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Pentosan polysulfate, a sulfated oligosaccharide, is a potent and selective anti-HIV agent in vitro

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Summary

Several sulfated oligo- or polysaccharides such as pentosan polysulfate, fucoidan, dextran sulfate, heparin and ι-, κ- and λ-carrageenans proved to be potent and selective inhibitors of human immunodeficiency virus type 1 (HIV-1) in vitro. The most potent anti-HIV-1 activity was recorded for the oligosaccharide pentosan polysulfate, its 50% antiviral effective dose (ED_{50}) being 0.19 μg/ml in MT-4 cells. It inhibited HIV-1 antigen expression in HUT-78 cells at an ED_{50} of 0.02 μg/ml, and complete inhibition of HIV-1 antigen expression was obtained at a concentration of 4.0 μg/ml. No toxicity for MT-4 cells was observed with pentosan polysulfate at a concentration of 2500 μg/ml. The anticoagulant activity of pentosan polysulfate was more than ten-fold lower than that of heparin (14.4 and 177 U/mg, respectively). In fact, pentosan polysulfate achieved its anti-HIV-1 activity at a concentration that is 370-fold below its anticoagulant threshold (1 U). Pentosan polysulfate inhibits virus adsorption to the cells, as was demonstrated by monitoring the association of radiolabeled HIV-1 virions with MT-4 cells.

Pentosan polysulfate; Sulfated polysaccharide; Human immunodeficiency virus

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Introduction

There are a number of compounds that show inhibitory activity in vitro against human immunodeficiency virus type 1 (HIV-1), the causative agent of acquired immunodeficiency syndrome (AIDS) (De Clercq, 1976). 3′-Azido-2′,3′-dideoxythymidine (azidothymidine) (Mitsuya et al., 1985) is the first drug that has been approved for clinical use in patients suffering from AIDS or AIDS-related complex (ARC). Since the antiviral effect of azidothymidine and related compounds (i.e., 2′,3′-dideoxynucleosides) (Mitsuya and Broder, 1986), is based upon the inhibition of the retrovirus-encoded reverse transcriptase (RT) following phosphorylation of the compounds by cellular enzymes to the corresponding 5′-triphosphates (Furman et al., 1986), toxicity for the host, caused by the compounds' metabolites (Cooney et al., 1986), seems to be an inevitable drawback associated with the long-term use of these drugs (Richman et al., 1987; Yarchoan et al., 1988). It is mandatory, therefore, to search for more potent and less toxic compounds and preferably compounds other than nucleoside analogues. We have now explored the inhibitory effect of various polysaccharides on HIV-1 replication in vitro, their anticoagulant activity and the mechanism of anti-HIV-1 action of the compounds.

Materials and Methods

Cells

MT-4, a T4 lymphocyte cell line carrying HTLV-I (Harada et al., 1985), and HUT-78, a T4 lymphocyte cell line, not carrying HTLV-I (Levy et al., 1984), were used for the anti-HIV-1 assay. The cells were mycoplasma negative. The cell lines were grown and maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 IU/ml penicillin G and 20 μg/ml gentamicin (culture medium).

Viruses

HIV-1 was obtained from the culture supernatant of a HUT-78 cell line persistently infected with HTLV-III_B (HUT-78/HTLV-III_B). The titer of the virus stock was 2×10^5 50% cell culture infective dose ($CCID_{50}$) per ml.

Radiolabeled HIV-1 particles were obtained from the supernatant of HUT-78/HTLV-III_B cultures. Briefly, HUT-78/HTLV-III_B cells were cultured with 1 mCi [5-^{3}H]Urd (30 Ci/mmol, Amersham, Great Britain). After 3 days, the supernatant was collected, centrifuged at low speed and then ultracentrifuged at 100000 × *g* for 2 h. Pellets were resuspended in culture medium, and the HIV-1 particles were further purified by isopycnic ultracentrifugation on a 15-60% sucrose gradient. Radioactivity and virus titer of the final preparation were 1.1×10^6 cpm/ml and 1×10^7 $CCID_{50}$/ml, respectively.

Compounds

Pentosan polysulfate, Fucoidan, dextran sulfate – approximate molecular weight (MW): 5000 – dextran (MW 90000) and ι-, κ- and λ-carrageenans were purchased from Sigma Chemical Co., St. Louis, MO. Sodium heparin (MW 15000) and dermatan sulfate were kindly provided by Dr. Godtfredsen, Leo Pharmaceutical Products Ltd., Ballerup, Denmark. Chondroitin sulfate was obtained from Organon, Oss, The Netherlands, and N-desulfated heparin (MW 8800) was described previously (Irimura et al., 1986).

Antiviral assay

The procedure for measuring anti-HIV-1 activity in MT-4 cells has been described previously (Pauwels et al., 1987). Briefly, MT-4 cells (input cell number: 3×10^4 cells/well) were cultured in microtray wells in the presence of various concentrations of the test compounds added immediately after infection with 100 $CCID_{50}$ of HIV-1 ($CCID_{50}$ being the 50% cell culture infective dose). After 5 days 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, which is based on the conversion of a yellow tetrazolium dye by living cells to a blue formazan product (Pauwels et al., 1988). Antiviral activity and cytotoxicity of the test compounds are expressed as ED_{50} and CD_{50}, which correspond to the doses required to reduce the number of viable cells in the virus- and mock-infected cell cultures by 50%, respectively.

Anti-HIV-1 activity of the compounds was also determined by monitoring viral antigen expression in HUT-78 cells. HUT-78 cells were infected with HIV-1 at a multiplicity of infection of 0.4, and immediately thereafter varying concentrations of the test compounds were added. Every 4 days, three quarters of the culture medium were replenished. At day 12, viral antigen expression was measured, using indirect immunofluorescence (with polyclonal antibody as probe) and laser flow cytofluorography (FACSTAR®, Becton Dickinson), as previously described (Pauwels et al., 1987).

Anticoagulant test

Anticoagulant activity of the compounds was measured according to the clotting procedure for the quantitative determination of heparin in plasma (Sigma Technical Bulletin No. 870, Sigma Chemical Co.) and is expressed in international units.

Reverse transcriptase assay

HIV-1 reverse transcriptase (RT) was obtained from the disrupted virions which had been partially purified by centrifugation of the supernatant of HUT-78/HTLV-III_B, followed by filtration (0.45 μ) and ultracentrifugation. The inhibitory effect of the compounds on HIV-1-RT was determined with poly(rA)·oligo(dT) (0.01 OD_{260}) as the template-primer, as previously described (Nakashima et al., 1987).

Virus adsorption assay

Two million MT-4 cells were suspended in medium (final volume: 500 μl) con-

taining 25 μg/ml pentosan polysulfate, fucoidan or no compound. Ten microlitre of the virus suspension was added, and the samples were incubated at 37°C for 0, 30, 60 or 120 min, upon which the cells were collected and washed three times with phosphate-buffered saline so as to remove unadsorbed virus particles. Cell-associated acid-insoluble material was analyzed for radioactivity after precipitation with 5% trichloroacetic acid.

Results

When these compounds were evaluated for their inhibitory effects on the cytopathogenicity of HIV-1 in MT-4 cells, several sulfated oligo- and polysaccharides were found to be potent and selective inhibitors of HIV-1 replication; viz. pentosan polysulfate, fucoidan, dextran sulfate, heparin and ι-, κ- and λ-carrageenans (Table 1). In contrast, chondroitin sulfate was only slightly active, whereas dextran, N-desulfated heparin and dermatan sulfate were totally inactive. The most potent anti-HIV-1 activity was recorded for pentosan polysulfate, its 50% antiviral effective dose (ED_{50}) being as low as 0.19 μg/ml. Dextran sulfate, λ-carrageenan and heparin also inhibited HIV-1 replication at very low concentrations, their ED_{50} being 0.30, 0.54 and 0.58 μg/ml, respectively. Fucoidan and κ-carrageenan were 10-fold less active than pentosan polysulfate. Except for fucoidan, the compounds did not show any in vitro toxicity for the host MT-4 cells at the highest concentration assayed (2500 μg/ml for pentosan polysulfate, dextran sulfate and heparin; 625 μg/ml for the ι-, κ- and λ-carrageenans). For fucoidan, its 50% cytotoxic dose (CD_{50}) was about 1000 μg/ml.

When pentosan polysulfate and fucoidan were further evaluated for their inhib-

TABLE 1

Anti-HIV-1 activity and anticoagulant activity of polysaccharides

Compound	ED_{50}[a] (μg/ml)	CD_{50}[b] (μg/ml)	Anticoagulant activity (U/mg)
Pentosan polysulfate (MW 3100)	0.19 ± 0.12	> 2500	14.4
Fucoidan	1.4 ± 0.43	1060 ± 210	2.6
Dextran sulfate (MW 5000)	0.30 ± 0.10	> 2500	14.7
Dextran (MW 90000)	> 625	> 2500	< 0.01
Heparin (MW 11000)	0.58 ± 0.14	> 2500	177
N-desulfated heparin (MW 8800)	> 625	> 2500	0.6
Dermatan sulfate	> 625	> 2500	< 0.01
Chondroitin sulfate	230 ± 14	> 2500	0.5
ι-Carrageenan	12 ± 1.0	> 625	3.2
κ-Carrageenan	2.5 ± 0.30	> 625	2.9
λ-Carrageenan	0.54 ± 0.02	> 625	4.2

[a] 50% Antiviral effective dose, based on the inhibition of HIV-1 induced cytopathogenicity in MT-4 cells. Data represent mean values with standard deviations for three separate experiments.

[b] 50% Cytotoxic dose, based on the reduction of the viability of mock-infected MT-4 cells. Data represent mean values with standard deviations for three separate experiments.

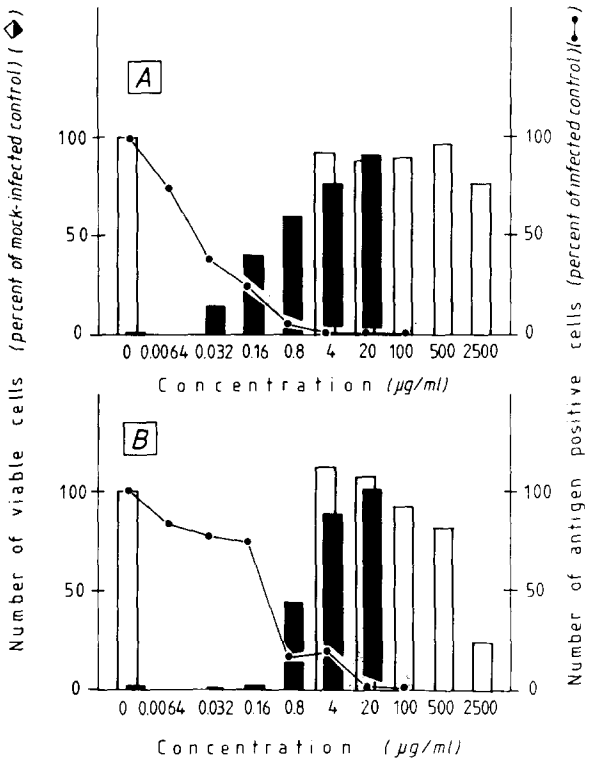

Fig. 1. Inhibitory effect of pentosan polysulfate (panel A) and fucoidan (panel B) on HIV-1-induced cytopathogenicity in MT-4 cells and HIV-1 antigen expression in HUT-78 cells. Viability of virus-infected (■) and mock-infected (□) MT-4 cells was measured by the MTT method (see Materials and Methods) at day 5 after infection. The number of viable cells was expressed as percent of mock-infected untreated control cells. HIV-1 antigen-positive HUT-78 cells were detected by indirect immunofluorescence and laser flow cytofluorography, using polyclonal antibody as probe. The number of HIV-1 antigen-positive cells (●——●) was expressed as percent of virus-infected untreated control cells.

itory effect on HIV-1 antigen expression in HUT-78 cells, pentosan polysulfate and fucoidan achieved a greater than 50% reduction in the number of antigen positive cells at a concentration of 0.032 μg/ml and 0.16 μg/ml, respectively (Fig. 1). Neither pentosan polysulfate nor fucoidan affected growth or viability of HUT-78 cells at a concentration up to 100 μg/ml (the highest concentration used in the indirect immunofluorescence assays) (data not shown).

Since sulfated polysaccharides are known to interfere with the blood coagulation process, it was very important to compare their anticoagulant activities with their anti-HIV-1 effects in view of their potential clinical applicability in AIDS patients. As shown in Table 1, anticoagulant activity was detected for all compounds found active against HIV-1. The highest anticoagulant activity was achieved by heparin (177 U/mg). The anticoagulant effects of the other sulfated polysaccharides were more than 10 times lower that that of heparin. When the ED_{50} values

for anti-HIV-1 activity in MT-4 cells (Table 1) were converted from μg/ml to U/ml, the following values were obtained: pentosan polysulfate, 2.7×10^{-3} U/ml; fucoidan, 3.6×10^{-3} U/ml; ι-carrageenan, 3.8×10^{-2} U/ml; κ-carrageenan, 7.3×10^{-3} U/ml; λ-carrageenan, 2.3×10^{-3} U/ml; dextran sulfate, 4.4×10^{-3} U/ml; heparin, 1.0×10^{-1} U/ml. Thus, except for heparin and ι-carrageenan, all compounds are active against HIV-1 at concentrations that are more than 100-fold lower than the anticoagulant threshold (1 U). In this respect, pentosan polysulfate and λ-carrageenan show the highest selectivity index (370 and 430, respectively).

Sulfated polysaccharides have been reported to inhibit retrovirus-associated reverse transcriptase (RT) activity (Nakashima et al., 1987; Sydow and Kröcking, 1987). Therefore, pentosan polysulfate, fucoidan, dextran sulfate, heparin and dextran were evaluated for their inhibitory effect on HIV-1 RT. The 50% inhibitory doses (ID_{50}) of pentosan polysulfate, fucoidan, dextran sulfate and heparin were 19.1, 29.5, 32.9 and 410 μg/ml, respectively. The ID_{50} of dextran was > 1000 μg/ml. The corresponding ID_{50} value found for suramin, a well-known inhibitor of reverse transcriptase (De Clercq, 1979), was 17.8 μg/ml. Whereas for suramin the minimum concentration required to block HIV-1 replication coincides with its ID_{50} for HIV-1 RT (De Clercq, 1987a), the concentrations of the sulfated polysaccharides that are needed to inhibit HIV-1 RT were considerably higher than those required for inhibition of HIV-1 replication in cell culture (Table 1, Fig. 1). Furthermore, the inhibitory effect of the sulfated polysaccharides, but not that of suramin, on HIV-1 RT, were annihilated if serum bovine albumin (25 μg/ml) was added to the reaction mixture (data not shown).

Previous studies have suggested that anionic polysaccharides may interfere with

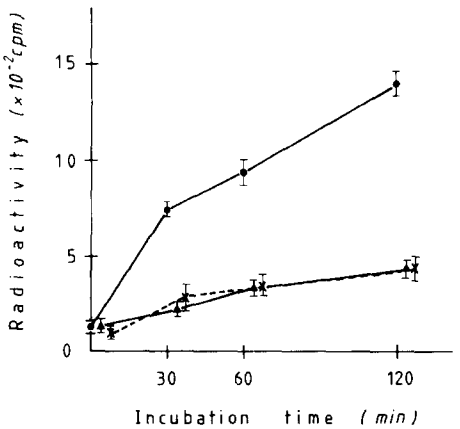

Fig. 2. Effect of pentosan polysulfate and fucoidan on the adsorption of radiolabeled HIV-1 particles to MT-4 cells. Suspensions of MT-4 cells with the test compounds – 25 μg/ml pentosan polysulfate (▲), fucoidan (x) or no compound (•) – and [5-^{3}H]uridine-labeled purified HIV-1 particles were prepared. The samples were incubated at 37°C for 0, 30, 60 or 120 min, upon which the cells were collected and washed three times with phosphate-buffered saline to remove unadsorbed virus particles. Cell-associated acid-insoluble material was analyzed for radioactivity after precipitation with 5% trichloroacetic acid. Radioactivity background (in the absence of radiolabeled virus) was less than 30 cpm.

virus adsorption to the host cells (De Somer et al., 1968). To determine whether the sulfated oligo- and polysaccharides may owe their anti-HIV-1 activity to such a mode of action, we examined the effects of pentosan polysulfate and fucoidan on the HIV-1 adsorption process, using radiolabeled purified HIV-1 particles. When [5-^{3}H]uridine-labeled virus (11 000 cpm) was incubated with a MT-4 cell suspension in the absence of the test compounds, cell associated radioactivity increased with the incubation time (Fig. 2). In contrast, when pentosan polysulfate or fucoidan had been added to the cell suspension at a concentration of 25 μg/ml – a concentration that was slightly higher than that required for complete protection of MT-4 cells against HIV-1 cytopathogenicity (Fig. 1), – only a very small increase in cell-associated radioactivity was observed (Fig. 2). These results indicate that pentosan polysulfate and fucoidan prevented the adsorption of HIV-1 to MT-4 cells. This conclusion was confirmed using indirect immunofluorescence and laser flow cytofluorographic (FACSTAR ®) analysis of MT-4 cells which had been exposed to HIV-1 virions in the presence or absence of the compounds: the sulfated oligo(poly)saccharides completely blocked virus attachment to the cells (data not shown).

Discussion

Virus adsorption has been proposed as a possible target, and polyanionic substances as a possible approach, in the search for chemotherapeutic agents against AIDS (De Clercq, 1986). That polyanionic substances may have considerable potential as anti-HIV-1 agents is supported by the finding of Ito et al. (1987) and Ueno and Kuno (1987), who showed that dextran sulfate and heparin inhibited HIV-1 replication in vitro at non-toxic concentrations. A major problem that should be addressed if these compounds are to be used in AIDS patients is their anticoagulant activity. The results presented here clearly indicate that several sulfated oligo(poly)saccharides are active against HIV-1 at a concentration which is considerably lower than that which interferes with blood coagulation. In this respect, pentosan polysulfate is superior to heparin and other sulfated polysaccharides in that it combines potent anti-HIV-1 activity with weak anticoagulant activity (Table 1). Moreover, the mean molecular weight of pentosan polysulfate is approximately 3 100, as determined by high speed permeation chromatography (Nakajima et al., 1984), and this molecular weight is only twice that of suramin (MW 1429.2). Suramin can be taken up by the cells and presumably acts intracellularly as a RT inhibitor (De Clercq, 1979, 1987a). Like suramin (De Clercq, 1979), pentosan polysulfate also behaves as a competitive inhibitor for the template-primer in the reverse transcriptase reaction (data not shown). To be active as a RT inhibitor pentosan polysulfate needs to be taken up by the cells, and whether this actually occurs remains subject of further study. In the abeyance of such data in view of the marked inhibition of virus-cell attachment observed in the presence of pentosan polysulfate, it seems logical to attribute its anti-HIV-1 activity to an inhibitory effect on the virus adsorption process.

In view of the severity of the AIDS problem and the relatively serious toxicity shown by the compounds (azidothymidine, dideoxycytidine) used so far in the treatment of AIDS (Richman et al., 1987; Yarchoan et al., 1988), other therapeutic modalities should be urgently pursued, and in this respect pentosan polysulfate merits, because of its highly potent and selective activity against HIV-1, due consideration.

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References

Cooney, D.A., Dalal, M., Mitsuya, H., McMahon, J.B., Nadkarni, M., Balzarini, J., Broder, S. and Johns, D.G. (1986) Initial studies on the cellular pharmacology of 2′,3′-dideoxycytidine, an inhibition of HTLV-III infectivity. Biochem. Pharmacol. 35, 2065–2068.

De Clercq, E. (1979) Suramin: a potent inhibition of the reverse transcriptase of RNA tumor viruses. Cancer Lett. 8, 9–22.

De Clercq, E. (1986) Chemotherapeutic approaches to the treatment of the acquired immune deficiency syndrome (AIDS). J. Med. Chem. 29, 1561–1569.

De Clercq, E. (1987a) Suramin in the treatment of AIDS: mechanism of action. Antiviral Res. 7, 1–10.

De Clercq, E. (1987b) New selective antiviral agents active against the AIDS virus. Trends Pharmacol. Sci. (TIPS) 8, 339–345.

De Somer, P., De Clercq, E., Billiau, A., Schonne, E. and Claesen, M. (1968) Antiviral activity of polyacrylic and polymethacrylic acid. I. Mode of action in vitro. J. Virol. 2, 878–885.

Furman, P.A., Fyfe, J.A., St. Clair, M.H., Weinhold, K., Rideout, J.L., Freeman, G.A., Nusinoff-Lehrman, S., Bolognesi, D.P., Broder, S., Mitsuya, H. and Barry, D.W. (1986) Phosphorylation of 3′-azido-3′-deoxythymidine and selective interaction of the 5′-triphosphate with human immunodeficiency virus reverse transcriptase. Proc. Natl. Acad. Sci. USA 83, 8333–8337.

Harada, S., Koyanagi, Y. and Yamamoto, N. (1985) Infection of HTLV-III/LAV in HTLV-I carrying cell MT-2 and MT-4 cells and application in a plaque assay. Science 229, 563–566.

Irimura, T., Nakajima, M. and Nicolson, G.L. (1986) Chemically modified heparins as inhibitors of heparan sulfate specific endo-β-glucuronidase (heparanase) of metastatic melanoma cells. Biochemistry 25, 5322–5328.

Ito, M., Baba, M., Sato, A., Pauwels, R., De Clercq, E. and Shigeta, S. (1987) Inhibitory effect of dextran sulfate and heparin on the replication of human immunodeficiency virus (HIV) in vitro. Antiviral Res. 7, 361–367.

Levy, J.A., Hoffman, A.D., Kramer, S.M., Landis, J.A., Shimabukuro, J.M. and Oshiro, L.S. (1984) Isolation of lymphocytopathic retroviruses from San Francisco patients with AIDS. Science 225, 840–842.

Mitsuya, H., Weinhold, K.J., Furman, P.A., St. Clair, M.H., Nusinoff-Lehrman, S., Gallo, R.C., Bolognesi, D.P., Barry, D.W. and Broder, S. (1985) 3′-Azido-3′-deoxythymidine (BW A509U): an antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus in vitro. Proc. Natl. Acad. Sci. USA 82, 7096–7100.

Mitsuya, H. and Broder, S. (1986) Inhibition of the in vitro infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus (HTLV-III/LAV) by 2′,3′-dideoxynucleosides. Proc. Natl. Acad. Sci. USA 83, 1911–1915.

Nakajima, M., Irimura, T., Di Ferrante, N. and Nicolson, G.L. (1984) Metastatic melanoma cell heparanase. Characterization of heparan sulfate degradation fragments produced by B16 melanoma endoglucuronidase. J. Biol. Chem. 259, 2283–2290.

Nakashima, H., Kido, Y., Kobayashi, N., Motoki, Y., Neushul, M. and Yamamoto, N. (1987) Purification and characterization of an avian myeloblastosis and human immunodeficiency virus reverse transcriptase inhibitor, sulfated polysaccharides extracted from sea algae. Antimicrob. Agents Chemother. 31, 1524–1528.

Pauwels, R., De Clercq, E., Desmyter, J., Balzarini, J., Goubau, P., Herdewijn, P., Vanderhaeghe, H. and Vandeputte, M. (1987) Sensitive and rapid assay on MT-4 cells for detection of antiviral compounds against the AIDS virus. J. Virol. Methods 16, 171–185.

Pauwels, R., Balzarini, J., Baba, M., Snoeck, R., Schols, D., Herdewijn, P., Desmyter, J. and De Clercq, E. (1988) Rapid and automated tetrazolium-based colorimetric assay for the detection of anti-HIV compounds. J. Virol. Methods 20, 309–322.

Richman, D.D., Fischl, M.A., Grieco, M.H., Gottlieb, M.S., Volberding, P.A., Laskin, O.L., Leedom, J.M., Groopman, J.E., Mildvan, D., Hirsch, M.S., Jackson, G.G., Durack, D.T. and Nusinoff-Lehrman, S. (1987) The toxicity of azidothymidine (AZT) in the treatment of AIDS and AIDS-related complexes. A double-blind, placebo-controlled trial. N. Engl. J. Med. 317, 192–197.

Sydow, G. and Kröcking, H.-P. (1987) Effect of pentosan polysulfate (SP 54) on the reverse transcriptase activity of several retroviruses. Biomed. Biochem. Acta 46, 527–530.

Yarchoan, R., Perno, C.F., Thomas, R.V., Klecker, R.W., Allain, J.-P., Wills, R.J., McAtee, N., Fischl, M.A., Dubinsky, R., McNeely, M.C., Mitsuya, H., Pluda, J.M., Lawley, T.J., Leuther, M., Safai, B., Collins, J.M., Myers, C.E. and Broder, S. (1988) Phase I studies of 2′,3′-dideoxycytidine in severe human immunodeficiency virus infection as a single agent and alternating with zidovudine (AZT). Lancet i, 76–81.

Ueno, R. and Kuno, S. (1987) Dextran sulfate, a potent anti-HIV agent in vitro having synergism with zidovudine. Lancet i, 1379.