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Inhibition of human immunodeficiency virus replication by the sulfonated stilbene dye resobene

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Abstract

The anti-HIV sulfonated dye, resobene, was found to be a potent inhibitor of the attachment of HIV to target cells, the fusion of envelope- and CD4-expressing cells, and the cell-to-cell transmission of virus. Resobene inhibited the infection of phenotypically distinct, established human cell lines and fresh human peripheral blood lymphocytes and macrophages by laboratory-derived isolates of human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2), and a panel of biologically diverse primary clinical isolates, including syncytium-inducing and non-syncytium-inducing viruses and strains representative of the various virus clades found worldwide. The compound was also active against all drug-resistant virus isolates tested. Cell-based and biochemical mechanism of action studies demonstrated that the compound inhibits the attachment of infectious virus and fusion of virus-infected cells to uninfected target cells by binding to the cationic V3 loop of the envelope glycoprotein. Resobene effectively inhibited the infection of cell populations which do and do not express cell surface CD4. Resobene prevented infection of the cervical epithelial cell line ME180, suggesting the compound may effectively act as a topical microbicide to prevent the sexual transmission of HIV.

Keywords: Human immunodeficiency virus; Resobene; Antiviral

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1. Introduction

The interaction of human immunodeficiency virus (HIV) with the specific receptor on human cells is the earliest occurring event in the HIV replication cycle, and this step in HIV infection has been the target of many antiviral agents. These compounds include dextran sulfate, pentosan polysulfate and other sulfated polysaccharides, aurintricarboxylic acid and cosalane derivatives, sulfonated dyes, soluble CD4, the phosphorothioate oligonucleotide ISIS 5320, polyoxometalates and other membrane-reactive compounds and antagonists of gp120–CD4 binding and cell fusion (for review see De Clercq, 1994, 1995).

The broad range of antiviral activity of the sulfonated compounds such as the sulfated polysaccharides (De Clercq, 1993), sulfonic acid polymers (Mohan et al., 1992), sulfonated dyes (Clanton et al., 1992), and naphthalene sulfonic acid derivatives (Clanton et al., 1995; Mohan et al., 1991) has been reported. These compounds inhibit attachment of virus to target cells, fusion of HIV-infected cells with CD4-expressing cells (syncytium-formation) and cell-to-cell transmission of virus (Clanton et al., 1995; Mohan et al., 1992). Of these compounds, only the sulfonated polymers, poly(4-styrenesulfonic acid) sodium salt (PSS) and poly(anetholesulfonic acid) sodium salt (PAS), inhibit the attachment of virus by binding to cell surface CD4 (Mohan et al., 1992). However, several of these compounds, including the sulfonated polymers and ISIS 5320, have been shown to potently inhibit virus attachment to target cells by interacting specifically with the V3 loop of viral gp120 (Buckheit et al., 1994b; Mohan et al., 1992). Interest in naphthalenesulfonic acid compounds as inhibitors of HIV replication began with the discovery that the compound suramin effectively prevented reverse transcription and HIV replication in vitro (De Clercq, 1979). Although suramin failed in clinical trial, the synthesis of analogs of the basic molecule has continued in an effort to increase potency and bioavailability. The effectiveness of this class of compounds as inhibitors of HIV infection and subsequent replication has been reported (Mohan et al., 1991).

The National Cancer Institute (NCI) operates a high-capacity antiviral drug screen to evaluate diverse compounds from worldwide sources for anti-HIV activity. The antiviral activity of a number of naphthalenesulfonic acid dyes evaluated by this program, most notably Chicago Sky Blue (CSB), has been previously reported (Clanton et al., 1992). Although CSB was particularly active in vitro, the compound had several properties which precluded further clinical development. Most importantly, this azo-benzidine compound was metabolized to carcinogenic aromatic amines in vivo (Bowman et al., 1983), and strong discoloration of the eyes and testicles was evident when the compound was administered intravenously into laboratory animals. Congener synthesis to develop compounds possessing a pharmacophore with an electron distribution similar to Chicago Sky Blue led to the development of quinobene (Gruszecka-Kowalik et al., 1992), where the biphenyl moiety of CSB was replaced with a stilbene, and of resobene. Quinobene did not yield carcinogenic metabolites in vivo, retained high potency against HIV in vitro and had reduced color. However, this compound formed colloidal suspensions in water, was poorly bioavailable, and caused a severe thrombocytopenia in dogs. Resobene (Burgess et al., 1995) possessed improved solubility characteristics, less color, and enhanced anti-HIV activity. Although the structures of CSB, quinobene and resobene appear to be quite different, comparison of their spatial electron density distributions from an atomic electrostatic multipole comparison revealed they were quite similar (Burgess et al., 1995). This was especially true for quinobene and resobene.

In this report, a detailed characterization of the range of activity of resobene against a number of laboratory-derived and clinical isolates of HIV, including primary isolates representative of clades of HIV type 1 (HIV-1) found worldwide, in a variety of phenotypically distinct human cell lines is presented. Further, cell-based and biochemical mechanism of action assays, which demonstrate the specificity of resobene as an inhibitor of virus attachment, syncytium formation and cell-to-cell transmission, are reported.

2. Materials and methods

2.1. Chemistry

Resobene (4,4′-bis(2-carboxy-4,6-dihydroxyphenylazo)stilbene-2,2′-disulfonic acid tetrasodium salt) (molecular weight 789) was synthesized and provided to the NCI by Dr Leon Zalkow. The structure and chemical properties of resobene have been previously described (Burgess et al., 1995). Resobene was synthesized as follows: to a suspension of 4,4′-diaminostilbene-2,2′-disulfonic acid (3.70 g, 10 mmol) in 30 ml of water was added solid $NaHCO_3$ (1.68 g, 200 mmol). The solution was cooled to 0°C and concentrated hydrochloric acid (4.1 ml, 46.8 mmol) was added. After stirring for 15 min, an aqueous solution (20 ml) of $NaNO_2$ (1.45 g, 20 mmol) was added below the liquid surface. The diazotization mixture was then stirred for 30 min at 0°C. A solution of 3,5-dihydroxybenzoic acid (6.2 g, 40 mmol) was stirred in 50 ml of water containing $NaHCO_3$ (11 g, 130 mmol). To this was added, all at once, the solution of the diazonium salt. The solution became red at first and then turned dark violet. Stirring was continued overnight. The solvent was then removed at reduced pressure. The residual solid was refluxed in 150 ml of 95% ethanol and then filtered while hot. The solid was then stirred in 150 ml of methanol, treated with charcoal and filtered. The methanol filtrate was concentrated at reduced pressure then triturated with 500 ml of diethyl ether. The precipitated product was collected by filtration and dried at 80°C at reduced pressure for 12 h to give 2.3 g of a dark red solid. Purification using preparative CPC was performed in ascending mode to remove polar, polymeric impurities. The solvent system was *n*-butanol/water/trifluoroacetic acid (1000:1000:1, v/v). A homogeneous stock solution of 2.0 g of the crude dye was dissolved in 60 ml of the stationary phase (aqueous, lower phase). A 15-ml sample of this solution was injected into the equilibrated CPC. The initial void volume (100 ml) was discarded and the following 2000 ml of eluent was collected. Water was passed through to elute the retained polymeric material, which was discarded. The separated dye was evaporated at reduced pressure at 45°C and dried under vacuum at 80°C. From five injections of the stock solution, a final yield of 1.1 g of material was recovered. Due to the acidic nature of the mobile phase, the dye was in the form of its disodium salt. ^{1}H-NMR (dimethyl sulfoxide (DMSO)-d_6)δ, ppm: 11.03 (s, 1H, OH), 8.58 (s, 1H), 8.48 (s, 1H), 8.10 (s, 2H), 6.86 (d, 1H, $J = 2.4$ Hz), 6.70 (d, 1H, $J = 2.4$ Hz). ^{13}C-NMR (DMSO-d_6)δ, ppm: 169.08, 162.26, 156.26, 148.81, 147.49, 138.56, 137.29, 129.10, 129.02, 127.11, 122.88, 120.55, 108.35, 104.43. Fourier transform infrared (FT-IR) (KBr, cm^{-1}) 3400, 2756, 1721, 1696, 1612, 1590, 1474, 1214, 1076. Anal: $C_{28}H_{18}N_4S_2Na_2 \cdot 8.5H_2O$. Calc: C, 37.46; H, 3.93. Found: C, 37.24; H, 3.82. The disodium salt was converted to the tetrasodium salt (resobene) by addition of the dye to a solution of $NaHCO_3$ (0.206 g, 2.46 mmol) in 50 ml of water. The mixture was stirred for 30 min, filtered, and the solvent was evaporated at reduced pressure at 45°C. The product was vacuum dried over KOH for 16 h. ^{1}H-NMR(D_2O)δ, ppm: 7.87 (d, 1H, $J = 8.7$ Hz), 7.84 (s, 1H), 7.56 (dd, 1H, $J =$ 2.1, 8.7 Hz), 6.01 (d, 1H, $J = 2$ Hz), 5.51 (d, 1H, $J = 1.8$ Hz). FT-IR (KBr, cm^{-1}) 3413, 1603, 1588, 1476, 1395, 1187, 833, 630.

2.2. Cells and viruses

The established human cells, laboratory-derived virus isolates (including drug-resistant virus isolates) and low passage clinical virus isolates used in these evaluations have been previously described in detail (Buckheit et al., 1994a). Virus isolates representative of the various clades found worldwide and HeLa-CD4-LTR-β-galactosidase cells were obtained from the NIAID AIDS Research and Reference Reagent Program. Fresh human cells were obtained from the American Red Cross (Baltimore, MD, USA). Cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml).

2.3. Materials

All experimental antiviral agents were obtained from the Developmental Therapeutics Program, Division of Cancer Treatment, Diagnosis and Centers, NCI. Crystalline stock materials were stored at −70°C and solubilized in 100% DMSO. All stocks were diluted at least 400-fold prior to performing drug susceptibility assays. The reference anti-HIV compound used in these studies was 3′-azido-3′-deoxythymidine (AZT, NSC 602670). Enzyme-linked immunosorbent assay (ELISA) plates were obtained from Coulter Cytometry (Hialeah, FL). Materials required for the performance of reverse transcriptase (RT) inhibition assays, anti-HIV assays, and for the growth and maintenance of established and fresh human cells have been previously described (Buckheit et al., 1994a). AZT triphosphate was obtained from Sierra Bioresearch (Tuscon, AZ).

2.4. Anti-HIV assays

Evaluation of the antiviral activity of test compounds was performed as previously described (Buckheit et al., 1994a). Antiviral and toxicity data are reported as the concentration of drug required to inhibit 50% of virus-induced cell killing or virus production (EC_{50}) and the concentration of drug required to reduce cell viability by 50% (IC_{50}). In selected anti-HIV assays, the multiplicity of infection (MOI) was varied to evaluate the effects of MOI on drug efficacy. Endpoint titration was utilized to quantitate the amount of infectious virus added to each of these assays and was determined as previously described (Buckheit et al., 1994a). Confirmatory RT assay, p24 ELISA, and CEM-SS infectivity assay were performed as previously described (Buckheit et al., 1993). The effect of compounds on chronically infected cells was evaluated using H9 cells infected with IIIB virus. These cells were cultured in the presence of serial, one-log dilutions of antiviral compounds. Cell-free supernatant samples were obtained on a daily basis and analyzed for virus content by RT assay, p24 ELISA and CEM-SS infectivity assay as described. Toxicity was quantitated by the addition of the tetrazolium dye XTT and by spectrophotometrically measuring the conversion of the dye to the colored formazan product by metabolically active cells.

2.5. Binding and fusion inhibition assays

HeLa-CD4-LTR-β-galactosidase cells, which employ a Tat protein-induced transactivation of the β-galactosidase gene driven by the HIV-1 long terminal repeat (LTR) promoter, were used to quantitate both the binding of infectious virions to cells and cell–cell fusion events. Both of these assays have been previously described (Buckheit et al., 1994b).

2.6. Cell-to-cell fusion and virus transmission assay

Evaluation of the ability of resobene to inhibit cell-to-cell fusion and virus transmission was evaluated as previously described (Buckheit et al., 1994b).

2.7. Topical microbicide assay

ME180 cervical epithelial cells were plated in the interior wells of a 96-well flat-bottom microtiter plate at a density of 5×10^3 cells per well and incubated overnight. Chronically infected H9 cells (H9-SK1) were treated with 200 μg/ml mitomycin C (Sigma, St. Louis, MO) in complete medium for 1 h, washed extensively and resuspended at 4×10^5 per ml. The concentration of mitomycin C used resulted in the killing of the chronically infected cells within 48 h of treatment, allowing sufficient time for cell–cell transmission of virus to the ME-180 cells while assuring that the virus endpoint quantification would not include a contribution from the chronically infected cells. Antiviral compounds and chronically infected cells (2×10^4 cells) were added to each well containing ME180 cells and incubated for 6 h. Following co-cultivation the monolayer was washed extensively and fresh medium added. Medium was removed and fresh medium added at 24 and 48 h post-infection to remove dead lymphocytes. On day 6 post-infection, supernatant samples were removed and analyzed for virus content by p24 ELISA.

2.8. Binding and enzymatic assays

The binding of gp120 to CD4 was analyzed using an antigen capture ELISA (DuPont, Wilmington, DE), which was performed according to the manufacturer's instructions. The effect of compounds on the in vitro activity of recombinant RT was determined as previously described (Buckheit and Swanstrom, 1991). The RT inhibition assay measures the incorporation of [^{3}H]TTP using a homopolymer poly(rA)·oligo(dT) template/primer system. Following reaction, samples were blotted onto DE81 paper, washed extensively with 5% dibasic sodium phosphate and then quantitated on a Packard Matrix 9600 Direct Beta Counter. 3′-Azido-3′-deoxythymidine-5′-triphosphate served as a positive control for inhibition of RT. HIV protease activity was quantitated by a reverse-phase high-performance liquid chromatography (HPLC) assay utilizing the Ala-Ser-Glu-Asn-Tyr-Pro-Ile-Val-Glu-Amide substrate (Multiple Peptide Systems, San Diego, DA) as described in Wondrak et al. (1990).

2.9. Antibody-binding inhibition assays

Quantitation of the ability of resobene to competitively inhibit the binding of antibodies to the envelope V3 loop and to CD4 was performed using standard flow cytometric techniques. Briefly, for CD4 antibody-binding inhibition, 10^6 CEM-SS cells were incubated with or without compound for 15 min at room temperature. Anti-CD4 monoclonal antibody (20 μl, 3 μg/ml) (Becton-Dickinson, San José, CA) was added, and cells were incubated at 4°C for 40 min. Cells were then washed twice with phosphate buffered saline (PBS), resuspended in 1% paraformaldehyde, and analyzed using a Becton-Dickinson FACSort flow cytometer. For gp120 V3 loop antibody binding, 10^6 HL2/3 cells were incubated with compound for 15 min at room temperature. Anti-gp120 V3 loop antibody (50 μl, 10 μg/ml) (Intracel) was added, and the cells were incubated at 4°C for 40 min. Cells were washed twice with PBS, resuspended in 1% paraformaldehyde, and analyzed using a Becton-Dickinson FACSort flow cytometer. For the PB1 sub2-CN1 ELISA, 50 μl of gp120 (5 μg/ml) (Intracel) was added to each well of a 96-well flat-bottom plate and incubated at 4°C overnight. Residual unbound antibody was removed from each well and each well was then blocked with 1% bovine serum albumin (BSA) in PBS for 30 min at room temperature. Following washing with 0.2% Tween-20 in PBS, compound (50 μl) (or PBS control) was added, and the plate was incubated at room temperature for 15 min. Antibody (50 μl) was then added, and the plate was incubated at room temperature for 1 h. Following washing, 100 μl of streptavidin-alkaline phosphatase (Boehringer-Mannheim, Indianapolis, IN) was added, and the plate was incubated for 1 h at room temperature. Following washing, 100 μl of PNPP substrate (Pierce, Rockford, IL) was added. The plate was evaluated spectrophotometrically at 570 nm using a V_{max} plate reader.

2.10. High MOI assays

In order to identify the stage(s) of HIV replication affected by the antiviral compound, a high MOI acute infection time-of-addition assay (TOA) was performed (Cushman et al., 1994). CEM-SS cells (1×10^5) were preincubated with HIV-1_{IIIB} (MOI = 1.0) at 0–4°C for 1 h to allow attachment of virus to cells without fusion. Samples were then washed three times with ice-cold media to remove unbound virus, after which the samples were rapidly warmed to 37°C (at time zero, t_0), allowing the viral replication cycle to proceed. Compound (100 μg/ml) was included during the preincubation step only (Pre), or during the preincubation step and then added back at t_0 (Pre/t_0) following removal of residual virus, or added to samples only at t_0 or at various times after warming to 37°C ($t = 0.5$, 1, 2 or 4 h post-warming). Dextran sulfate (100 μg/ml) and ddC (10 μM) served as controls for inhibitors of virus attachment and RT, respectively. After 24 h incubation, the cells were collected by centrifugation, lysed in QuickLyse Buffer (10 mM Tris (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.1 mg/ml gelatin, 0.45% Nonidet P-40, 0.45% Tween-20) containing 100 μg/ml proteinase K, incubated at 56°C for 2 h and boiled for 20 min. Products of viral reverse transcription were amplified by polymerase chain

Table 1
Range of activity of resobene in different human cell lines

Cell line	Phenotype[a]	Assay[b]	EC_{50} (μg/ml)[c]	IC_{50} (μg/ml)
CEM-SS	T	XTT	3.2	127
MT2	T (HTLV-I+)	XTT	14.5	110
AA5	B (EBV+)	XTT	3.0	112
174 × CEM	B × T	XTT	9.0	137
U937	M	RT	5.8	154
PBL	Lymphocyte	RT	2.6	125
Macrophage	Macrophage	p24	0.3	160
H9-III_B	T (HIV-1+)	RT	>100	135

[a] T, T cell; B, B cell; M, macrophage; HTLV-I, human T-cell leukemia virus type-I; EBV, Epstein-Barr virus;
[b] Drug-induced inhibition of virus replication was quantitated by XTT assay in cytopathically infected cells and by RT activity assay or p24 ELISA in noncytopathically infected cells.
[c] HIV-1_{IIIB} was utilized in all antiviral testing involving established cell lines; fresh peripheral blood lymphocytes and macrophage cells were infected with clinical HIV-1 isolates.

reaction (PCR) using LTR/*gag* primer pairs (M667/M661, Recombinant DNA Laboratory, Program Resources, NCI-FCRDC, Frederick, MD). Products of the β-globin gene were amplified using primer pairs as previously described (Zack et al., 1991). Amplified products were analyzed by electrophoresis on 2% gels and visualized by ethidium bromide staining. The specificity of products was verified by restriction enzyme cleavage and by Southern blot hybridization.

3. Results

3.1. Range of anti-HIV activity of resobene

Resobene exhibited anti-HIV activity in CEM-SS cells at an effective concentration (EC_{50}) of 3.2–4.5 μg/ml (4.0–5.7 μM) when evaluated in the microtiter antiviral XTT assay. Drug-treated cells showed no evidence of toxicity at concentrations more than 30-fold higher (IC_{50} = 127 μg/ml, 161 μM). The cytoprotective effect of resobene was confirmed by microscopic observation of individual microtiter plate wells. Evidence of HIV-induced cytopathic effects, including syncytium formation, was absent in microtiter wells protected by resobene. RT, p24 and infectious virus assays documented the reduction in virus production associated with the drug's cytoprotective effect.

A variety of studies were performed to further define the range of antiviral activity of resobene. The compound was found to be active in phenotypically distinct human cell lines, including the T-cell lines CEM-SS and MT2, the B-cell line AA5, the monocyte–macrophage cell line U937 and the T-cell:B-cell hybrid cell line 174 × CEM (Table 1). Resobene had no effect on virus production from cells chronically infected with HIV-1 (Table 1). The compound was also found to be active when evaluated against a variety of laboratory HIV-1 isolates, including isolates resistant to nucleoside and nonnucleoside RT inhibitors, and HIV-2 ROD (Table 2). Resobene was not active against the $Delta_{B670}$ strain of simian immunodeficiency virus (SIV) in 174 × CEM cells. These evaluations indicated that resobene remained nontoxic in all cell lines and fresh cells until concentrations greater than 100 μg/ml were reached (Table 1).

The anti-HIV activity of resobene was further evaluated against a panel of primary HIV-1 strains, including both syncytium-inducing (SI) and non-syncytium-inducing (NSI) isolates and isolates representative of the various clades of virus found worldwide. These low passage clinical virus isolates were obtained from patients by cocultivation with freshly isolated human cells and have never been passaged in established human cell lines. Resobene effectively inhibited replication of these clinical isolates at concentrations

ranging from 1.8 to 9.0 μg/ml, showing no preference for virus isolates with SI or NSI phenotype (Table 2). The inhibitory activity of the compound was also demonstrated in fresh human monocyte–macrophages infected with the clinical strain Ba-L ($EC_{50} = 0.3$ μg/ml) (Table 1). These experiments with freshly isolated human cells indicated that resobene is capable of inhibiting clinical strains of HIV-1 with no evidence of cytotoxicity at concentrations up to 100 μg/ml.

To further evaluate the activity of resobene, CEM-SS cells were infected in the presence of resobene or AZT at increasing MOI. While the antiviral activity of AZT rapidly declined as the MOI was increased, resobene remained effective at inhibiting virus replication in cells infected at high MOI (Table 3). At an MOI 128 times higher than the MOI routinely used in the XTT assay (standard MOI = 0.01), the EC_{50} concentration of resobene was only 48-fold higher than the value obtained when evaluated at the standard MOI. In contrast, AZT was unable to inhibit virus replication at this high MOI at more than 500 times its EC_{50} concentration at the standard MOI.

Table 2
Activity of resobene against various strains of virus

Cell line	Virus (isolate)	EC_{50} (μg/ml)	EC_{50} (μM)
		Resobene	AZT
CEM-SS	HIV-1 (RF)	4.5	0.027
MT2	HIV-1 (A17)[a]	11.3	0.007
CEM-SS	HIV-1 (4× AZT)[b]	1.3	0.145
CEM-SS	HIV-1 (N119)[c]	0.5	0.3
CEM-SS	HIV-2 (ROD)	11.9	0.005
174×CEM	SIV ($Delta_{B670}$)	>100	0.04
PBMC	HIV-1 (WEJO)[d]	2.6	0.003
PBMC	HIV-1 (JOGA)[d]	9.0	0.02
PBMC	HIV-1 (BAKI)[d]	1.8	0.003
PBMC	HIV-1 (WOME)[d]	2.3	0.002
PBMC	HIV-1 (VIHU)[e]	2.2	0.008
PBMC	HIV-1 (Clade A)	3.2	0.02
PBMC	HIV-1 (Clade C)	1.5	0.003
PBMC	HIV-1 (Clade D)	0.5	0.002
PBMC	HIV-1 (Clade F)	10.1	0.02
Macrophage	HIV-1 (Ba-L)	0.3	0.04

[a] Pyridinone-resistant isolate.
[b] AZT-resistant isolate.
[c] Nevirapine-resistant isolate.
[d] Syncytium-inducing (SI) clinical isolate.
[e] Non-syncytium-inducing (NSI) clinical isolate.

Table 3
Efficacy of resobene when challenged at increased MOI

MOI[a]	EC_{50} (fold increase)[b]	
	Resobene	AZT
1.28	48.3	>312
0.64	17.5	140.1
0.32	16.1	62.5
0.16	16.1	43.8
0.08	6.5	21.9
0.04	4.8	3.1
0.02	3.9	1.0
0.01	1.0	1.0

[a] MOI was determined by endpoint titration of the virus pool used for infection as previously described (Buckheit and Swanstrom, 1991). The standard MOI used in the anti-HIV assay was 0.01.
[b] Fold-increase values are based upon at least two replicate determinations of the respective EC_{50} values at each of the different MOI values.

3.2. Mechanism of anti-HIV activity of resobene

A high-MOI, single cycle of virus replication assay was performed to determine the phase(s) of HIV replication affected by resobene. Resobene, or control reagent, was added during viral attachment or at various times post-attachment during the replication cycle. The extent of intracellular proviral DNA synthesis was then determined for each condition by PCR-based amplification and utilized as an indicator of infection. As illustrated in Fig. 1, resobene prevented HIV-1 DNA synthesis when present during the virion attachment phase (preincubation of cells with virus, Pre condition), when present during the attachment phase and then added back immediately following re-

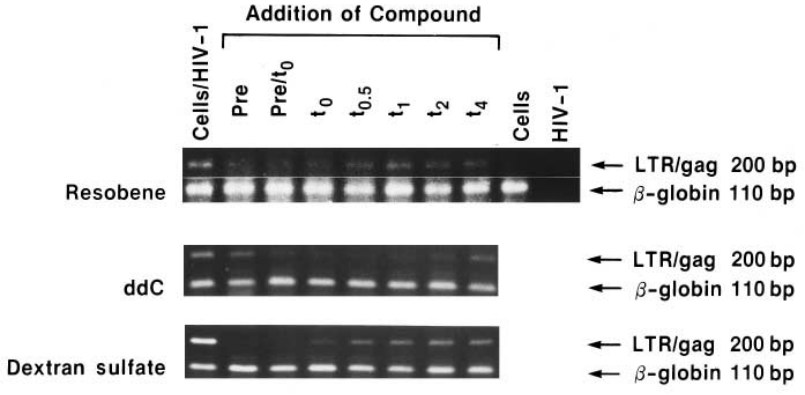

Fig. 1. Time course analysis of resobene-induced HIV inhibition. CEM-SS cells were preincubated with HIV-1 (MOI = 1) for 1 h at 0–4°C to allow virion attachment. The unattached virus was then washed away and the cultures were warmed to 37°C. Resobene (or control reagent) was added during the preincubation step only (Pre), during the preincubation and then added back after removal of unbound virus and warming (Pre/t_0), or at the indicated time after warming (t_0, $t_{0.5\ h}$, $t_{1\ h}$, $t_{2\ h}$, or $t_{4\ h}$). Cells were collected after 24 h and processed as described in Section 2. Compound concentrations employed were as follows: resobene, 100 μg/ml; dextran sulfate, 100 μg/ml; ddC, 10 μM.

moval of unbound virus (Pre/t_0 condition), or when only added immediately after attachment had occurred (t_0). No inhibition of infection was detectable when resobene was added more than 0.5 h ($t_{0.5}$) after virion attachment. Thus, the anti-HIV activity of resobene was observed only when the compound was present during the virion attachment and fusion steps of HIV infection. This finding was consistent with data obtained in parallel for the sulfated polysaccharide dextran sulfate and indicated that the compound acts at an early stage of virus replication. Conversely, addition of the RT inhibitor ddC could be delayed 4–6 h and still effectively inhibit HIV replication (Fig. 1).

The co-cultivation of chronically infected CEM-SS cells with uninfected CEM-SS cells results in rapid syncytium formation. When added at the time of co-cultivation, resobene inhibited syncytium formation at concentrations greater than 5 μg/ml (data not shown), indicating that resobene effectively inhibits the virus-induced fusion of cells. These data were confirmed by observing the effect of resobene on the fusion of HIV envelope-expressing cells (HL2/3 cells) and HeLa-CD4-LTR-β-galactosidase cells. Mixing of these cells results in fusion and syncytium formation, and induction of the Tat-responsive β-galactosidase gene in the syncytia. Resobene was able to effectively inhibit both syncytium formation and β-galactosidase expression at concentrations above 5 μg/ml (Fig. 2(A)).

The co-cultivation of chronically infected cells (CEM-SK1) with uninfected cells (CEM-SS) also results in the rapid transmission of infectious virus from the infected cells to the uninfected cells (cell-to-cell transmission), yielding a burst of virus production in the well. Resobene effectively inhibited the high level of virus production associated with cell-to-cell transmission at concentrations greater than 10 μg/ml. (Fig. 2(B)). The inhibitory activity of resobene on cell-to-cell transmission was also determined in an assay designed to measure the transmission of infectious virus from chronically infected H9 cells (H9-SK1) to CD4(−) cells derived from the cervical epithelium (ME180). Resobene inhibited this cell-to-cell transmission of virus in a dose-responsive manner at concentrations greater than 10 μg/ml (Fig. 2(B)).

Additional experiments were conducted to determine the ability of resobene to inhibit the attachment of virus to both CD4-expressing and CD4-non-expressing cells. The attachment of virus to the HeLa-CD4-LTR-β-galactosidase cell line results in productive infection and the forma-

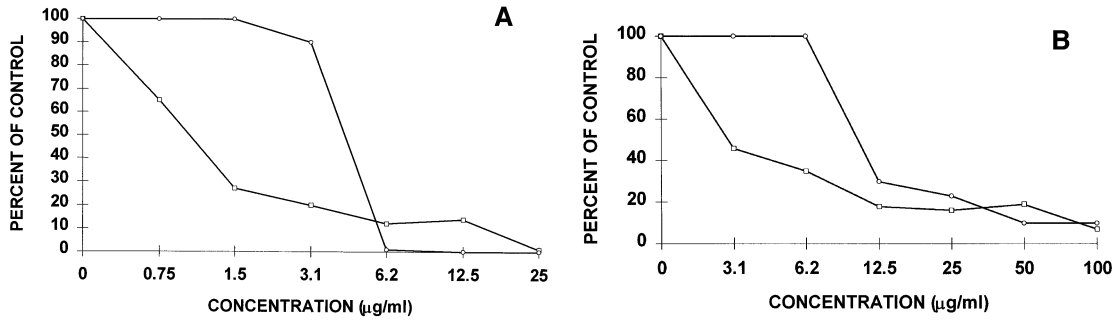

Fig. 2. Mechanism of antiviral action of resobene. (A) Inhibition of cell fusion (○) and virus attachment (□) by a range of concentrations (μg/ml) of resobene in HeLa-CD4-LTR-β-galactosidase cells. (B) Inhibition of cell-to-cell virus transmission from chronically infected lymphocytes to CEM-SS (□) or ME180 (○) cells. Detailed protocols for the performance of each of these assays are presented in Section 2.

tion of syncytia which can be quantitated by staining with X-gal. Resobene, at concentrations greater than 5 μg/ml, inhibited virus attachment when present at the time of infection (Fig. 2(A)). Further experiments were performed to determine the effect of delayed addition of resobene on the inhibition of virus attachment and post-attachment virus-to-cell fusion using HeLa-CD4-LTR-β-galactosidase cells. Data from these assays indicate that the compound can be added to HeLa-CD4-LTR-β -galactosidase cells up to 1.5 h post-infection without any significant loss of inhibitory activity (data not shown). At time points greater than 1.5 h, this inhibitory action is rapidly lost. Although the ME180 cells do not express CD4, these cells are susceptible to cell-free HIV infection by an as yet undetermined CD4-independent route of infection. Resobene was also able to inhibit the attachment of virus to these CD4-nonexpressing cells, but at an EC_{50} concentration approximately 3 times greater than that observed for the inhibition of attachment to CD4-expressing cells (data not shown).

Resobene was found to be totally inactive in the inhibition of virus production from chronically infected lymphocytes (Table 1). These results provided initial evidence that the mechanism of anti-HIV action of the compound was limited to early occurring events, such as attachment and internalization. Confirmatory biochemical inhibition assays performed with resobene indicated that the compound did not inhibit RT, integrase, or protease (data not shown).

To further delineate the specific mechanism employed by resobene to prevent virus attachment or cell fusion, antibody-binding inhibition assays were performed using a purified anti-CD4 monoclonal antibody and two distinct antibodies specific for the V3 loop of the viral envelope glycoprotein gp120. Resobene had no effect on the binding of the anti-CD4 antibody to CD4 expressed on 174 × CEM cells as determined by flow cytometric analysis at concentrations up to 50 μg/ml. However, the compound did effectively inhibit the binding of both gp120 V3 loop antibodies in a dose-responsive manner. Binding of the PB1 sub2-CN1 antibody (a polyclonal antibody specific for amino acids 295–333) to the V3 loop of gp120, as evaluated by ELISA, was completely blocked at resobene concentrations at or above 0.8 μg/ml ($EC_{50} = 0.02$ μg/ml). A second antibody (a monoclonal antibody specific for amino acids 307–327 of gp120) was also inhibited from binding to the V3 loop of gp120 in the presence of resobene as determined by flow cytometric analysis. Resobene inhibited the binding of this antibody to gp120 at concentrations at or above 1 μg/ml ($EC_{50} = 5$ μg/ml), although complete blockade of antibody binding required higher concentrations of resobene (50 μg/ml). Although resobene interacts directly with the viral envelope glycoprotein, the compound must be continuously present to be active and did not directly inactivate virions (data not shown).

4. Discussion

Based on the previously reported properties of several members of the naphthalenesulfonic acid class of compounds (Mohan et al., 1990; Gruszecka-Kowalik et al., 1992; Clanton et al., 1992), resobene was synthesized as a sulfonated dye with improved solubility characteristics, less color and enhanced anti-HIV activity. Unlike CSB, resobene does not yield carcinogenic metabolites when administered in vivo (unpublished observations). Resobene was found to exhibit a broad range of antiviral activity in phenotypically distinct human cells, including T-cells, B-cells and monocyte–macrophages. Antiviral activity was demonstrated against several laboratory strains of HIV-1, HIV-1 isolates resistant to nucleoside and nonnucleoside RT inhibitors, and HIV-2. The clinical potential of resobene is highlighted by its ability to inhibit the replication of low passage lymphotropic and macrophage-tropic isolates, including both syncytium-inducing and non-syncytium-inducing isolates, in fresh human peripheral blood lymphocytes and macrophages. Resobene was determined to be active against virus isolates representative of clades of HIV-1 found throughout the world. This antiviral activity against clinical virus isolates is noteworthy in light of the preclinical and clinical experiences with soluble CD4, which emphasized the critical nature of demonstrating the ability of a virus attachment/fusion inhibitor to inhibit clinical strains of virus when the selection of the inhibitor was performed using laboratory-derived isolates (Moore et al., 1993; Daar et al., 1990). All of the clinical virus isolates used in these antiviral assays were determined to be sensitive to AZT, ddC, ddI, and nevirapine and were phenotypically defined in terms of their growth potential in culture (slow/low versus rapid/high), their syncytium-forming ability in fresh human cells, and their ability to grow in established human cells such as MT-2 (syncytium-inducing versus non-syncytium-inducing isolates). Resobene inhibited each of these phenotypically distinct virus isolates. Consistent with previous findings on the combination anti-HIV activity of attachment and fusion inhibitors (Busso and Resnick, 1990; Anand et al., 1990), the combination of resobene with either AZT or ddI yielded additive inhibition of HIV replication without evidence of antagonism or synergistic toxicity (R.W. Buckheit, unpublished observations). To date, efforts to select for resobene-resistant viruses have been unsuccessful.

Flow cytometric and ELISA analysis indicated that resobene inhibited HIV replication by binding to the cationic V3 loop of the HIV envelope glycoprotein, gp120. The hypervariable V3 loop has a functional requirement for cationic amino acid residues and maintains a high percentage of positively charged amino acids across all strains of HIV (Callahan et al., 1991). The V3 loop is required for binding of the virion to target cells and for cell–cell fusion (Fouchier et al., 1992; De Jong et al., 1992). The extent of virus-mediated cell fusion and rapid viral replication has been correlated with increased cationic composition of the V3 loop (Fouchier et al., 1992). The V3 loop is also considered to be the principal neutralizing domain of the envelope glycoprotein, eliciting type-specific neutralizing antibodies that block viral infection by inhibition of cell fusion (Kenealy et al., 1989; Javaherian et al., 1989). Resobene was found to inhibit the attachment of infectious virus to human cells, to inhibit post-attachment virus-to-cell fusion, to inhibit the fusion of chronically-infected and uninfected CEM-SS cells, and to inhibit the fusion of indicator cell lines expressing CD4 with cells expressing gp120. In addition, resobene inhibited the cell–cell transmission of virus to both CD4-expressing and non-CD4 expressing cell lines, indicating that the compound inhibits HIV replication through a CD4-independent mechanism. Resobene was not found to interfere with the binding of a specific monoclonal antibody to cell surface CD4, but did prevent antibody binding to gp120 in both ELISA and cell-based systems. Specifically, resobene inhibited the binding of two distinct antibodies specific for the V3 loop of gp120. The V3 loop is also the site of action of both the sulfated polysaccharide dextran sulfate and the phosphorothioate oligonucleotide ISIS 5320, which similarly inhibit virus binding and fusion (Buckheit et al., 1994b; Callahan et al., 1991). Resobene did not directly inactivate HIV.

Further evidence that resobene interacts with the glycoprotein V3 loop was obtained from results indicating that resobene did not exhibit activity against the SIV isolate $Delta_{B670}$. Similar results were obtained with the V3-loop-specific, phosphorothioate oligonucleotide ISIS 5320 (Buckheit et al., 1994b). It has been reported that isolates of HIV-1, HIV-2 and SIV differ in their conformational response to the binding of soluble CD4 (sCD4) (Sattentau et al., 1993) and that the V3 loop region of the HIV-2 and SIV envelope glycoprotein was less exposed in its native conformation, resulting in resistance to neutralization by sCD4 (Looney et al., 1990; Clapham et al., 1992). The binding of sCD4 to HIV-2 and SIV induced increased exposure of the V2 and V3 loops of the surface glycoprotein (Sattentau et al., 1993). It has also been reported that the V3 loop of SIV_{agm} may be cryptic and exposed only after the binding of sCD4 (Allan et al., 1990). These studies, as well as the results presented here, indicate that the binding and fusion events required for virus infection may be different for SIV, and that the target site for resobene may not be available until the virus has completed that step of infection that is inhibited by the compound.

The results of variable MOI experiments and combination antiviral activity assays provide support for the potential therapeutic use of resobene. In contrast to results obtained with AZT, resobene was relatively insensitive to changes in MOI. This suggests that sufficiently high concentrations of resobene might completely suppress the binding of virus to infected cells, thus preventing de novo infection of cells. Based on its range and mechanism of anti-HIV action, resobene was also evaluated in assays designed to identify potential inhibitors of sexual HIV transmission. In this assay system, chronically infected lymphocytes or cell-free virus are co-cultured with uninfected cervical epithelial ME180 cells, which do not express cell surface CD4. Resobene effectively inhibited the transmission of virus to these cells, indicating that the compound may be useful as a topical microbicide. Other compounds such as dextran sulfate and nonoxynol-9 have been previously proposed for use as inhibitors of HIV sexual transmission. These studies, as well as those demonstrating the activity of resobene when challenged at high MOI, indicate that resobene may be used at high concentration as topical microbicides and prevent the attachment of virus to target cells irrespective of their level of CD4 expression.

In summary, resobene exhibits potent and specific inhibitory activity against HIV infection and replication. The broad range of anti-HIV activity and mechanism of action suggests that resobene may be useful in both preventing infection in uninfected individuals and suppressing virus spread in individuals infected with HIV. As with other members of this pharmacologic class, anticoagulation, protein binding and oral bioavailability issues remain to be resolved. Resobene was determined to be effective at inhibiting HIV replication in a hollow fiber murine animal model system when the fiber was implanted near the site of intravenous resobene administration (M. Hollingshead, personal communication). Fibers located at distal sites were not protected from HIV replication at the highest dose tested (100 mg/kg, tid). In light of the data presented, resobene may be useful as a topical microbicide to prevent the sexual transmission of HIV.

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