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Derivatives of 4-Amino-3,6-Disulfonato-1,8-Naphthalimide Inhibit Reverse Transcriptase and Suppress Human and Feline Immunodeficiency Virus Expression in Cultured Cells

Darryl Rideout, Raymond Schinazi, C. David Pauza, Kathleen Lovelace, Lin-Chang Chiang, Theodora Calogeropoulou, Michael McCarthy, and John H. Elder

Department of Molecular Biology, Research Institute of Scripps Clinic, La Jolla, California 92037 (D.R., K.L., L.-C.C., T.C., M.M., J.H.E.); Department of Pediatrics, Emory University School of Medicine, Laboratory of Biochemical Pharmacology and Veterans Affairs Medical Center, Decatur, Georgia 30033 (R.S.); Department of Pathology and Laboratory of Medicine, School of Medicine, University of Wisconsin-Madison, Madison, Wisconsin 53706 (C.D.P.)

Abstract We have developed a series of 4-amino-3,6-disulfonato-1,8-naphthalimide (ADSN) derivatives in an attempt to create nontoxic compounds effective against lentivirus infections. The ADSN derivative Lucifer Yellow CH ([N-(hydra zinocarbonyl)amino]-4-amino-3,6-disulfonato-1,8-naphthalimide) (LYCH) was chosen as a parent compound because of its low toxicity in vivo and in vitro and its tendency to accumulate in monocyte/macrophages, a major reservoir for lentiviruses in vivo. Several ADSN derivatives inhibited reverse transcriptases (RTs) from human immunodeficiency virus type 1 (HIV-1) and feline immunodeficiency virus (FIV). Viral expression in HIV-infected human peripheral blood mononuclear cells was inhibited by noncytotoxic concentrations of two ADSN derivatives, designated *A4* (biphenyl-4,4'-dicarboxaldehyde, Lucifer Yellow CH monohydrazone; EC_{50} = 29 μM after 6 days) and *H4* (biphenyl-4,4'-dicarboxaldehyde, Lucifer Yellow CH dihydrazone; EC_{50} = 5.61 μM). *A4* effectively suppressed the expression of FIV in infected Crandall feline kidney fibroblasts (CRFK) at 46.2 μM, reducing the RT levels by 97% after 19 days under conditions allowing direct cell-to-cell transmission of the virus. The viability of drug-treated FIV-infected CRFK cells increased significantly in the presence of *A4* relative to the viability of untreated virus-infected cells. In contrast to *A4* and *H4*, LYCH (which lacks the appended aromatic rings characteristic of *A4* and *H4*) had no inhibitory effects on either virus and did not inhibit RT ex vivo. However, flow cytometry studies showed that both *A4* and LYCH accumulate in two cell types that can support lentiviral infections: U937 human monocytic leukemic cells that have been induced to differentiate by using tetradecanoyl phorbol acetate, and CRFK cells. © 1993 Wiley-Liss, Inc.

Key words: polysulfonic acid, anti-retroviral, FIV, Lucifer Yellow CH, polyanion, AZT, suramin, monocyte, HIV, hydrazone

Currently, the primary treatment of acquired immune deficiency syndrome (AIDS) involves the use of 3'-azido-3'-deoxythymidine (AZT). After conversion to its 5'-triphosphate, AZT is selectively incorporated into viral DNA chains by the reverse transcriptase (RT) of human immunodeficiency virus type 1 (HIV-1), the causative agent of AIDS [Mitsuya et al., 1985; Sandstrom, 1989; Yarchoan et al., 1988]. Treatment with AZT can lead to partial remission of many signs and symptoms of AIDS, including aspects of dementia [Sandstrom, 1989; Yarchoan et al., 1988]. Unfortunately, AZT is inherently toxic to bone marrow and other tissues. This can severely compromise its clinical usefulness, especially in immunosuppressed patients [Richman et al., 1987]. In addition, viruses sometimes become resistant to AZT, which may also decrease its usefulness [Larder and Kemp, 1989; Larder et al., 1989]. Therefore, it is necessary to

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Theodora Calogeropoulou's current address is: Institute of Organic and Pharmaceutical Chemistry, National Hellenic Research Foundation, Athens, Greece.

Address reprint requests to Darryl Rideout, Department of Molecular Biology, Research Institute of Scripps Clinic, 10666 N. Torrey Pines Road, La Jolla, CA 92037.

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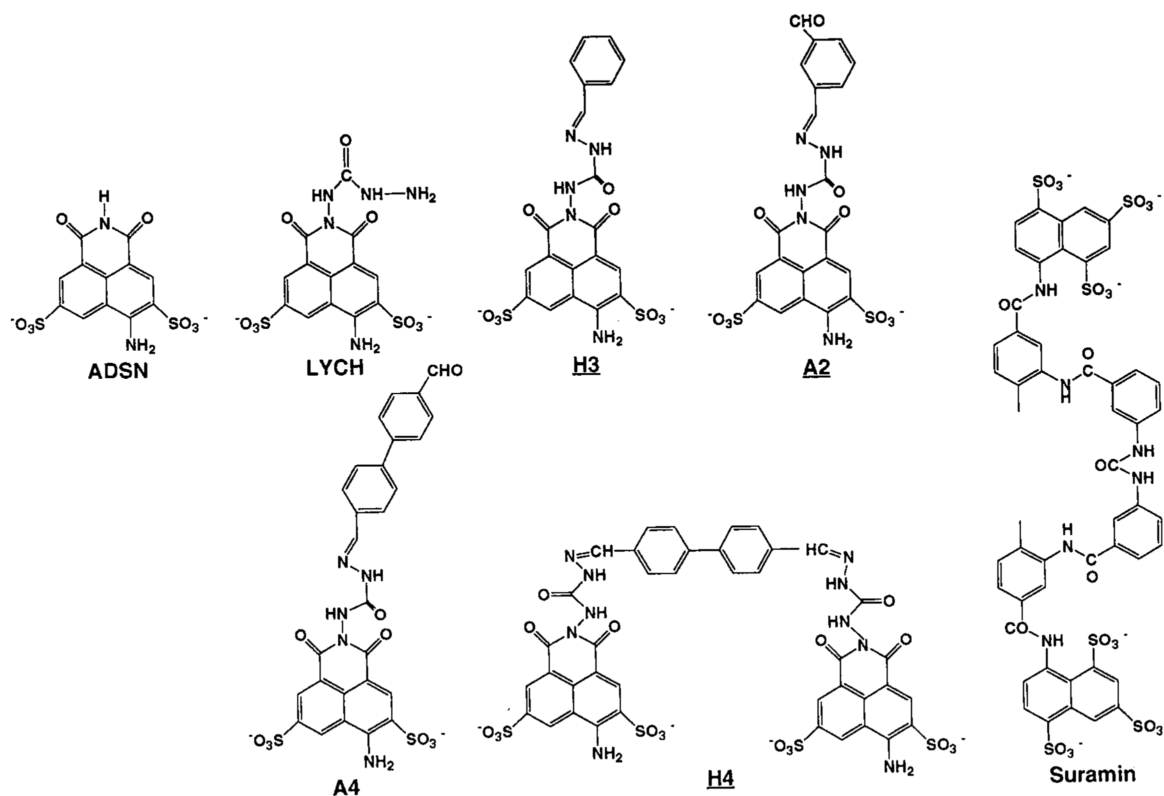

Fig. 1. Chemical structures of ADSN, five ADSN derivatives (LYCH, *A2*, *A4*, *H3*, and *H4*), and suramin.

develop other compounds, distinct from AZT, that will suppress lentivirus infections with a minimum of harmful side effects.

The fluorescent dye Lucifer Yellow CH (LYCH) (Fig. 1), a derivative of 4-amino-3,6-disulfonato-1,8-naphthalimide (ADSN) (Fig. 1), provides a unique starting point for the development of new antiviral agents active against lentiviruses. Structurally, LYCH resembles polysulfonatonaphthalene derivatives that inhibit HIV-1 [Mohan et al., 1991; Sandstrom, 1989; Jentsch et al., 1987]. LYCH is not very toxic to cultured human cells [Swanson et al., 1985] or to animals at high doses [Silverstein, personal communication]. It accumulates with some selectivity in macrophages [Scieszka and Cho, 1988; Swanson et al., 1985]. Anti-retroviral compounds that act preferentially in monocyte/macrophage cells might provide a direct way to inhibit HIV in this important reservoir in vivo [Fauci, 1988; Ho et al., 1987; Klatzman and Gluckman, 1986; Pauza, 1988]. LYCH and the other ADSN derivatives shown in Figure 1 bear no structural resemblance to AZT; their anti-retroviral activities should be affected little if at all by AZT resistance due to alterations in nucleoside transport, kinase activity, or the ability of RT to incorporate AZT triphosphate into DNA. In addition, the phototoxic properties of LYCH [Yamasaki and Katoh, 1988] suggest that ADSN derivatives can be used for photochemotherapeutic inhibition of HIV.

With these considerations in mind, we developed a series of ADSN derivatives (*A2*, *A4*, *H3*, and *H4*) that are structurally more similar than LYCH to known anti-retroviral agents [Jentsch et al., 1987; Mohan et al., 1991]. These derivatives were assayed for their effects on RT from HIV-1 and from the feline immunodeficiency virus (FIV). The lentivirus FIV is an increasingly important animal model for development of drug therapies against lentivirus infection [Egberink et al., 1990; North et al., 1989; Pedersen et al., 1987]. As will be shown, these derivatives inhibit RT from both viruses. *A4* and *H4* inhibit the proliferation of HIV in human peripheral blood mononuclear cells (PBMC) at noncytotoxic concentrations, as shown by a decrease in the levels of RT. Noncytotoxic concentrations of *A4* also inhibit the replication and cytopathic effect of FIV in cultured feline kidney fibroblasts.

METHODS

Materials

Lucifer Yellow CH (dilithium salt) and 4-aminonaphthalene-1,8-dicarboxylic anhydride (disodium salt) were either purchased (from Molecular Probes, Eugene, OR, or Aldrich Chemical Co., Milwaukee, WI, respectively) or synthesized according to the literature (see Scalera and Forster [1948] for sulfonation and Stewart [1981] for the remainder of the preparation). The sulfonation procedure was modified, as detailed in the following. Benzaldehyde, DIBAL (1.5 molar in toluene), and metaphthalaldehyde were purchased from Aldrich Chemical Company (Milwaukee, WI). Tetradecanoyl phorbol acetate was purchased from Sigma Chemical Company (St. Louis, MO). Basal Medium Eagle (BME) vitamins (100× stock) were purchased from Hazleton Biologics (Lanexa, KS). Gentamycin solution was purchased from ESI (Cherry Hill, NJ). Phosphonoformate (PFA) was provided by Astra Lab (Sodertalje, Sweden).

^{1}H nuclear magnetic resonance (NMR) and ^{13}C NMR spectra of new compounds were measured in D_2O by using a Brucker 300 MHz NMR spectrophotometer. Exact masses for the sulfonic acids were determined by fast atom bombardment negative-ion mass spectroscopy with a VG ZAB-VSF double-focusing high-resolution mass spectrometer. Positive-ion mass spectroscopy was used to determine the mass of biphenyl-4,4′-dicarboxaldehyde.

Synthetic Procedures

Biphenyl-4,4′-dicarboxaldehyde. Biphenyl-4,4′-dicarbonitrile (0.679 g, 3.33 mmoles) was dissolved in 100 ml dry tetrahydrofuran (THF) and cooled to −78°C under nitrogen. DIBAL (7 ml of a 1.5 molar solution in toluene, 10.5 mmol) was added dropwise via syringe, and the mixture was allowed to warm to ambient temperature and was stirred for 18 h. The reaction progress was followed by treating small aliquots with 10% aqueous H_2SO_4 and then saturated aqueous $NaHCO_3$. Aliquots were reduced with excess $NaBH_4$, and thin layer silica gel chromatography with 40:60 EtOAc/hexanes was used to detect the 4,4′-bis(hydroxymethyl)biphenyl and unreacted dinitrile (the dialdehyde and dinitrile are almost inseparable on silica gel). After the reaction was complete, the mixture was cooled to 0°C, and 10% aqueous H_2SO_4 was added cautiously with vigorous stirring until all the salts were dissolved. The organic layer was separated, and the aqueous layer was washed with $CHCl_3$ several times. The combined organic extracts were washed with saturated aqueous $NaHCO_3$ and then brine and finally dried over $MgSO_4$ and stripped of solvent to yield 85% of the desired product. ^{1}H NMR ($CDCl_3$): δ 10.09 (s, 2H), δ 8.00 (d, 4H, J = 8.3 Hz), δ 7.81 (d, 4H, J = 8.3 Hz). HRMS: m/e = 211.0761 (observed), m/e = 211.0759 (expected for $[C_{14}H_{10}O_2]^+$).

4-Amino-1,8-naphthalic anhydride-3,6-disulfonic acid, disodium salt. This molecule is an intermediate in the synthesis of LYCH. The following is a modification of an existing procedure [Scaler and Forster, 1948]. A 25 ml flask was charged with 3.3 ml of fuming H_2SO_4 (13 mmol SO_3) and cooled to 0°C. Then 4-amino-1,8-naphthalic anhydride (1 g, 4.7 mmol) was added in portions with stirring. The dark brown mixture was heated to 95°C for 1.8 h. Then the mixture was cooled to ambient temperature and added carefully to 170 ml of doubly distilled water at 5°C. NaCl was added until the solution became saturated (about 75 g). The mixture was filtered, and the residue was dried in vacuo, yielding 91% of the desired product. ^{1}H NMR (D_2O): δ 8.72 (s, 1H), δ 8.54 (s, 1H), δ 8.44 (s, 1H). HRMS: 371.9484 (found); 371.9488 (expected for $[C_{12}H_5NS_2O_9]^-$). Conversion of this anhydride to LYCH dilithium salt was carried out as described by Stewart [1981]. HRMS for the synthetic LYCH: m/e = 443.9929 (found); m/e = 443.9920 (calculated for $[C_{13}H_{10}N_5S_2O_9]^-$).

A2. Metaphthalaldehyde (190 mg, 1.42 mmol) was dissolved in a mixture of 15 ml water and 8 ml methanol. A solution of LYCH dilithium salt (25 mg, 54 μmol) dissolved in 10 ml water was added dropwise over 90 min at 25°C. The reaction mixture was stirred for 16 h at 25°C and then extracted repeatedly with chloroform to remove the metaphthalaldehyde. The water was removed in vacuo, and the product was purified by using semipreparative C_{18}-silica high-pressure liquid chromatography (HPLC) and a gradient from 100% water to 12% acetonitrile/88% water (25 min, flow rate 2 ml/min). Yield: 43%. ^{1}H NMR: δ 9.51 (s, 1H), δ 8.54 (s, 1H), δ 8.49 (s, 1H), δ 8.44 (s, 1H), δ 7.52 (m, 1H), δ 7.33 (m + s, 3H), δ 7.00 (m, 1H). HRMS: m/e = 560.0165 (found), 560.0182 (expected for $[C_{21}H_{14}N_5S_2O_{10}]^-$).

A4. Biphenyl-4,4′-dicarboxaldehyde (1.55 g, 7.4 mmol) was dissolved in 300 ml DMF and heated to 40°C. A solution of LYCH (disodium salt, 1.7 g, 4.1 mmol) in 60 ml dilute aqueous

HCl (pH adjusted to 5.0) was added dropwise over 18 h at 40°C. After the solvent was evaporated, the product was dissolved in water and extracted with chloroform to remove excess dialdehyde.

The aqueous layer was stripped of solvent, redissolved in 800 ml doubly distilled water, treated with 20 milliequivalents of Dowex-50W cation exchange resin (Li^+ form), and filtered through Celite. After evaporation, 2.2 g of crude product was obtained. The product was purified by using a full preparative reverse-phase diphenylsilica HPLC column (2.5 cm outside diameter, Vydac #219TP1022) with water as a solvent under isocratic conditions. After drying in vacuo, the yield of the purified product was 0.57 g (21% based on LYCH). ^1H NMR (D_2O): δ 9.67 (s, 1H), δ 8.43 (s, 1H), δ 8.37 (s, 1H), δ 8.33 (s, 1H), δ 7.59 (broad singlet, 3H), δ 7.29 ppm (d, J = 7.3 Hz, 2H), δ 7.22 (m, 2H), δ 7.06 (d, J = 7.4 Hz, 2H). ^{13}C-NMR (D_2O/DMSO as standard): δ 197.6, δ 165.2, δ 164.7, δ 157.9, δ 151.3, δ 145.0, δ 141.2, δ 139.4, δ 136.2, δ 135.9, δ 134.8, δ 132.1, δ 131.9, δ 131.1, δ 128.9, δ 127.43, δ 127.39, δ 123.1, δ 122.4, δ 121.7, δ 108.1. HRMS: 636.0482 (found), 636.0495 (calculated for $[C_{27}H_{18}N_5S_2O_{10}]^-$).

H3. LYCH (260 mg, 0.5 mmol) was dissolved in 2 ml dilute aqueous HCl (pH 5.4), and benzaldehyde (0.211 ml, 2 mmol) was added. The mixture was stirred at 42°C for 30 h and extracted repeatedly with chloroform to remove the benzaldehyde. After the water was removed in vacuo, the residue was purified by using semipreparative C_{18}-silica reverse-phase HPLC (see the *A2* preparation procedure for details). The yield, based on LYCH, was 76%. ^1H NMR: δ 8.67 (s, 1H), δ 8.71 (s, 1H), δ 8.59 (s, 1H), δ 7.52 (s, 1H), δ 7.28 (m, 2H), δ 7.05 (m, 3H). HRMS: 532.0249 (found), 532.0233 (calculated for $[C_{20}H_{14}N_5S_2O_9]^-$).

H4. A solution of *A4* (5.5 mg, 8.6 μmol) and LYCH (8 mg, 17 μmol) in dilute aqueous HCl (1.5 ml, pH 5.3) was stirred overnight at 35°C. The product was purified by using a semipreparative reverse-phase C_{18} silica column with H_2O as the solvent (isocratic conditions). The product *H4* elutes later than LYCH and earlier than *A4*. The yield was 52% based on *A4*. ^1H NMR in D_2O δ 7.5 (broad singlet, 4H), δ 7.57 (broad singlet, 4H), δ 7.83 (s, 2H), δ 8.65 (s, 2H), δ 8.7 ppm (s, 2H), δ 8.88 (s, 2H). HRMS: 1063.0356 (found), 1063.03887 (calculated for $[C_{40}H_{25}S_4N_{10}O_{18}]^-$).

Cells

Continuous cell lines were obtained from the American Type Culture Collection (Rockville, MD). Crandall feline kidney fibroblasts (CRFK cells) were grown as monolayers in Delbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum, 1% sodium pyruvate, 50 units/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, 1× BME vitamins, 40 μg/ml gentamycin at 37°C. CRFK cells used for anti-retroviral assays and isolation of FIV RT were grown in 5% CO_2; those used in the flow cytometry studies were grown in 7% CO_2 to more closely match the culture conditions for U937. The U937 human leukemic cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum and 2 mM glutamine without antibiotics at 37°C, in a 7% CO_2/air atmosphere. Untransformed monkey kidney epithelial cells (CV-1) were grown in RPMI 1640 supplemented with 5% fetal bovine serum and 2 mM glutamine without antibiotics at 37°C, in a 7% CO_2/air atmosphere. Human PBMC were isolated from individuals seronegative for hepatitis B virus and HIV-1 and stimulated with phytohemagglutinin as described previously [Schinazi et al., 1988].

FIV Reverse Transcriptase Inhibition Assays Ex Vivo

FIV Mg^{2+}-dependent RT activity was measured using a previously published procedures [Pederson et al., 1987; Talboltt et al., 1989], modified as follows: one to four milliliters of virus-containing tissue culture supernatant was centrifuged at 100,000 *g* for 90 min to pellet the virus. The supernatant was discarded, and the tube was drained thoroughly to avoid contamination of the pellet. The pellet was resuspended in 75 μl 1.5× reaction A buffer (30 mM KCl, 30 mM dithiothreitol, 60 mM Tris-HCl, pH 8.1; 0.3% Nonidet-P-40), and the virus was disrupted by multiple cycles of freezing and thawing on a dry ice bath. Inhibitor stock or buffer was added to make a final volume of 100 μl, and the samples were incubated on ice for 45 min. Portions (25 μl) of the sample were added to an equal volume of reaction mixture B (20 mM KCl, 60 mM Tris, pH 8.1; 40 μg/ml $(rA)_n(dT)_{12\text{-}18}$; 0.8 mM $MgCl_2$; 30 mM NaCl; 100 μCi/ml Me^3H-dTTP) or to reaction mixture C (20 mM KCl, 60 mM Tris-HCl, pH 8.1; 0.8 mM $MgCl_2$, 30 mM NaCl; 100 μCi/ml Me^3H-dTTP) with or without

drug and incubated for 60 min at 37°C. The portions were then spotted onto DE81 filters and washed with 0.1 M disodium pyrophosphate. The filters were further washed with 0.3 M ammonium formate and then with ethanol, dried under a heat lamp, and placed in Safety-Solv (Research Products International). The amount of radioactivity was determined by using a liquid scintillation counter (Beckman). Values were then calculated for RT activity and expressed as cpm/ml culture fluid. (This same procedure was carried out, in the absence of added drugs, to monitor viral expression in FIV-infected CRFK cells.)

HIV-1 Reverse Transcriptase and DNA Polymerase-α Assays Ex Vivo

Assays of the effects of ADSN derivatives on RT in vitro in pelleted HIV were carried out by using a modification [Pauza et al., 1988] of the method of Popovic et al. [1984]. The LAV1/bru strain of HIV was maintained in the T-lymphoblastoid cell line CEM as described previously [Pauza et al., 1988]. Cell-free supernatants from infected CEM cultures were combined in a 7:3 ratio with solutions of 30% PEG (8,000) in 0.3 M NaCl. The mixture was incubated overnight at 4°C and centrifuged for 5 min in a microcentrifuge at 14,000 rpm. The supernatant was discarded, and the pellet was resuspended in 50 ml of 0.025% Triton X-100, TBS buffer (0.9% NaCl, 20 mM Tris-HCl, pH 7.5). Enzyme activity was measured by quantifying ^{32}P incorporation into $(rA)_n(dT)_{12}$ from α-^{32}P-ATP as described previously [Pauza et al., 1988]. The EC_{50} values for RT inhibition by *A4* and suramin were determined by using the median effect equation [Chou, 1991].

Recombinant p66/p51 HIV-1 RT was obtained from Biotechnology General (Rehovot, Israel). The specific activity of the enzyme was 5,600 U/mg of protein. One unit of enzyme is defined as the amount required to incorporate 1 nmol of dTTP in 10 min, per milligram of protein at 37°C. The standard reaction mixture (100 μl) contained 100 mM Tris-HCl (pH 8.0), 50 mM KCl, 2 mM $MgCl_2$, 5 mM dithiothreitol, 0.05 U/ml of $(rA)_n$. $(dT)_{12-18}$, 1 μM [^{3}H] dTTP (DuPont, NEN Products, Boston, MA; specific activity 50 Ci/mmol) and 10 μl of HIV-1 RT (0.84 U/well). The reaction mixtures were incubated and processed as previously described [Bardos et al., 1992; Eriksson et al., 1989]. *Escherichia coli* DNA polymerase 1 was obtained from Boehringer Manheim Corp. (Indianapolis, IN). The material had a specific activity of 331,439 U/mg as determined in our laboratory under standard condition (see following). One unit is defined as the amount required to incorporate 1 pmol of dTTP in 1 h at 37°C. Standard assay conditions were as follows: the reaction mixture (100 μl) contained 100 mM Tris-HCl (pH 8.0); 6 mM $MgCl_2$; 0.2 mg/ml activated DNA; 0.1 mM dATP, dCTP, dGTP; 1 μM [^{3}H]-dTTP (Dupont, NEN Products; specific activity 50 Ci/mmol); 5 mM dithiothreitol; and 10 μl of enzyme containing 700 U/ml (7 U/well).

Cytotoxic and Systemic Toxic Effects

The cytotoxic effects of ADSN derivatives on human PBMC were determined by comparing ^{3}H-thymidine uptake in treated and untreated cells during a 24-h exposure [Bardos et al., 1992] and by comparing viability, as shown by trypan blue exclusion, after a 6-day exposure. The antiproliferative effects on CV-1 cells were determined after a 48-h exposure by using cell monolayers in microtiter plates and Gentian violet staining, as described previously [Rideout et al., 1989].

Aqueous solutions of *A4* (0.2 ml × 12.72 mg per ml) were injected intravenously (tail vein) into two 6-week-old female Balb/C mice (dose 127 mg/kg). Mice were weighed on days 0, 7, and 16. After 16 days, mice were sacrificed and examined for gross histological damage. Sections of liver, spleen, lung, and kidney were fixed, stained with hematoxylin and eosin, and examined for microscopic histological damage.

Anti-Retroviral Effects Against HIV-1

Human PBMC were stimulated with 6 μg/ml phytohemagglutinin for 3 days, and 2 × 10^6 cells in 5 ml of medium (RPMI 1640 supplemented with 1.5 mM L-glutamine, 15% heat-inactivated fetal bovine serum, 100 units/ml of streptomycin, 100 U/ml penicillin, and 4 mM sodium bicarbonate) were added evenly in each of several 25 cm^2 culture flasks as needed. HIV-1 (strain LAV-1) was added (MOI = 0.01) to achieve a mean RT activity of greater than 50,000 dpm/ml by day 6 after infection. The medium contained interleukin-2 (Chiron Corp., Emeryville, CA), anti-interferon-α antibody, and DEAE-dextran as described [Schinazi et al., 1988]. This is 100-fold higher than the dose required for 50% tissue culture infection, as determined by a standard limiting dilution

method. In order to test the activity of a drug concentration x, aliquots of 5 ml of medium containing concentration 2x were added to one infected and one uninfected flask. After incubation for 6 days (5% CO_2, 37°C), virus was concentrated by centrifugation, and cultures were assayed for RT activity as described previously [Schinazi et al., 1988].

Anti-Retroviral Effects Against FIV in Cultured Feline Cells

CRFK cells transfected with a clone of the Petaluma strain of FIV and uninfected CRFK cells were grown in DMEM supplemented with 10% fetal bovine serum as described previously [Talbot et al., 1989]. Cells were maintained at 37°C in a 5% CO_2 atmosphere and split 1:5 or 1:10 at required intervals. For drug treatment experiments, uninfected CRFK cells were seeded in six-well tissue culture plates (Falcon) at a density of 3×10^4 cells/well. The cells were allowed to grow for 48 h, and then drug (*A4* or LYCH) was added. The cells were infected 48 h later with 200 μl of viral supernatant containing 4×10^5 cpm/ml of RT activity and simultaneously treated with 10 μg/ml of polybrene. Noninfected controls also received polybrene. Polybrene was used only at the time of infection. Supernatants were removed from the culture wells on the days indicated and assayed for the presence of Mg^{2+}-dependent RT, as described earlier. Cells were counted by hemacytometry, and viability was assessed by using trypan blue exclusion.

Uptake of LYCH and *A4* by U937 Monoblastoid Leukemia Cells and CRFK Feline Kidney Fibroblasts

U937 human leukemic cells in log phase were diluted to 1.67×10^5 cells/ml in medium (RPMI 1640 supplemented with 10% fetal bovine serum and 2 mM glutamine). Cells were treated with 0.1% TPA (tetradecanoylphorbol acetate, added as a 1 mg/ml DMSO stock) and immediately plated out on 21 cm^2 tissue culture dishes at 2.4×10^4 cells/cm^2 (the cells become adherent after differentiation). After 1 h (37°C, 7% CO_2) the TPA-containing medium was removed and replaced with drug-free medium, and the cells were incubated for 48 h. CRFK cells (an adherent line) or differentiated U937 cells were left untreated, treated with LYCH (0.88 mM), or treated with *A4* (0.88 mM) for 1 h. After trypsinization, cells were washed with cold phosphate buffered saline (4°C) and resuspended in 0.5 ml cold PBS. Flow cytometric analysis was carried out by using a Becton-Dickson FACS 440 fluorescence activated cell sorter, with an excitation wavelength of 457 nm and an emission wavelength range of 515–545 nm. Fluorescence spectra of LYCH and *A4* (1 μM aqueous solutions) were determined by using an excitation wavelength of 457 nm with a Shimadzu RF5000 U spectrofluorometer.

RESULTS

Enzyme Inhibition Studies (Ex Vivo)

The activity of virus-derived FIV RT was inhibited 44 ± 12% by 52.4 μM *A4*, and 100% by 524 μM *A4*. *A4* was somewhat more potent than suramin, which caused only 35% inhibition at 70 μM. *A2* was considerably less potent than suramin, inhibiting RT only 32.6 ± 0.08% at 698 μM, and LYCH caused no significant inhibition even at 3,300 μM. Each determination was an average of two to four measurements, and error limits represent the range.

The dose-response curves for inhibition of cloned HIV RT are depicted in Figure 2a. The EC_{50} values for cloned HIV RT inhibition were 0.04 μM for *H4*, 1.43 μM for *A4*, 2.2 μM for *A2*, > 100 μM for LYCH, and 0.53 μM for phosphonoformate (used as a positive control). The dose-response curves for inhibition of bacterial (*E. coli*) DNA polymerase are depicted in Figure 2b. EC_{50} values for inhibition of DNA polymerase were 0.105 μM for *H4*, 4.81 μM for *A4*, and 5.45 μM for *A2*.

Plots representing the ex vivo inhibition of HIV-1 as determined by measuring RT obtained from pelleted virus are shown in Figure 3. The EC_{90} values were 465.5 μM for *A4*, 652 μM for suramin, 146.9 μM for *H4*, > 1,740 μM for *H3*, and > 1,740 μM for *A2*. Although *H3* and *A2* were weak inhibitors, LYCH did not significantly inhibit HIV-1 RT, even at 1,740 μM (Fig. 3). Variability between runs for individual data points was typically better than ± 12%. With the exception of *H4*, the order of potency is the same for HIV-1 and FIV (*A4* > suramin > *A2* > LYCH).

Cytotoxic and Systemic Toxic Effects

A4 was nontoxic to mice when administered intravenously at 127 mg/kg. The compound did not cause weight loss during a 16-day observation. At the end of the 16 days, no gross histolog-

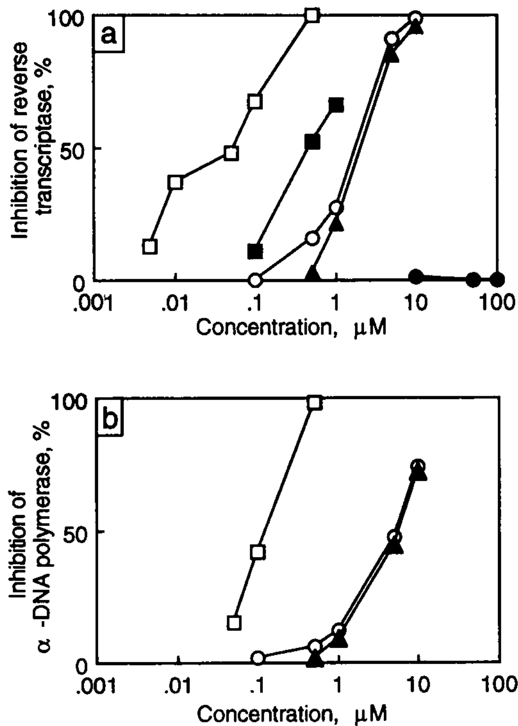

Fig. 2. Cell-free inhibition by cloned polymerases. Each point represents an average of duplicate observations. **a:** Inhibition of cloned HIV-1 RT activity (p66/51). **b:** Inhibition of bacterial (*E. coli*) DNA polymerase. Symbol key for a and b: □, inhibition by *H4*; ■, inhibition by phosphonoformate (positive control); ○, inhibition by *A4*; ▲, inhibition by *A2*; ●, inhibition by LYCH. Phosphonoformic acid did not affect DNA polymerase when evaluated up to 100 μM.

ical changes in internal organs were seen. Microscopic examination of liver, lung, spleen, and kidney sections stained with hematoxylin and eosin did not reveal any damage due to *A4*.

Cytotoxic effects on human PBMC were determined by comparing uptake of tritiated thymidine in control and drug-treated cells during a 24-h exposure and trypan blue exclusion after a 6-day exposure. By these criteria, *A2*, *A4*, and *H4* were nontoxic with IC_{50} values greater than 100 μM. Proliferation of CV-1 untransformed monkey kidney epithelial cells in 96-well microtiter plates was unaffected by 800 μM LYCH and by 800 μM *A2* after a 48-h exposure. With CRFK cells, no significant antiproliferative effects were seen during 49 days of exposure to 49.6 μM *A4*.

Inhibition of FIV Proliferation and Cytopathic Effect in CRFK Cells

The degree of inhibition of FIV replication in high-density CRFK cells by exposure to hydrazone *A4* for 19 days is shown in Figure 4. Cells were split every 7–10 days. *A4* was reintroduced at each split to maintain a constant level. Viable cells were counted at each split in wells free of drug and FIV, FIV-infected wells free of drug, and FIV-infected wells with drug in order to assess the influence of *A4* on the cytopathic effect of the virus (Fig. 5). Viability was assessed by using trypan blue exclusion. Cell counts in the FIV-infected, drug-free wells were 55.5% of the cell counts in uninfected wells on day 19 after infection, indicating a cytopathic effect

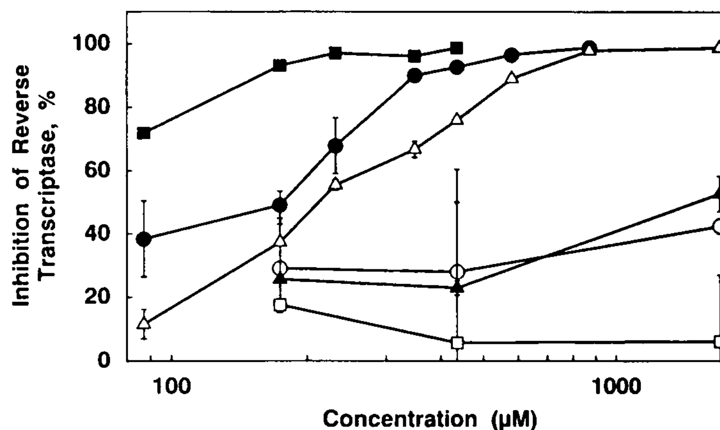

Fig. 3. Inhibition of virion particle derived HIV RT. Each point represents an average of two (LYCH, *A2*, *H3*) or three (*A4*, *H4*, suramin) sets of triplicate observations. Error bars represent the ranges of the triplicate set averages. No error bar is shown when the width of the symbol exceeds the range. Key: ●, inhibition by *A4*; ■, inhibition by *H4*; △, inhibition by suramin; □, inhibition by LYCH; ▲, inhibition by *H3*; ○, inhibition by *A2*. The anti-retroviral activities of *A4* and *H4* are discussed in Results.

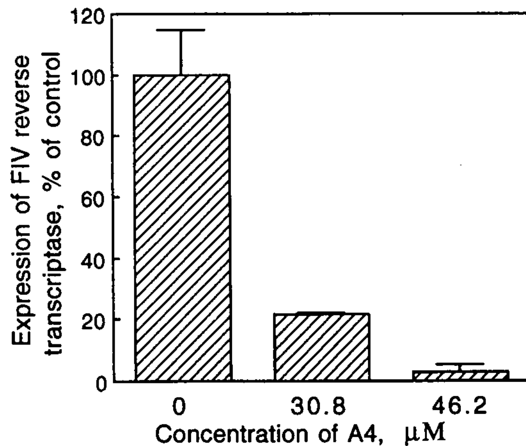

Fig. 4. Inhibition of FIV replication in cultured cells. CRFK fibroblasts in six-well plates were seeded at 1.5 × 10^5 cells per well and exposed 48 h after seeding to various concentrations of *A4* (0–46.2 µM). At 96 h after seeding, 200 µl of 4 × 10^5 cpm/ml of viral supernatant were added to each well. When cells were split (every 7–10 days), fresh *A4* was added to maintain its concentration in the drug-treated wells. The RT levels in the supernatant were determined 19 days after infection. Data is expressed as mean ± standard error (N = 2). The control supernatant RT level was 186,000 counts per milliliter per minute, expressed in terms of the amount of ^{32}P bound to the filter per ml of supernatant. Inhibition of FIV RT by *A4* ex vivo is discussed in Results.

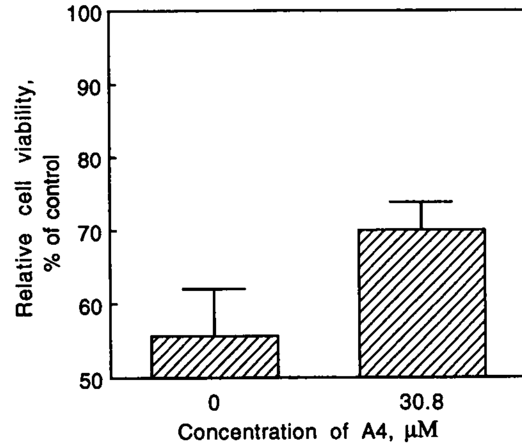

Fig. 5. Inhibition of FIV cytopathic effect in CRFK cells (see Fig. 4 legend for conditions). Cells were counted by hemacytometry, and viability was determined by trypan blue exclusion 19 days after infection. The number of viable cells in cultures of infected cells without *A4* and with 30.8 µM *A4* is expressed as a percentage of the number of cells in cultures of uninfected cells (without *A4*) determined simultaneously under identical cell culture conditions. Control viable cell counts were 3.8 × 10^6 cells/well. Data is expressed as mean ± standard error (N = 2).

throughout the experiment. This cytopathic effect was inhibited by *A4* at 30.8 µM: the viable cell counts relative to uninfected controls were higher in the drug-treated well.

Inhibition of Virus Replication in HIV-1–Infected Human PBMC

The degree of HIV infection was determined in human PBMCs incubated with various concentrations of ADSN derivatives for 6 days. An HIV-1 RT assay with concentrated virus obtained from supernatant was used for this purpose. The EC_{50} values, determined by using the median effect method [Chou, 1991] are shown in Table I. Variability of the calculated EC_{50} value between runs was typically less than ±30%. AZT, used as a positive control, gave an average EC_{50} value of 5.3 nM.

Uptake of *A4* and LYCH by U937 and CRFK Cells

U937 monocytoid human leukemia cells (5 × 10^5 per experiment) were treated with TPA for 1 h, washed, and incubated for 48 h to induce differentiation to an adherent form resembling a monocyte/macrophage [Harris and Ralph, 1985]. Cells (differentiated U937 or CRFK) were treated with 0.88 mM LYCH, 0.88 mM *A4*, or drug-free medium for 1 h. After trypsinization, the cells were analyzed by flow cytometry by using an excitation wavelength (457 nm) and an emission range (515–545 nm) close to the optimal excitation (428 nm) and emission (540 nm) wavelengths for LYCH [Haugland, 1989]. Fluorescence histograms with logarithmic fluorescence intensity scales are depicted in Figure 6A–F.

In order to allow meaningful comparison between fluorescence histograms of LYCH-treated

TABLE I. Anti-HIV-1 Activity*

Compound	EC_{50}, µM	EC_{90}, µM	IC_{50}, µM
A2	22.8	95.9 (slope = 1.53)	>100
A4	21.8	93.4 (slope = 1.51)	>100
H4	5.6	13.5 (slope = 2.5)	>100
LYCH (K+)	>100	>100	>100

*Inhibition of HIV-1 proliferation (EC_{50} and EC_{90}) by ADSN derivatives in phytohemagglutinin-stimulated human PBMC. Exposure time was 6 days. Cytotoxicity (IC_{50}) was evaluated both by measuring ^3H-thymidine uptake during 24 h and by determing trypan blue exclusion after 6 days of exposure. Slopes were calculated for the median-effect plots according to $y = \log[(f_a)^{-1} - 1]^{-1}$ vs. $x = \log(D/D_m)$, where f_a is the fractional decrease in viral expression relative to controls, D is the concentration of drug, and D_m (= EC_{50}) is the median-effect dose [Chou, 1991].

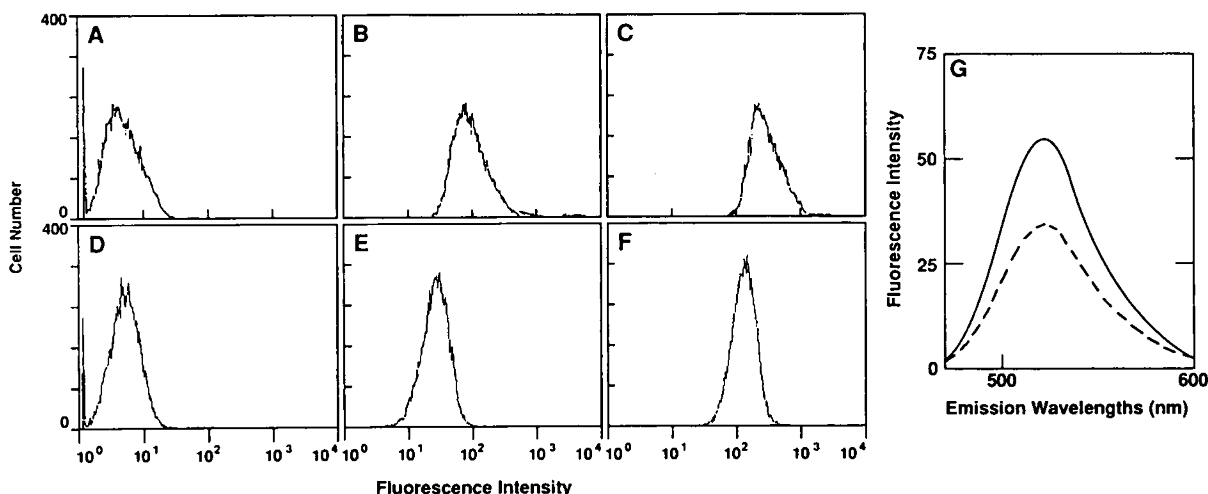

Fig. 6. Flow cytometric histograms of differentiated U937 human monoblastoid leukemic cells and CRFK cells; fluorescence emission spectra of LYCH and *A4*. U937 human leukemic cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum and 2 mM glutamine without antibiotics at 37°C, 7% CO_2. These U937 cells were treated with 0.1% tetradecanoylphorbol acetate for 1 h, washed, and allowed to differentiate at 37°C in 7% CO_2 for 48 h. During this time they became adherent because of differentiation to a macrophage-like cell. CRFK cells were grown as monolayers in DMEM supplemented with 10% fetal bovine serum, 1% sodium pyruvate, 50 units/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, 1× BME vitamins, 40 μg/ml gentamycin at 37°C in 7% CO_2. The cell monolayers were treated with 0.88 mM *A4*, 0.88 mM LYCH, or drug-free medium for 1 h. The monolayers were than washed 2× with cold (4°C) phosphate buffered saline and trypsinized. Flow cytometric analysis was carried out by using a Becton-Dickson FACS 440 fluorescence activated cell sorter. Excitation: 457 nm; emission: 515–545 nm. **A:** U937 control cells (no drug). **B:** U937 cells exposed to 0.88 mM LYCH for 1 h. **C:** U937 cells exposed to 0.88 mM *A4* for 1 h. **D:** CRFK control cells (no drug). **E:** CRFK cells exposed to 0.88 mM LYCH for 1 h. **F:** CRFK cells exposed to 0.88 mM *A4* for 1 h. **G:** Fluorescence emission spectra of 1 μM LYCH (solid line) and 1 μM *A4* (dashed line) in water. Excitation: 457 nm.

cells and *A4*-treated cells, fluorescence spectra were determined for 1 μM aqueous solutions of LYCH and *A4* by using the same excitation wavelength (457 nm) as that used in the flow cytometry experiments (Fig. 6G). The fluorescence emission intensity of *A4* is 28% less than that of LYCH at equal concentrations. For both of these ADSN derivatives, the fluorescence emission intensity was linear with respect to concentration between 0.1 and 12 μM ($r^2 > 0.99$).

DISCUSSION

ADSN derivatives *A2*, *A4*, *H3*, and *H4* (Fig. 1) were synthesized as potential anti-retroviral agents. These resemble the variety of naphthalenepolysulfonic acid derivatives that inhibit HIV replication in cultured human cells and, in some cases, RT activity in vitro [Jentsch et al., 1987; Mohan et al., 1991; Sandstrom, 1989]. In addition, *A2*, *A4*, *H3*, and *H4* are closely related to Lucifer Yellow CH (LYCH) (Fig. 1), a highly fluorescent dye that is not very toxic for cultured cells and animals [Silverstein, personal communication; Swanson et al., 1985]. LYCH also accumulates selectively in activated macrophages relative to fibroblasts and neutrophils [Scieszka and Cho, 1988; Swanson et al., 1985]. This observation is relevant to drug design in that macrophages are believed to represent a significant reservoir for HIV [Fauci, 1988; Ho et al., 1987; Klatzman and Gluckman, 1986; Pauza, 1988].

Some preliminary conclusions about structure/activity relationships can be made by comparison of the ex vivo RT inhibitory activity of the ADSN derivatives depicted in Figure 1. The aldehyde moiety does not contribute significantly to the inhibition of HIV RT. For example, *H3* is slightly more active than aldehyde *A2*; when pelleted virus RT is used the degree of inhibition at 1,749 μM is 53% for H3 vs. 42% for *A2* (Fig. 3). *H4* is slightly more active than aldehyde *A4* (when either pelleted virus RT or cloned RT is used). The ability to inhibit RT increases with the number of aromatic rings appended to LYCH. Thus LYCH itself (no appended phenyl ring) lacks significant RT inhibitory activity even at 1,750 μM; the ADSN derivatives *A2* and *H3* (one appended phenyl ring) have slight activity at 1,750 μM with EC_{90} values > 1,750 μM. *A4* and *H4* (two appended phenyl rings) inhibit the virus particle-derived

enzyme with IC_{90} < 500 μM. A similar trend is observed when cloned HIV RT is used, although the compounds were more inhibitory to cloned RT than to RT from pelleted virus (Fig. 2a). *A4* is also a much more potent inhibitor than *A2* of FIV RT. The dianionic ADSN derivative *A4* is somewhat more potent than the hexaanionic suramin as an inhibitor of RT in pelleted virus, both for human RT (Fig. 3) and the FIV RT (see Results). This comparison shows that the number of sulfonate groups is not a crucial factor in the inhibition of RT by naphthalenepolysulfonic acid derivatives. These preliminary structure/activity relationship observations will aid the design of new ADSN derivatives that are more potent inhibitors than *A4* and *H4*.

Compounds *A4, A2,* and *H4* were all active against HIV in cultured PBMC, with EC_{50} values less than 30 μM when a standardized RT-based assay of HIV in cultured cells was used as a measure of virus yield [Schinazi et al., 1988]. *A4* also had significant anti-retroviral activity against FIV in cultured CRFK cells, as judged by the drop in RT relative to infected, drug-free controls (Fig. 4). The EC_{50} for *H4* against HIV-1 (5.6 μM; 6.8 μg/ml) is comparable to the best values observed for a variety of other polyanions [Baba et al., 1990; Cushman et al., 1991a,b; Jentsch et al., 1987; Mohan et al., 1991; Moriya et al., 1991; Weeks et al., 1992]. The anti-retroviral activity was selective in that the IC_{50} values for cytotoxic effects against PBMC were considerably higher (> 100 μM). In addition, *A4* was nontoxic when administered intravenously to mice at 127 mg/kg. The low systemic toxic effects of LYCH [Silverstein, personal communication] and *A4* make the ADSN derivatives more attractive as antiviral drug candidates.

In the HIV-1 assays, LYCH is the least potent and *H4* is the most potent ADSN derivative tested for anti-retroviral assays against HIV-1 in human PBMC and for inhibition of RT activity ex vivo (for both virion-derived and cloned RT). These results suggest that RT inhibition may be important in the mechanism of anti-retroviral action of these derivatives. However, if selective inhibition of RT relative to cellular DNA polymerases does play a significant role, it is unlikely to be due to differences in the K_i values for the different enzymes. The ADSN derivatives show little selectivity against cloned HIV RT as compared with DNA polymerase (Fig. 2). Selective inhibition of the virus might be explained in terms of subcellular biodistribution if the ADSN derivatives have access to RT but not to cellular DNA polymerase. Alternately, a mechanism that does not involve RT may be involved. The observation that LYCH only has access to endosomes and lysosomes in macrophages [Swanson et al., 1985] may be related to the selective anti-retroviral activity of ADSN derivatives. These compounds also could have anti-fusion activity. This possibility is under investigation.

The anti-retroviral effects of *A4* against FIV occur in CRFK cells grown at high densities as semiconfluent to confluent monolayers. In other words, *A4* can prevent FIV spread through direct cell-to-cell contact. In this respect, *A4* may resemble the polyanion dextran sulfate, which prevents HIV-1 transmission through direct contact from infected HUT-78 to uninfected MOLT-4 cells [Baba et al., 1990]. In addition, *A4* may be superior to AZT in this respect, as AZT does not prevent direct cell-to-cell transmission of HIV-1 [Baba et al., 1990].

Preliminary studies compared the uptake of LYCH to that of *A4* in CRFK cells and in U937 human monocytoid leukemia cells induced to differentiate with TPA. After TPA-induced differentiation, U937 cells resemble macrophages in many respects, including susceptibility to infection by HIV-1 [Harris and Ralph, 1985; Pauza et al., 1988]. For both cell lines, uptake of *A4* is somewhat greater than uptake of LYCH. Apparently, substantial modification of the hydrazine group in LYCH can be made without the derivative (*A4*) losing its ability to accumulate in U937 and CRFK cells.

In conclusion, *A4, H4,* and other derivatives of 4-amino-3,6-disulfonato-1,8-naphthalimide (ADSN) are members of a promising new class of polyanionic molecules with selective anti-lentiviral activity in vitro against HIV-1 and the feline immunodeficiency virus (FIV). *A4* can suppress FIV viral titers by more than 97% in cultured feline cells. The measurements were made under conditions allowing direct cell-to-cell transmission. *H4,* with an EC_{50} of 5.6 μM against HIV, is among the most potent anti-retroviral polyanions. Inhibition of RT ex vivo was noted using enzymes derived from HIV-1 and FIV RT. However, the nonselectivity of the ADSN derivatives for these enzymes suggests that other targets, such as HIV-1 gpl20 or the CD_4 receptor, may be involved.

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