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Novel sulfonated and phosphonated analogs of distamycin which inhibit the replication of HIV

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Abstract

A series of novel distamycin-related polyanionic compounds were compared for their anti-HIV activity. Several were highly potent inhibitors of HIV virus-induced cell killing and viral replication of a wide variety of laboratory isolates, as well as a monocytotropic virus and a clinical isolate in human peripheral blood lymphocytes. These compounds are structurally different from other sulfonic acid containing compounds reported to be potent inhibitors of the human immunodeficiency virus (HIV) in two respects: (1) they are structurally related to the non-toxic minor groove DNA binder distamycin; and (2) a number of them contain the aromatic phosphonic acid group. The compounds that were evaluated can be categorized into monomeric or dimeric ureido structural classes incorporating the bisamido-*N*-methylpyrrolenaphthalene-sulfonic acid group, with differences in the number and position of the sulfonic acids on the naphthalene rings. Broader

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structure–activity studies were made possible through the synthesis and evaluation of the compounds containing only a single *N*-methylpyrrole unit, those incorporating the *N*-methylpyrazole structure, and compounds having the isosteric phosphonic acid group substituted for the sulfonic acid group. One of the most potent of the inhibitors was 2,2′[4,4′[[aminocarbonyl]amino]bis[*N*,4′-di[pyrrole-2-carboxamide-1,1′-dimethyl]]-4,6,8 naphthalenetrisulfonic acid] hexasodium salt, NSC 651015. This compound, the phosphonic acid analog NSC 662162, and the monomeric compound NSC 651018 were studied to determine the mechanism of their inhibitory activity. Mechanistic studies revealed that inhibition was due to the disruption of virus attachment to CD4 + -susceptible cells and a further restraint on fusion of virus and cell membranes. The relative tolerance of these compounds in mice suggests that sufficient antiviral concentrations could be reached in vivo and thus may prove valuable in the treatment of AIDS patients.

Keywords: Human immunodeficiency virus; Antiviral activity; Distamycin analog

1. Introduction

It has long been believed that the causative agent of acquired immune deficiency syndrome (AIDS) is the human immunodeficiency virus, or HIV (Barré-Sinoussi et al., 1983; Gallo et al., 1984; Levy et al., 1984). The search for an effective antiviral compound to combat this virus has been discouraging. To date, only 4 drugs, all reverse transcriptase inhibitors, have been approved for therapy. AZT has shown limited usefulness in the clinic (Lagakos et al., 1993) and, more recently, the emergence of resistant variants of HIV to ddC, ddI, and D4T has reduced their effectiveness (Yarchoan et al., 1991, Lacey and Larder, 1994). Furthermore, the severe toxicities associated with protracted use of these compounds have further diminished their value.

The consensus among researchers in the field of HIV chemotherapy is that a single drug (monotherapy) holds little promise due to the high rate of drug resistant mutations that occur. A more practical approach most likely will be a combination of compounds that attack an essential feature in HIV replication to the point that the virus cannot mutate (convergent combined therapy), or a series of compounds that target several aspects of replication. Since the discovery that combinations of several drugs targeted against the reverse transcriptase of HIV have resulted in mutant viruses that can replicate in the presence of the drugs (Larder, 1993), it is important to continue the pursuit of compounds that interfere with steps in the virus replication cycle other than the reverse transcriptase.

The drug discovery program of the National Cancer Institute operates a high capacity antiviral drug screen which is used to test diverse synthetic and natural product compounds from worldwide sources for anti-HIV activity. We have previously reported on the effects of a variety of naphthalenesulfonic acid dyes on the binding and fusion of HIV to cells (Clanton et al., 1992) and continue to pursue this approach to antiviral therapy. A group of analogs of distamycin (Fig. 1), submitted to the NCI by Pharmacia/Farmitalia Carlo Erba (Milan, Italy), have been extensively studied as potential agents for clinical trials. The properties of these compounds are described.

2. Materials and methods

2.1. Compounds

The compounds used in this study were provided to the NCI for anti-HIV evaluation by Pharmacia/Farmitalia Carlo Erba (Milan, Italy).

2.2. Cell lines and viruses

The CEM-SS lymphocytic cell line was obtained from Peter Nara (Nara and Fischinger, 1988). Chronically infected cell lines CEM_{SK1}, $H9_{SK1}$, $MT2_{SK1}$, $U937_{SK1}$ (Buckheit et al., 1992) were used. The chronically infected cell lines U1 and ACH-2 were obtained from the AIDS Research and Reference Reagent Program, AIDS Program, NIAID, NIH. MT-4 and MT-2 cells were also obtained from the AIDS Research and Reference Reagent Program, a contribution of Douglas Richman. All cells were maintained in RPMI 1640 medium with phenol red and supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 50 μg of gentamicin per ml. The same media without phenol red was used in the primary screen.

The HIV isolates included the common laboratory HIV-1 and -2 strains; $HIV\text{-}1_{RF}$, $HIV\text{-}1_{IIIB}$, $HIV\text{-}2_{ROD}$ have been previously described (Cloyd and Moore, 1989). HIV-1 clinical isolates were obtained from patients at The University of Alabama at Birmingham Children's Hospital. The monocytotropic virus isolate $HIV\text{-}1_{BaL}$, as well as the AZT sensitive (AO12 G762-3) and AZT-resistant (AO12 G691-2, mutations at amino acids 67, 70, 215, and 219). HIV-1 isolates were obtained from the AIDS Research and Reference Reagent Program, and the pyridinone resistant HIV-1 A17 isolate with mutations at amino acids 103 and 181 in the viral RT domain (Nunberg et al., 1991) was obtained from Emilio Emini at Merck Sharpe and Dohme Laboratories. Both the nevirapine resistant mutant (N119, mutation at amino acid 181) and SIV_{MAC251} virus isolates were also obtained from the AIDS Research and Reference Reagent Program. Cells and viruses utilized in the performance of Rauscher MuLV assays have been previously described (Shannon et al., 1974).

2.3. Assays for anti-HIV activity in cultured cells

The initial anti-HIV screening was performed as previously described (Weislow et al., 1989). This microtiter assay quantitates drug-induced protection from the killing of CD4 + lymphoid cells by $HIV\text{-}1_{RF}$. Briefly, test compounds and controls, 3′-azido-3′-de-

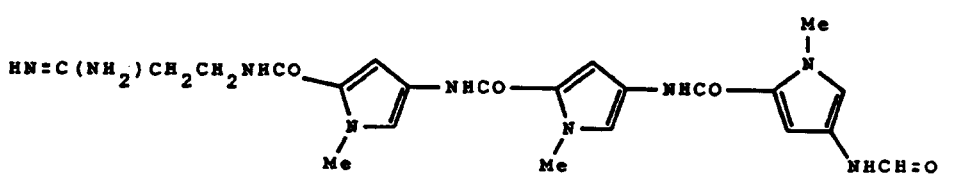


Fig. 1. The structure of distamycin.

oxythymidine (AZT, NSC 602670) or dideoxycytidine (ddC, NSC 606170), were serially diluted in complete medium and added to 96-well test plates. Exponentially growing CEM-SS cells were pelleted, suspended in complete medium, and added at 5000 cells per well. Frozen virus stock solutions were thawed immediately before use, suspended in complete medium to yield the desired multiplicity of infection (m.o.i. ≈ 0.1), and added to the microtiter wells. Test plates were incubated at 37°C in 5% CO_2 for 6 days. On day 6, aliquots of cell-free supernatant fluid were removed from each well and analyzed for reverse transcriptase (RT) activity, p24 antigen, and infectious virions (Gulakowski et. al., 1991). Using the XTT assay, antiviral and toxicity data is reported as the concentration of drug required to inhibit 50% of virus induced cell killing (EC_{50}) and the concentration of drug required to reduce cell viability by 50% (IC_{50}).

The activity of compounds in chronically infected cell lines was evaluated as previously described (Buckheit and Swanstrom, 1991). Cell-free supernatant samples were collected daily and analyzed for the presence of virion-associated RT.

Phytohemagglutinin-stimulated human peripheral blood mononuclear cells (PBMCs) were prepared according to the method of Saag (Saag et al., 1991). Antiviral assays in PBMCs were performed as previously described (Rice et al., 1993). Culture supernatant fluids were assayed for HIV RT and/or p24 by an antigen capture ELISA as described above. Fresh monocyte-macrophage cultures were prepared from blood of uninfected donors by electroelution (Gerrard et al., 1983). The cells were incubated for 7 days in complete medium containing 1000 U/ml M-CSF (kindly provided by Genetics Institute, Cambridge, MA) and then washed twice with phosphate buffered saline (PBS). The cultures were treated with various concentrations of antiviral compound for 2 h prior to infection with the Ba-L strain of HIV-1 (m.o.i. ≈ 0.5). Following 7 days in culture at 37°C in complete medium the supernatant levels of p24 were determined as described above.

2.4. Time course experiments

In order to identify the stage(s) of HIV replication that is affected, the compounds were evaluated in a high m.o.i. acute phase time-of-addition assay (TOA) (Cushman et al., 1994). CEM-SS cells (1×10^5) were preincubated with HIV-1_{IIIB} (m.o.i. = 1.0) at 0–4°C for 1 h to allow attachment of virus to cells, but not fusion. Samples were then washed 3 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 infectious cycle to proceed. Compound (25 μM) 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 postwarming). Dextran sulfate (100 μg/ml) and ddC (10 μM) served as controls for inhibitors of virus attachment and reverse transcriptase, 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 PCR

using LTR/*gag* primer pairs (M667/M661, Recombinant DNA Laboratory, Program Resources, Inc., NCI-FCRDC, Frederick, MD) and products of the β-globin gene were amplified using primer pairs as previously described (Zack et al., 1990). Amplified products were analyzed by electrophoresis in 2% agarose gels and visualized by ethidium bromide staining. The specificity of products was verified by restriction enzyme cleavage and by Southern blot hybridization.

2.5. Virus attachment and fusion assays

Binding of HIV-1_{RF} to PBMCs was measured by a p24-based assay (Rice et al., 1993). Briefly, 5×10^5 PBMCs were incubated with a concentrated stock of virus for 30 min at 37°C, the unbound virus was washed away, and the cell-associated virus was solubilized in 1% Triton X-100, 1% BSA and analyzed by the p24 antigen-capture assay as described above.

HeLa CD4 + cells containing a β-galactosidase gene attached to the HIV-1 promoter were used to study HIV-specific membrane fusion interactions (Kimpton and Emerman, 1992). HeLa cells (HL2/3) expressing HIV-1 envelope protein on the cell surface, as well as tat protein in the cytoplasm, can fuse with the CD4 + /β-gal cells, activating β-galactosidase in the syncytium (Ciminale, et al., 1990). Effects of compounds on infectivity and fusion interactions were examined by exposing the appropriate cells to increasing concentrations of the drug before mixing with the HL2/3 cells. Foci of fused cells expressing β-galactosidase can be observed microscopically after staining with the reagent X-gal.

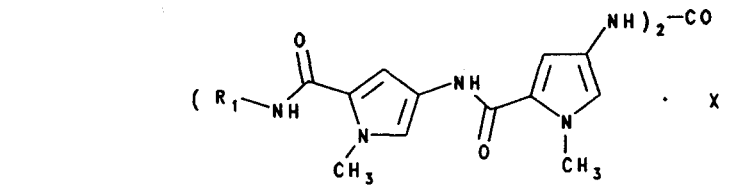
2.6. Binding and enzymatic assays

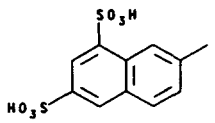
The binding of gp120 to CD4 was analyzed using an antigen capture ELISA (DuPont). All steps of the assay were carried out according to the manufacturer's protocols. The effect of drugs on the in vitro activity of recombinant RT was determined by a previously described method (Buckheit and Swanstrom, 1991). The assay measures the incorporation of [^{3}H]TTP onto the artificial poly(rA):oligo(dT) homopolymer primer/template. Samples (5 μl) 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; the absence of RT served as the negative control. HIV protease activity was quantitated by a reverse-phase HPLC assay utilizing the Ala–Ser–Glu–Asn–Tyr–Pro–Ile–Val–Glu–Amide substrate (Multiple Peptide Systems, San Diego, CA) as described (Wondrak et al., 1991).

2.7. Combination antiviral analysis

Analysis of drug combination assays was performed utilizing the XTT assay described above, with statistical evaluations performed according to the method of Prichard and Shipman (Prichard et al., 1990). Combination antiviral XTT assays were performed with CEM-SS cells utilizing HIV-1_{RF}.

Table 1
Structures and anti-HIV activities of the ureido analogs





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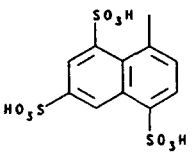

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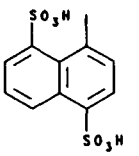

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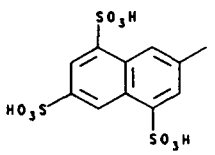

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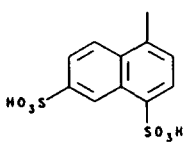

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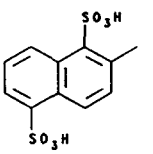

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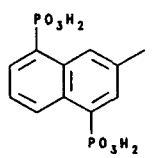

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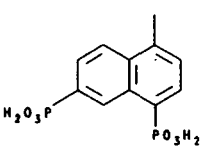

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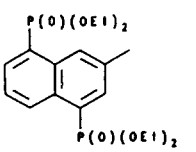


No.	NSC no.	R_1	X	EC_{50} (μM)	IC_{50} (μM)
2	645793	A	4K	29.6	> 200
3	645794	B	6Na	31.6	> 200
4	645795	C	4Na	Inactive	> 200
5	651015	D	6Na	4.6	> 200
6	651016	E	4Na	12.3	> 200
7	651017	F	4Na	23.0	153
8	658434	G	4Na	6.6	> 200
9	662162	H	4Na	3.9	> 200
10	658433	I	–	Inactive	> 200

2.8. Pharmacokinetic studies

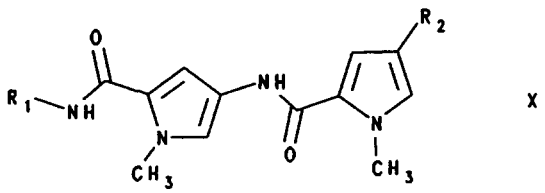
Plasma samples and tissue homogenates were extracted with CH_3CN/MeOH (1 : 1, v/v) and the resultant supernatant fluids analyzed with a Waters C-18 Nova-pack reverse phase column (300 × 3.9 mm) using a phosphate buffer (pH 6.5): CH_3CN/MeOH mobile phase containing 5 mM tetrabutylammonium phosphate at a flow rate of 1.2 ml/min. Suramin (Germanin, Bayer) was used as an internal standard.

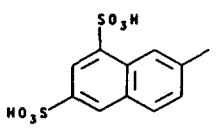
3. Results

3.1. Structure–activity relationships

For structure activity comparisons, the 27 distamycin analogs were grouped into 4 broad structural types. The majority of the compounds fell into the dimeric ureido structural class incorporating the bisamido-*N*-methylpyrrolenaphthalenesulfonic (or phosphonic) acids (Table 1), with a small subgroup of monomeric analogs (Table 2).

Table 2
Structures and anti-HIV activities of the monomeric analogs with two *N*-methylpyrrole groups





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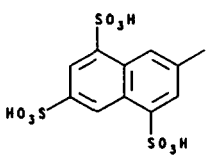

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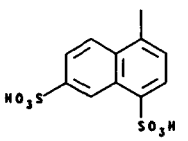
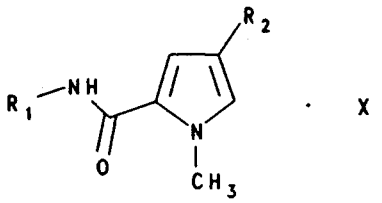
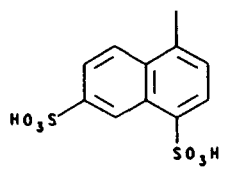


E

No.	NSC no.	R_1	R_2	X	EC_{50} (μM)	IC_{50} (μM)
11	651018	A	NH_2	K	52.8	256
12	655717	D	NO_2	3Na	256	> 200
13	655718	D	NH_2	3Na, HCl	155.7	> 200
14	655721	E	NO_2	2Na	Inactive	> 200
15	655722	E	NH_2	2Na, HCl	34.9	313

Table 3
Structures and anti-HIV activities of the monomeric analogs with a single *N*-methylpyrrole group





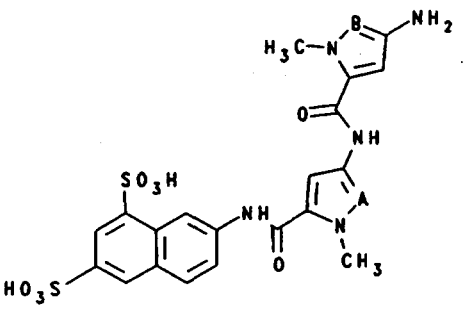
E

No.	NSC no.	R_1	R_2	X	EC_{50} (μM)	IC_{50} (μM)
16	655719	E	NO_2	2Na	Inactive	> 500
17	655720	E	NH_2	2Na, HCl	Inactive	> 300

Congeners were also synthesized that contained a single *N*-methylpyrrole unit (Table 3), or the isosteric *N*-methylpyrazole structure (Table 4). All congeners were evaluated from a high test concentration of 200 μM for their 50% effective antiviral concentration (EC_{50}) and their concentrations that caused 50% cell death (IC_{50}) utilizing the XTT cytoprotection assay and for each congener.

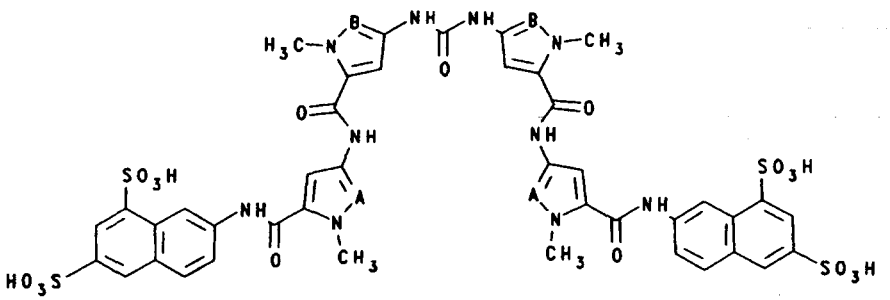
Comparison of the chemical structure of these compounds with their anti-HIV activity revealed that the dimeric ureido structural class of compounds were more potent than the corresponding monomeric units (Tables 1 and 2, **2** versus **11**, **5** versus **12** and **13**, and **6** versus **14** and **15**). Within the monomeric series (Table 2), the nitro-substituted compounds were considerably less active than the corresponding amino compounds. Compounds containing only a single *N*-methylpyrrole unit were inactive, even with an amino substituent (Table 3, **16** and **17**). As in the *N*-methylpyrrole series (Tables 1 and 2), the dimeric compounds containing the *N*-methylpyrazole ring were far more potent than the corresponding monomeric types (Table 4).

Table 4
Structures and anti-HIV activities of monomeric and ureido analogs containing the isosteric *N*-methylpyrazoles



· X

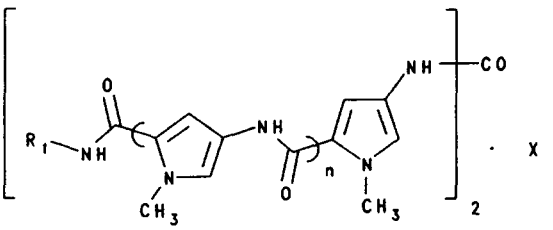
No.	NSC no.	A	B	X	EC_{50} (μM)	IC_{50} (μM)
11	651018	CH	CH	K	52.8	256
18	668531	N	N	2K, HCl	Inactive	> 200
19	668532	N	CH	2K, HCl	19.5	> 200
20	668533	CH	N	2K, HCl	Inactive	> 200

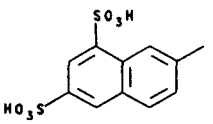


· X

No.	NSC no.	A	B	X	EC_{50} (μM)	IC_{50} (μM)
2	645793	CH	CH	4K	29.6	> 200
21	668535	CH	N	4Na	7.9	> 200
22	668536	N	N	4K	7.5	> 200
23	668537	N	CH	4K	6.5	> 200

Table 5
Structures and anti-HIV activities of the ureido analogs with varying numbers of *N*-methylpyrrole groups





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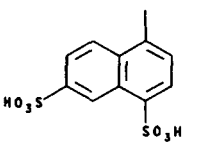

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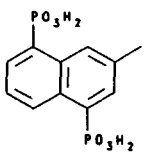

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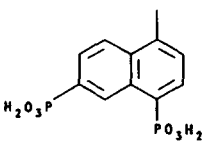

H

No.	NSC no.	R_1	$(n+1)$	X	EC_{50} (μM)	IC_{50} (μM)
24	664740	A	1	4Na	80.7	> 200
2	645793	A	2	4K	29.6	> 200
25	664739	A	3	4Na	1.0	86
6	651016	E	2	4Na	12.3	> 200
26	670886	E	3	4Na	0.2	> 200
8	658434	G	2	4Na	6.6	> 200
27	670887	G	3	4Na	0.3	> 200
9	662162	H	2	4Na	3.9	> 200
28	670888	H	3	4Na	1.4	> 200

Among the dimeric ureido compounds, substitution of a pyrrole carbon with nitrogen increased the antiviral potency (Table 4, **2** versus **21**, **22**, or **23**). The distance between the naphthalene ring and the ureido function, as determined by the number of linking *N*-methylpyrrole units, had an effect on the observed anti-HIV activity, as shown in Table 5. This was true whether or not the substituents on the naphthalene ring were sulfonic or phosphonic acids, although the difference is less apparent with the phosphonic derivatives.

A detailed SAR analysis of the promising dimeric ureido structural class of compounds (Table 1) highlighted several prominent features. The position of attachment of the naphthalene ring (α or ß) to the pyrrole unit affected potency, with the ß-position favored (**5** versus **3**).

The effect of the positioning of the sulfonic acid substituents on the naphthalene ring on the antiviral activity was also related to the position of attachment of the naphthalene group (α or ß) to the pyrrole unit. When the attachment was ß, there was very little effect (**2** versus **7**); if the attachment was α, on the other hand, the effect was profound (**4** versus **6**). The most potent compound in this series, **5** (NSC 651015), contained 3 sulfonic acid units. When the naphthalene ring was attached in the ß-position (in contrast to the α-position) the number of sulfonic acid groups in the molecule dramatically influenced activity. The replacement of sulfonic acid by the isosteric phosphonic acid enhanced the anti-HIV potency (**6** versus **9**), while esterification of phosphonic acid obliterated the anti-HIV activity (**8** versus **10**).

3.2. Range of activity

Three compounds were chosen for further testing. The naphthalenesulfonic (NSCs 651015 (**5**), and 651018 (**11**)) and phosphonic acid (NSC 662162 (**9**)) compounds were

Table 6
Range of activity of selected distamycin-related naphthalenesulfonic and naphthalenephosphonic acid compounds

Isolate	EC_{50} (μM) [a]			EC_{50} (nM)	
	651015	651018	662162	CSB	AZT
HIV-1					
IIIB	3.2	10.7	1.3	0.6	1.82
RF	0.4	7.1	1.7	1.7	1.71
A17	1.0	6.7	1.3	0.6	0.14
N119	0.4	7.0	0.3	0.5	4.4
G762-3	0.2	5.4	0.1	2.5	2.6
G691-2	0.1	3.6	35.0	3.9	> 100
Ba-L [b]	0.4	1.1	6.1	1.8	0.6
HIV-2					
ROD	4.2	120	Inactive	18.0	0.9
SIV					
MAC251	84	30.3	Inactive		32
WEJO [c]	2.7	56	0.4	2.9	3.0
Rauscher					
MLV [d]	2.3	ND [e]	ND	2.9	1.5

[a] Values shown are representative of results obtained from at least two replicate determinations; standard error measurements of replicate EC_{50} determinations typically average less than 10%; high test concentration of each assay was 200 μM.
[b] Cultured in monocyte/macrophages.
[c] In PBMCs, IC_{50}, p24 endpoint.
[d] UV-XC plaque reduction.
[e] ND, not done.

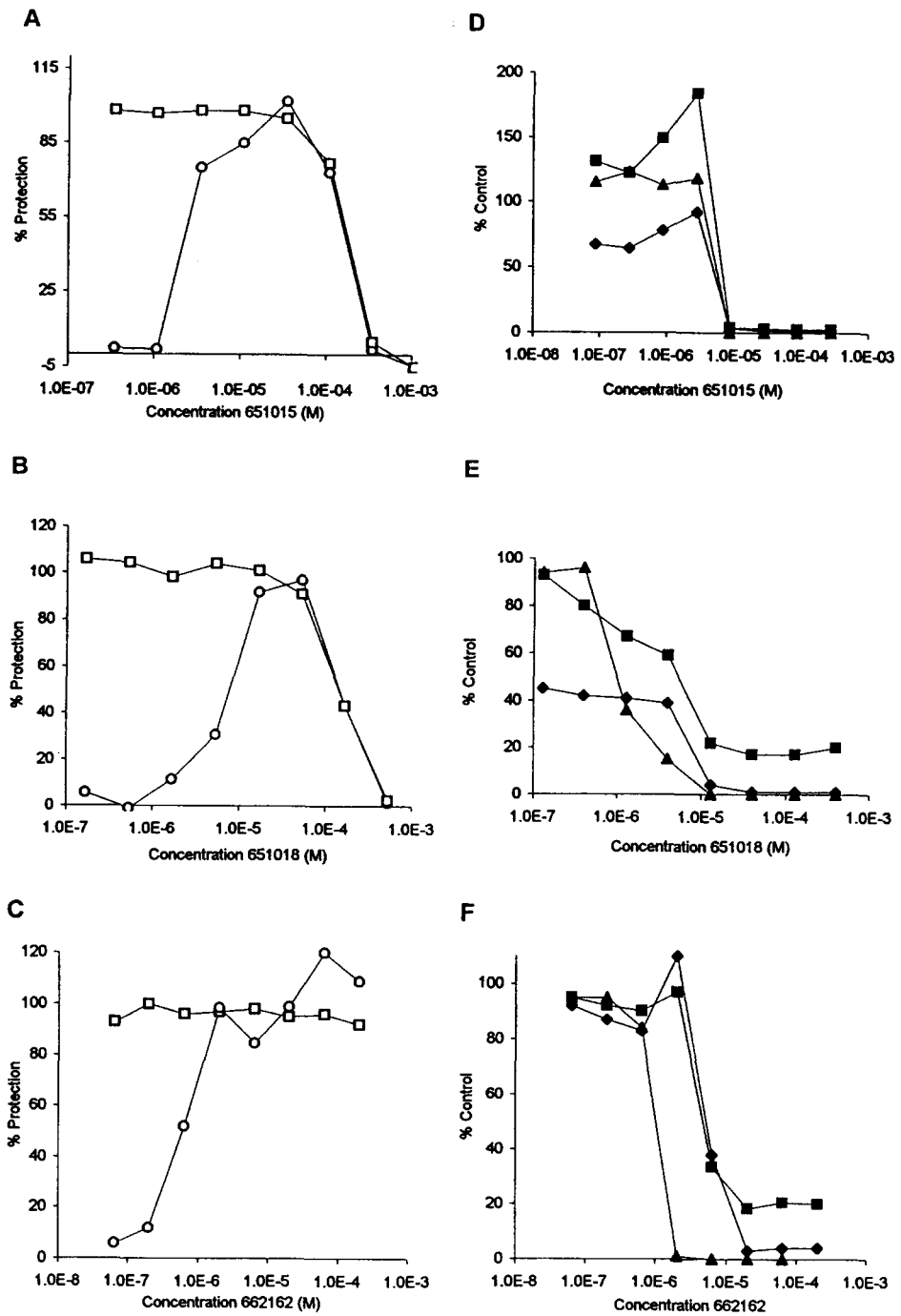

Fig. 2. Relative activities of NSCs 651015, 651018, and 662162. A–C represent the results of the XTT primary screen. ○, infected cells; □, uninfected cells. D–F are the corresponding indices of viral replication. ▲, p24; ◆, syncytia; ■, RT.

active against a wide range of HIV-1 isolates (Table 6). AZT and the sulfonated surface binding inhibitor Chicago Sky Blue (CSB) (Clanton et al., 1992) are shown for comparison. Specifically, these compounds protected cells from all HIV-1 and -2

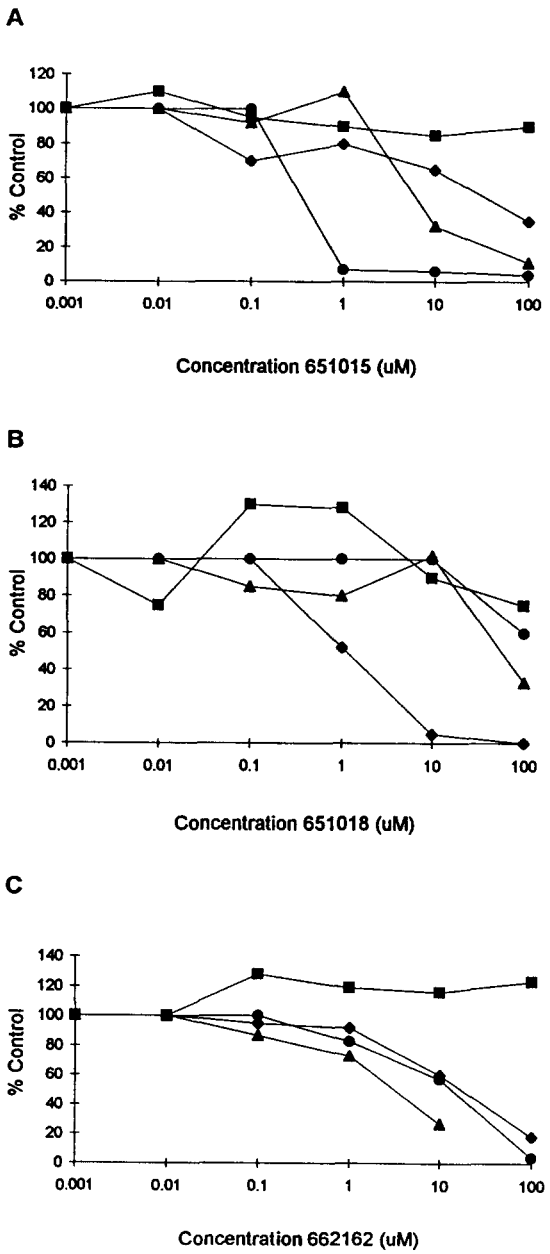

Fig. 3. Comparison of binding and enzymatic activities of (A) 651015, (B) 651018, and (C) 662162. ■, protease; ●, RT; ▲, gp120–CD4; ◆, attachment.

laboratory isolates at concentrations between 0.1 and 10 μM. These compounds could inhibit the replication of several drug resistant mutants, such as A17, N119, and G910-6 viruses at essentially the same level as HIV-1_{RF}. The compound was effective against the WEJO strain of HIV-1 in PBMCs, as well as the monocytotropic virus HIV-1_{BaL} in monocyte/macrophages. NSC 651015 also inhibited SIV and Rauscher MuLV at slightly higher concentrations. It is interesting to note that the phosphonated compound did not inhibit either HIV-2 or SIV.

The cell-free supernatant fluids from infected cells treated with NSCs 651015, 651018, or 662162 were found to be free of p24, reverse transcriptase, or infectious virus particles at cytoprotective concentrations. The 50% inhibitory concentrations (IC_{50}) of all 3 indices were similar to those obtained in the cytopathicity assay (Fig. 2D–F).

Chronically infected cell lines were tested at 0, 0.2, 2.0, and 20 μM to determine the effect of 651015 on production of virus by measuring virion-associated reverse transcriptase at 24, 48, and 72 h. Culture fluids were collected after treatment with drug. A continuous uninhibited production of RT was found over the time course of the experiment. Control experiments without supernatant fluids displayed very low (< 200 cpm) background activity (data not shown).

3.3. Mechanism of action studies

Mechanistic studies were performed to evaluate the effects of compounds on individual steps in the virus replication cycle. As shown in Fig. 3, each of the compounds inhibited the attachment of HIV-1 virions to target cells, with 651015 and 662162 being the most active. The binding inhibition was correlated with a concomitant blockage of the interaction between gp120 and CD4. None of the compounds inhibited the enzymatic

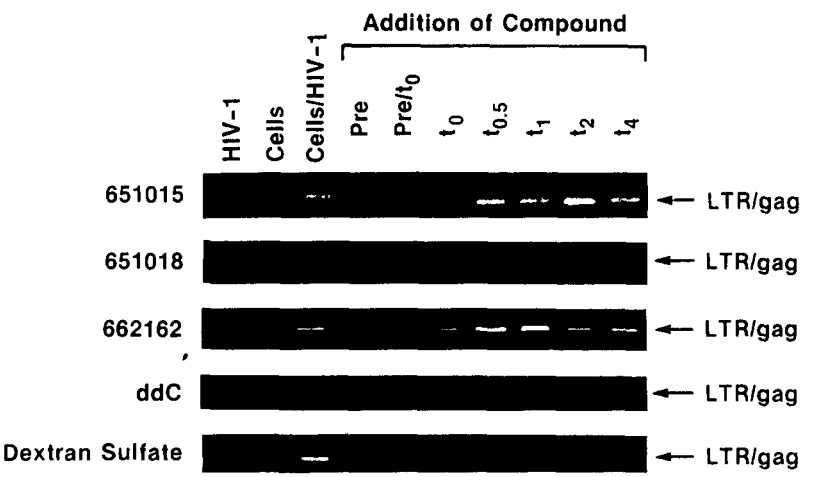

Fig. 4. Time of addition assay. Drugs were added as described in Materials and methods. The successful infection of cells was determined by PCR and separation of DNA products on agarose gels.

activity of the HIV-1 protease. The compounds did inhibited the in vitro enzymatic activity of purified HIV-1 RT, but the effect did not correlate with biological activity (see time course assays below).

To more clearly define the true antiviral mechanism, we performed acute infection time course studies that are designed to identify the stage(s) of the HIV-1 replication cycle in which a compound acts. As shown in Fig. 4, NSCs 651015, 651018, and 662162 all prevented intracellular HIV-1 proviral DNA synthesis when added during the attachment period of virus with cells (Pre and Pre/t_0), indicating that binding of virions was inhibited. Moreover, 651015 and 651018 appeared to inhibit the fusion process, as indicated by the prevention of proviral DNA synthesis when compounds were added at t_0 after viral attachment had already occurred. However, 662162 was ineffective in cells treated after virus attachment (t_0), since infection progressed through reverse transcription. Waiting 0.5 h or longer to add any of these compounds failed to inhibit DNA synthesis. In control experiments, ddC (a nucleoside inhibitor of reverse transcriptase) prevented DNA synthesis when added as late as 2 h after virus binding, as reverse transcription was not completed until approximately 4 h. This pattern of inhibition by an RT inhibitor was distinct from that of the ureido-based compounds. Dextran sulfate prevented DNA synthesis if present during the virus attachment phase (Pre), but not when added following virus binding.

A fusion inhibition assay was used to examine if the compounds had a direct effect on cells in the absence of virus production. HL2/3 cells expressing the viral envelope glycoproteins as well as the HIV-1 tat protein will fuse with HeLa cells expressing the CD4 receptor gene to form visible syncytia. These latter HeLa cells also contain a tat-inducible β-galactosidase gene, which facilitates observation and quantitation after staining the mixed cells with X-gal. Increasing concentrations of NSC 651015 and 651018 inhibited the fusion of cells in a dose-dependent manner (Fig. 5), and completely inhibited the formation of blue multinucleated cells at 5 μM, consistent with cytoprotec-

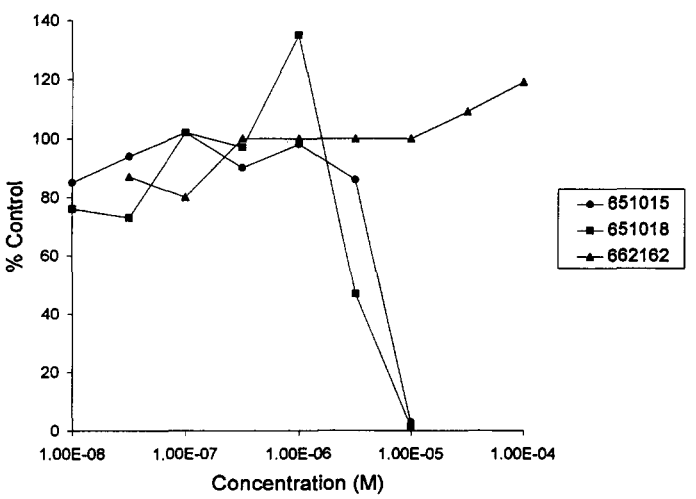

Fig. 5. Fusion inhibition assay. HeLa-CD4-LTR-β-gal cells were mixed with HL2/3 cells in the presence of increasing concentrations of drugs. After staining with X-gal, foci of fused cells were counted.

tion assays. However, the phosphonated compound NSC 662162 failed to prevent the formation of syncytia even at the highest concentration.

Finally, all compounds failed to directly inactivate infectious virions. The pretreatment of susceptible cells with NSC 651015 for 2 h followed by drug removal also failed to inhibit virus replication or the formation of syncytia (data not shown).

3.4. Drug combination studies.

Serial dilutions of NSC 651015 ranging from 0.2 to 125 μM were assayed in combination with AZT (0.2–120 nM) for activity against HIV-1_{RF} and HIV-1_{IIIB}. Only additive effects (confidence limits > 95%) were observed (data not shown). Synergistic toxicities were not observed.

Table 7
Summary of pharmacokinetics in mice: NSC 651015

	Time after administration	Concentration
Plasma	2 h	322 μM
	24 h	124 μM
	48 h	98 μM
	168 h	42 μM
Spleen	2 h	46 μg/g
	24 h	47 μg/g
	48 h	46 μg/g
	168 h	51 μg/g
Liver	2 h	13 μg/g
	24 h	23 μg/g
	48 h	28 μg/g
	168 h	14 μg/g
Lung	2 h	187 μg/g
	24 h	154 μg/g
	48 h	143 μg/g
	168 h	110 μg/g
Thymus	2 h	34 μg/g
	24 h	22 μg/g
	48 h	24 μg/g
	168 h	27 μg/g
Kidney	2 h	112 μg/g
	24 h	405 μg/g
	48 h	611 μg/g
	168 h	860 μg/g
LD_{50}	574 mg/kg	

All mice were dosed at 200 mg/kg i.v.

3.5. Preliminary pharmacokinetic studies

NSC 651015 was well tolerated in mice. Animals were dosed at 200 mg/kg administered intravenously. The amount of drug that accumulated in various tissues was determined by HPLC as described in the methods section. Inhibitory concentrations remained in the plasma for more than 7 days. Additionally, NSC 651015 accumulated in hematopoietic tissue while rapidly collecting in the kidney. The LD_{50} in mice dosed intravenously was 574 mg/kg. (Table 7).

4. Discussion

The sulfonic and phosphonic acid distamycin analogs described in this paper represent a new class of surface acting antiviral agents. The compounds had broad activity against many different strains of both HIV-1 and HIV-2, as well as a clinical isolate. Structure–function analysis suggests that the number of *N*-methylpyrrole groups plays a significant part in the inhibitory properties of these compounds, particularly for the sulfonated analogs. Whether this is due to a difference in spacing of the naphthalene moieties or is a direct function of the *N*-methlypyrrole group has yet to be determined. In the monomeric series, the nitro substituted compounds were certainly less active than the corresponding amino compounds, possibly due to the electron withdrawing effects of the nitro substituent.

Specifically, NSC 651015 (**5**) was shown to interfere with attachment of virus to cells, most likely due to a modest effect on CD4–gp120 interactions, as well as inhibiting the fusion process between virus and cell. This was most clearly seen in the time of addition study where adding drug during attachment (Pre), continuing drug treatment after attachment (Pre/t_0), or adding drug after attachment (t_0) prevented subsequent infection. The inhibition of binding/fusion was confirmed in the fusion assay where HL2/3 cells were prevented from fusing to HeLa CD4 + cells at drug concentrations similar to those effective in the XTT assay. This is also consistent with the ability of NSC 651015 to inhibit CD4–gp120 interactions in the ELISA assay ($IC_{50} \approx 5\ \mu M$). It is interesting to note that the phosphonic acid derivative NSC 662162 (**9**) did not prevent infection when added after attachment (t_0) in the time course assay. Consistent with these results are the data from the fusion assay, suggesting that the fusion-associated events were curtailed by the sulfonic acid derivatives, but not by the phosphonic acid derivatives. The phosphonic acid derivative also failed to inhibit both HIV-2 and SIV, isolates with remarkable homology of their envelop proteins (Hirsch et al., 1989). Studies are in progress to understand the selectivity of this unique compound.

It is unlikely that these highly polar compounds enter the cell to inhibit HIV replication by their effect on RT. There was no correlation between biological activity and enzymatic activity with this group of compounds, and the time course assay clearly show that inhibition is at the surface of the cell. Any inhibition of RT by these compounds in cells would have been if the compounds were added after time t_0.

We and others (Clanton et al., 1992; Gruszecka-Kowalik et al., 1992; see review, Mohan and Baba, 1993) have been studying the effects of sulfonic acid derivatives for

their potential use in clinical trials. It was shown that many of the polyanionic sulfonated compounds would bind to serum proteins and that mere changes in the position of the sulfonic acid moieties would alter both protein binding and antiviral activity. In most cases, the antiviral activity of these compounds was attributed to the inhibition of binding/fusion. The most commonly studied compounds, the naphthalenesulfonic acids, were required to be in bis formation to retain antiviral activity, although the naphthalene subunit did display anti-RT activity (Tan et al., 1992). As previously noted, the inhibition of virus infectivity by the naphthalenesulfonic acid compounds is non-competitive with respect to the inhibition of gp120–CD4 binding (Kozlowski and Watson, 1992). This may be a valuable asset in terms of clinical potential. Inhibition of this kind cannot be overcome by increasing viral load and complete inhibition of virus reproduction would be easier to achieve.

There is considerable debate over the use of combinations of drugs to circumvent the problems of mutagenesis. Approaches fall into two categories: using different drugs that target the same site of replication (convergent combined drug therapy), and using combinations of drugs that target different sites (divergent therapy). While convergent drug therapy initially appeared promising, more recent data in vitro and in vivo have shown that multiple drug resistance is a common occurrence (Larder et al., 1993). The use of divergent drug therapy has yet to be fully tested. It is therefore important to investigate drugs such as those presented here that have effects at different sites in the replication cycle of HIV.

The favorable features of the distamycin derived compounds are that they are well tolerated in animals, are of relatively low molecular weight, are colorless, and, unlike certain azo dyes such as Chicago Sky Blue, will not metabolize to carcinogens. Their potential usefulness in a clinical setting remains to be determined.

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