigen Determined by an Indirect Immunofluorescence Method

Concentration (μg/ml)	Percentage of IF-positive cells[a]			
	NF-86II-S-13.3		NF-86II-S-6.2	
	3d	6d	3d	6d
200	—	—	0	0
100	<0.2	<1	1	1
50	<0.2	<1	1	30
25	<0.2	<1	—	—
12.5	<0.2	<1	—	—
10	—	—	20	20
6.3	<0.2	<1	—	—
5	—	—	33	100
3.1	<0.2	86	—	—
0	73	>90	48	100

a) More than 500 cells were counted, and the percentage of IF-positive cells was calculated 3 and 6d after infection. —, not counted.

noma, but did not show any significant cytotoxicity against various mammalian cells in culture.[19]

Recently, it has been reported that sulfated polysaccharides have an inhibitory effect on HIV infection.[16] In the present study, we synthesized sulfated derivatives of the 5′-nucleotidase inhibitory polyphenol, NF-86II-S, and examined their inhibitory effect on HIV infection. The structure of NF-86II-S has not been established because of the high molecular weight. However, monomeric polyphenols ((+)-catechin, (−)-epicatechin), and their oligomeric compounds have been isolated from the seeds of *Areca catechu* L.[21,22] It is considered that the 5′-nucleotidase inhibitory polyphenol, isolated from this plant, was a polymeric compound of (+)-catechin and/or (−)-epicatechin. Therefore, hydroxyl-groups of the catechin unit of the inhibitor, NF-86II, were sulfated by chlorosulfonic acid.

NF-86II-S inhibited the cytopathic effect of HIV and the HIV-specific antigen expression in MT-4 cells. The anti-HIV activity of sulfated NF-86II depends mainly on the degree of substitution with sulfate groups of the phenolic molecule. NF-86II without sulfur did not show these activities: sulfated NF-86II lost its original 5′-nucleotidase inhibitory activity.

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