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Preferential Inhibition of the Magnesium-Dependent Strand Transfer Reaction of HIV-1 Integrase by α-Hydroxytropolones

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ABSTRACT

Integration is a crucial step in the life cycle of human immunodeficiency virus type 1 (HIV-1); therefore, inhibitors of HIV-1 integrase are candidates for antiretroviral therapy. Two 7-hydroxytropolone derivatives (α-hydroxytropolones) were found to inhibit HIV-1 integrase. A structure-activity relationship investigation with several tropolone derivatives from The National Cancer Institute compound repository demonstrated that the 7-hydroxy group is essential for integrase inhibition. α-Hydroxytropolones preferentially inhibit strand transfer and are inhibitory both in the presence of magnesium or manganese. Lack of inhibition of disintegration in the presence of magnesium coupled with results from different cross-linking assays suggests α-hydroxytropolones as interfacial inhibitors. We propose that α-hydroxytropolones chelate the divalent metal (Mg^{2+} or Mn^{2+}) in the enzyme active site. The most active compound against HIV-1 integrase in biochemical assays [2,4,6-cycloheptatrien-1-one, 2,7-dihydroxy-4-isopropyl (NSC 18806) IC_{50} = 4.8 ± 2.5 μM] exhibits weak cytoprotective activity against $HIV\text{-}1_{IIIB}$ in a cell-based assay. α-Hydroxytropolones represent a new family of inhibitors for the development of novel drugs against HIV infection.

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The screening and investigation of novel drugs against human immunodeficiency virus (HIV) remain critical because of the ongoing AIDS epidemics and because of the fast emergence of virus variants resistant to present antiviral therapy (Kellerman et al., 2005). The replication steps of HIV, a member of the retrovirus family, are well known and can therefore be targeted rationally (for general review, see De Clercq, 2005). After HIV binding to the host cell, viral single-stranded RNA genomes are released into the cell and serve as templates for the virus-encoded reverse transcriptase to synthesize double-stranded DNA copies bearing the long terminal repeats (LTRs) at both ends (Turner and Summers, 1999). The viral linear DNA is integrated into the host genome in a reaction catalyzed by the viral enzyme integrase (IN). Integration is essential for viral replication because integrated viral DNA (provirus) serves as a template for the synthesis of new viruses after processing by the host cell transcription-translation machines (Brown, 1990; Fesen et al., 1993; Asante-Appiah and Skalka, 1997; Van Maele and Debyser, 2005).

Antiviral therapy currently uses a combination of reverse transcriptase and HIV protease inhibitors. Inhibitors of virus fusion to the host cells have recently been developed (Barbaro et al., 2005; De Clercq, 2005). Because HIV integrase is crucial for virus replication, the search for integrase inhibitors has been ongoing (Fesen et al., 1993; Hazuda et al., 2000; Debyser et al., 2002; Deprez et al., 2004; Johnson et al., 2004; Pommier et al., 2005). Integrase inserts the proviral DNA into host chromosomes in two steps: 3′ processing (3′-P) and strand transfer (ST). 3′-P is an endonucleolytic cleavage reaction removing the 3′ ends of the viral LTR DNA (generally a dinucleotide pGpT for HIV-1) immediately 3′ from the conserved sequence (CA for HIV-1) (Fig. 1A). ST is the insertion of the processed 3′ ends of the viral DNA into the cell genome (Asante-Appiah and Skalka, 1997). The HIV-1 integrase catalytic site contains three essential amino acids: Asp64, Asp116, and Glu152 (D,D-35 E-motif) that coordinate at least one and probably two divalent cations (Mg^{2+} or Mn^{2+}) between the enzyme and its DNA substrates (Engelman and Craigie, 1992; Chiu and Davies, 2004).

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ABBREVIATIONS: HIV, human immunodeficiency virus; IN, integrase; 3′-P, 3′-processing; ST, strand transfer; LTR, long terminal repeat; DMSO, dimethyl sulfoxide; MOPS, 3-(*N*-morpholino)propanesulfonic acid; bp, base pair(s); PAGE, polyacrylamide gel electrophoresis; L-731,988, 4-(1-(4-fluoro-benzyl)-1*H*-pyrrol-2-yl)-2,4-dioxo-butyric acid; NSC 624404, 2-(4-{bis[2-hydroxy-5-(methylethyl)-3-oxocyclohepta-1,4,6-trienyl]methyl}phenoxy) acetic acid, sodium salt; NSC 310618, 1,2,3,4-tetrahydro-2-7-dihydroxy-9-methyl-2-(1-methylethenyl)-6*H*-benzocyclohepten-6-one.

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The ST inhibitors 5-chloroindolyltetrazolylpropenone and L-731,988 have been proposed to chelate the divalent metal cations (Mg^{2+} or Mn^{2+}) in the enzyme active site (Grobler et al., 2002; Marchand et al., 2003; Pommier et al., 2005). 5-Chloroindolyltetrazolylpropenone has been cocrystallized in the catalytic domain of HIV integrase and shown to bind in the DDE motif (Goldgur et al., 1999). The diketo acid derivative L-731,988 was shown to block binding of target DNA in the integrase active site (Espeseth et al., 2000). The selective inhibition of the strand transfer reaction by diketo acids has been proposed to be due to their interfacial inhibition on preformed integrase-viral DNA complexes (Pommier and Marchand, 2005).

We previously reported one derivative (NSC 624404) containing the characteristic seven-membered tropolone ring as novel inhibitor of HIV-1 integrase in a four-point pharmacophore analysis of the National Cancer Institute drug database (Neamati et al., 1997). While screening the National Cancer Institute chemical library for HIV-1 integrase inhibitors, we recently found additional positive hits with tropolone derivatives. We report here the structure-activity relationship of tropolones available in the National Cancer Institute compound repository on HIV integrase activities. Tropolone derivatives are present in cupressaceous trees from genus *Thuja* and are probably responsible for resistance of fungal and insect attack on the heartwood (Baya et al., 2001; Diouf et al., 2002; Lim et al., 2005). Our experiments demonstrate the ability of the monomer 7-hydroxytropolones (α-hydroxytropolones) to preferentially inhibit the ST reaction by interfering with the enzyme catalytic site. α-Hydroxytropolone derivatives are new lead inhibitors for HIV-1 integrase.

Materials and Methods

Compounds. All drugs were obtained from the National Cancer Institute chemical repository from the Developmental Therapeutics Program (National Institutes of Health, Bethesda, MD). Compounds were dissolved in 100% DMSO. Stock solutions (10 mM) were stored at −20°C.

Recombinant HIV Integrase and Oligonucleotide Substrates. Expression and purification of the recombinant HIV-1 integrase in *Escherichia coli* were performed according to Leh et al. (2000) and Marchand et al. (2001) with addition of 10% glycerol to all buffers. The preparation of the Q148C/SSS-mutant integrase is described in Johnson et al. (2006). The oligonucleotide substrates, except those used for the disulfide cross-linking (Fig. 5A), were purchased from Integrated DNA Technologies, Inc. (Coraville, IA) and purified by polyacrylamide gel. The sequences of DNA substrates are shown in Figs. 1A, 2A, 3A, and 6A. The single-stranded oligonucleotides were 5′ end-labeled with [γ-^{32}P]ATP (PerkinElmer Life and Analytical Sciences, Boston, MA) and T_4 polynucleotide kinase (New England BioLabs, Ipswich, MA). Unincorporated nucleotide was removed using mini Quick Spin Oligo columns (Roche Diagnostics, Indianapolis, IN). Substrates were obtained after annealing with complementary nonlabeled oligonucleotides. The thiol-modified substrate (Fig. 5A) for disulfide cross-linking assay was synthesized by W. Santos and G. Verdine (Department of Chemistry and Chemical Biology, Harvard University, Cambridge, MA) as described previously (He and Verdine, 2002).

Integrase Catalytic Assays. Reactions were performed in 10 μl with 300 nM recombinant IN, 20 nM 5′ end ^{32}P-labeled oligonucleotide substrates, and inhibitors at the indicated concentrations. We included 10% DMSO in controls. Reactions were incubated for 40 min at 37°C in a buffer containing a final concentration of 25 mM MOPS, pH 7.2, 25 mM NaCl, 14.3 mM β-mercaptoethanol, and 7.5 mM divalent cations ($MgCl_2$ or $MnCl_2$ as indicated). Reactions were stopped by addition of 20 μl of loading dye (10 mM EDTA, 98% deionized formamide, 0.025% xylene cyanol, and 0.025% bromphenol blue). Reactions were heated at 95°C for 1 min before electrophoresis in 20% polyacrylamide-7 M urea gels. Gels were dried, and reaction products were visualized and quantitated with a PhosphorImager (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Densitometric analyses were performed using ImageQuant from the Molecular Dynamics software. The concentrations at which enzyme activity was reduced by 50% (IC_{50}) was determined using Prism software (GraphPad Software Inc., San Diego, CA) for nonlinear regression to fit dose-response data to logistic curve models.

Integrase Binding to HIV DNA Using the Disulfide-Cross-Linking Assay. The disulfide cross-linking assay was described in detail previously (Johnson et al., 2006). In brief, 10 μM recombinant Q148C/SSS-mutant integrase was incubated with 10 μM DNA substrate (Fig. 5A) containing tethered thiols in the presence of 20 mM Tris, pH 7.4, 10% glycerol, and 7.5 mM divalent cations ($MgCl_2$ or $MnCl_2$ as indicated) for 20 min at 37°C. Reactions were stopped by the addition of 20 mM methylmethanethiosulfonate (capping reagent). Nonreducing gel loading buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 0.2% bromphenol blue, and 20% glycerol) was added, and samples were heated at 95°C before loading onto 16% Tricine gels (Invitrogen, Carlsbad, CA). Gels were stained with Microwave Blue according to manufacturer's recommendations (Protiga, Frederick, MD).

Otherwise, for dose-response experiments, 500 nM integrase was incubated with NSC 18806 as shown at Fig. 5C in the buffer described above for 20 min. DNA (20 nM) containing a 5′ ^{32}P label on one strand and a thiol-modified cytosine on the other strand was added, and reactions were capped with methylmethanethiosulfonate at 1 min. After capping, nonreducing gel loading buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 0.2% bromphenol blue, and 20% glycerol) was added, and samples were directly loaded on 16% Tricine gels (Invitrogen). Gels were dried, and reaction products were quantitated as described above.

Integrase Binding to HIV DNA Using the Schiff-Base Assay. The Schiff-base assay was performed as described previously (Mazumder and Pommier, 1995). In brief, 300 nM recombinant IN was incubated with inhibitors (at the indicated concentration) for 15 min at 37°C. Subsequently, 20 nM 5′ end-labeled substrate containing the abasic oligonucleotide (Fig. 6A) was added for 10 min at room temperature in reaction buffer described above for integrase catalytic assays. A freshly prepared solution of sodium borohydride (0.1 M final concentration) was added for 5 min. An equal volume (10 μl) of 2× SDS-polyacrylamide gel electrophoresis buffer (Invitrogen) was added in each reaction. Reaction products were heated at 95°C for 1 min before analysis by electrophoresis in 12 to 20% polyacrylamide gels (Invitrogen). Gels were dried, and reaction products were quantitated using the same method as described above.

Fluorimetric HIV-1 Protease Assay. The fluorescent HIV-1 protease substrate [RE-(EDANS)-SQNYPIVQK-(DABCYL)-R] was obtained from Molecular Probes (Eugene, OR). Substrate and buffer were prewarmed at 37°C for at least 20 min before use. The protease (25 nM final concentration) was incubated in the manufacturer's recommended assay buffer (100 mM sodium acetate, 1 M NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10% DMSO and 1 mg/ml bovine serum albumin, pH 4.7) at 37°C in the presence of 1 to 25 μM NSC 18806 for 10 min and then added to the warmed substrate solution (40 μM) containing the different treatments to initiate the reaction. Acetyl pepstatin (Sigma-Aldrich, St. Louis, MO) at 20 nM was used a positive HIV-1 protease inhibitor control. The total assay volume was 100 μl. Fluorescence was monitored for 30 min in a fluorescence microplate reader (FMAX; Molecular Devices, Sunnyvale, CA) with 355-nm excitation and 460-nm emission filters, and the rate of reactions was compared for the different conditions.

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Inhibition of HIV-Induced Cytopathic Effect in Cell Culture. The MT-2 cells were grown in RPMI 1640 medium with GlutaMAX, supplemented with 10% (v/v) heat-inactivated fetal bovine serum (both from Invitrogen). The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. Every 4 to 5 days, cells were spun down and seeded at 2×10^5 cells/ml in new cell culture flasks. HIV (HTLV-IIIB isolate) was obtained from Advanced Biotechnology Incorporated (Columbia, MD). The virus stock (3.2×10^4 50% cell culture infective dose per milliliter as determined for MT-2 cells) was stored at −70°C until used. Stock solutions of compounds were diluted using medium directly into 96-well assay plate (Corning, Corning, NY).

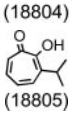
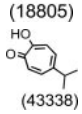
MT-2 cells (5×10^5 cells/ml) were pretreated for 2 h with test compounds at various concentrations as indicated in Fig. 7. Cells were then infected with 100 50% cell culture infective dose or mock-infected. The cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Four days after infection, the viability of mock- and HIV-infected cells was examined spectrophotometrically by the CellTiter 96 nonradioactive cell proliferation assay (Promega, Madison, WI) and also confirmed microscopically in a hemacytometer by the trypan blue exclusion method. The percentage of cell viability in drug-treated uninfected and infected cells was determined based on the viability of the uninfected control drug-treated cells. The concentration of drug required to inhibit approximately 50% of the HIV-1-induced cytotoxicity was calculated from the plot of compound concentration versus the percentage of viable cells.

Results

The 7-Hydroxy Group Is Essential for Inhibition of HIV-1 Integrase by Tropolone Derivatives. The tropolone derivatives tested in this study are shown in Table 1. Compounds were first screened for inhibition of HIV-1 integrase in the presence of Mn^{2+} as a cofactor using DNA substrates mimicking the U5 LTR viral DNA end (Fig. 1A). The derivative containing only the minimal tropolone core structure (NSC 89303; Table 1) showed only marginal inhibition ($IC_{50} > 333$ μM). Addition of an isopropyl group at positions 5 (NSC 43338), 4 (NSC 18804), and 3 (NSC 18805), and of a 3-methyl-2-butenyl group at position 5 along with an isopropyl at the 4 position (NSC 43339) failed to increase potency. However, addition of a hydroxy group at position 7 (α-hydroxytropolone) resulted in inhibitory activity against HIV-1 integrase (NSC 18806 and NSC 310618). NSC 18806 was the most inhibitory against integrase in strand transfer reaction ($IC_{50} = 4.8 \pm 2.5$ μM) compared with NSC 310618 ($IC_{50} = 11.7 \pm 5.2$ μM) (Table 1).

The Tropolone NSC 18806 Inhibits Preferentially Strand Transfer in the Presence of Magnesium. For detailed characterization of NSC 18806, we compared its effect on the three reactions catalyzed by HIV integrase. 3′ Processing, strand transfer, and disintegration can be independently measured in biochemical assays using specific oligonucleotides (Figs. 1A, 2A, and 3A) (Marchand et al., 2001). A divalent cation, either Mg^{2+} or Mn^{2+}, is required for integrase activity in vitro (Engelman and Craigie, 1995). Mg^{2+}, however, is the more likely cofactor in vivo. Because the integrase active site could be structurally different in the presence of Mg^{2+} or Mn^{2+} and inhibitors can act in different ways in Mg^{2+} or Mn^{2+} (Grobler et al., 2002; Neamati et al., 2002; Marchand et al., 2003), all assays were performed in the presence of either Mg^{2+} or Mn^{2+}.

TABLE 1
Inhibition by tropolones of the different activities of HIV-1 integrase
All data represent mean values and S.D. for at least three independent experiments.

Structure	Compound Number	Divalent Cation	In Vitro IC_{50} Values			
			21-bp Substrate		ST in Preprocessed Substrate	Disintegration
			3′-P	ST		
					μM	
 (18806)	NSC 18806	Mg^{2+}	117.3 ± 7.1	21.6 ± 3.4	18.1 ± 6.2	>333[b]
		Mn^{2+}	24.6 ± 3.9	4.8 ± 2.5	5.0 ± 2.9	23.8 ± 5.9
 (310618)	NSC 310618	Mg^{2+}	182.0 ± 42.5	71.1 ± 3.8	>333[c]	>333[b]
		Mn^{2+}	20.2 ± 8.9	11.7 ± 5.2	25.8 ± 6.6	98.3 ± 30.9
 (43339)	NSC 43339	Mg^{2+}	>333[b]	>333[c]	>333[b]	>333[a]
		Mn^{2+}	>333[b]	>333[c]	>333[c]	>333[a]
 (89303)	NSC 89303	Mg^{2+}	>333[a]	>333[c]	>333[b]	>333[a]
		Mn^{2+}	>333[b]	>333[c]	>333[b]	>333[a]
 (18804)	NSC 18804	Mg^{2+}	>333[c]	>333[b]	>333[b]	>333[a]
		Mn^{2+}	>333[c]	>333[b]	>333[b]	>333[a]
 (18805)	NSC 18805	Mg^{2+}	>333[b]	>333[a]	>333[a]	>333[a]
		Mn^{2+}	>333[b]	>333[a]	>333[a]	>333[a]
 (43338)	NSC 43338	Mg^{2+}	>333[a]	>333[a]	>333[a]	>333[a]
		Mn^{2+}	>333[a]	>333[a]	>333[a]	>333[a]

[a] Inhibition activity ≤5%.
[b] 5% < Inhibition activity <20%.
[c] 20% < Inhibition activity <40% at drug concentration of 333 μM.

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Figures 1 to 3 show the results of representative experiments for the different assays, and Fig. 4 and Table 1 summarize the results of these three assays.

NSC 18806 exhibited greater potency against 3′-P and ST using the standard 21-bp oligonucleotide duplex in the presence of Mn^{2+} than in the presence Mg^{2+} (Figs. 1B and 4; Table 1). The IC_{50} values for 3′-P were approximately 5-fold higher than the IC_{50} values for ST in the presence of either Mg^{2+} or Mn^{2+}. Therefore, NSC 18806 shows some selectivity for ST.

Because ST follows 3′-P in the reaction using the 21-bp DNA substrate shown in Fig. 1, independent measurement of ST was performed with a preprocessed substrate (Fig. 2A). This assay segregates the action of a compound against ST from a decrease of the integrase activity related to the 3′-P inhibition in the overall integration. Results from this assay show similar ST inhibition and comparable IC_{50} values for ST as were observed for overall integration (Figs. 1B, 2B, and 4; Table 1) with exception for NSC 310618.

Disintegration was suggested as a reverse reaction of ST (Chow et al., 1992) (Fig. 3A). Figure 3B shows the inability of NSC 18806 to inhibit disintegration in the presence of Mg^{2+}. Disintegration was only inhibited in the presence of Mn^{2+} at high drug concentration. These results indicate that NSC 18806 is a more potent inhibitor of ST compared with disintegration (Fig. 4; Table 1).

NSC 18806 Affects the HIV-1 Integrase Catalytic Site without Inhibiting Overall DNA Binding. For determination of the possible binding site of NSC 18806 within the integrase catalytic site, we evaluated the ability of NSC 18806 to inhibit a cross-linking reaction between the cytosine in the 5′-AC dinucleotides overhang of the viral DNA and integrase glutamine 148 (Fig. 5A). A Q148C mutant form of HIV-1 integrase allows specific covalent interaction with a thiol-modified cytosine in the 5′-AC dinucleotide overhang without nonspecific interference of other integrase cysteine residues (Johnson et al., 2006).

The results of this assay show metal-dependent inhibition of integrase-DNA disulfide-cross-linking by NSC 18806 (Fig. 5B). The importance of the α-hydroxy group in this inhibition is illustrated by the lack of inhibition observed for NSC 18804 (structural analog of NSC 18806 lacking the α-hydroxy group) (Fig. 5B). For quantitative analysis of dose-dependent inhibition of integrase-DNA disulfide-cross-linking by NSC 18806, we performed the assay using 5′ ^{32}P-labeled DNA substrate (Johnson et al., 2006). The sensitivity of this method allows reactions to be performed with the same ratio of integrase-DNA as in the assays measuring ST. Inhibition of integrase cross-linking by NSC 18806 was observed at concentrations similar to those required to inhibit ST (Fig. 5C). Integrase-DNA complexes were represented by several bands (Fig. 5C, left), with the lowest corresponding to integrase-monomer-DNA (confirmed by Western blot experiments; data not shown). The slower migrating bands correspond to integrase-multimer-DNA complexes (dimer, trimer, tetramer, and so on) because of nondenaturing electrophoresis conditions. The IC_{50} for cross-linking inhibition (32 μM) is comparable with the IC_{50} for the ST inhibition in the presence of Mg^{2+} (21.6 ± 3.4 μM; Table 1).

To determine whether cross-linking inhibition could be because of inhibition of overall binding of HIV-1 integrase to the viral DNA end, we investigated the effect of tropolone derivatives using a Schiff-base assay (Mazumder and Pommier, 1995) measuring cross-linking between integrase and a

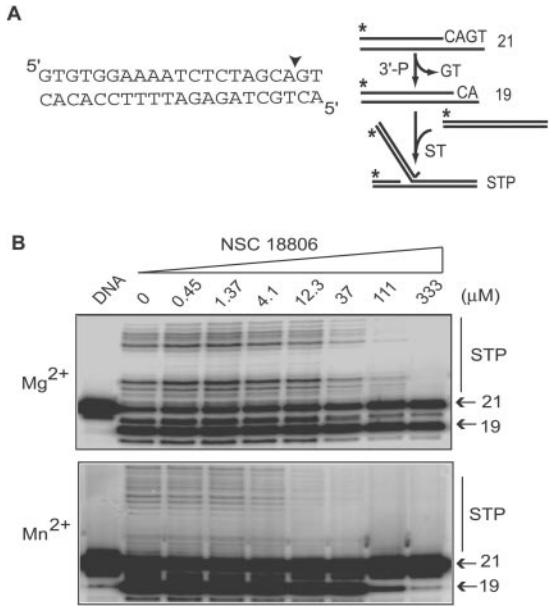

Fig. 1. Inhibition of HIV-1 integrase 3′-P and ST activities by NSC 18806. A, sequence of the 21-bp oligonucleotide duplex that corresponds to the terminal U5 sequence of the HIV-1 LTR used as a substrate, and schematic representation of the integrase reactions. Arrowhead represents the 3′-P site. Asterisks represent the 5′ ^{32}P label. The initial step involves cleavage of two bases from the 3′-OH end resulting in a 19-bp product. ST products (STP) result from the covalent joining of the 3′-processed duplex into another identical duplex that serves as the target DNA. B, PAGE analysis of HIV-1 integrase inhibition by NSC 18806 using the 21-bp duplex as substrate in the presence of Mg^{2+} or Mn^{2+}. Drug concentrations are shown above each lane.

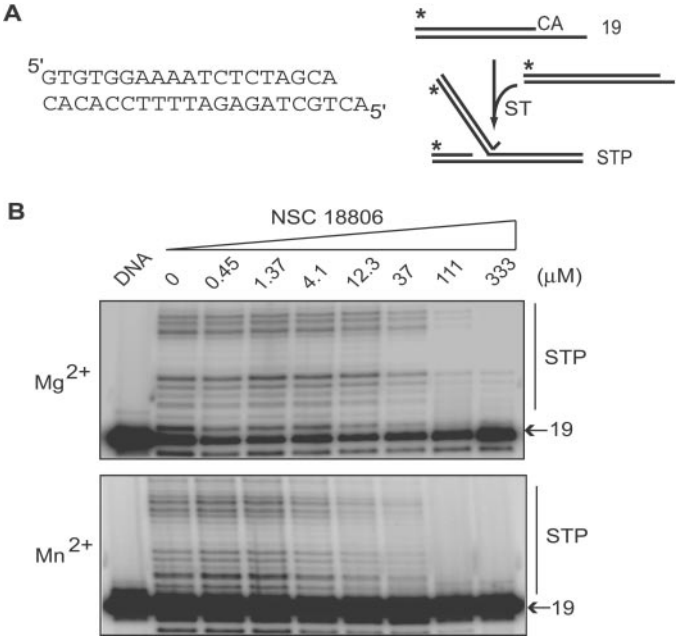

Fig. 2. Inhibition of HIV-1 integrase-catalyzed ST by NSC 18806 is independent of 3′-processing. A, sequence of the preprocessed (19/21) oligonucleotide duplex used as substrate and schematic representation of the ST assay. Asterisks represent the 5′ ^{32}P label. Strand transfer products (STP) result from the covalent joining of the 3′-OH end of the precleaved substrate into another identical substrate that serves as the target DNA. B, PAGE analysis of HIV-1 integrase inhibition by NSC 18806 in the presence of Mg^{2+} or Mn^{2+}. Drug concentrations are shown above each lane.

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DNA substrate mimicking the viral U5 LTR end and containing an abasic site corresponding to the adenine in the conserved CA-dinucleotide (Fig. 6A). Tropolone derivatives did not block the Schiff base IN-DNA interaction even at 1 mM concentration (Fig. 6B), indicating specific inhibition of disulfide cross-linking by NSC 18806.

NSC 18806 Exhibits Moderate Cytoprotective Activity against HIV-1 in Cell-Based Assay. The tropolone compounds shown in Table 1 were tested in an HIV infectivity assay (Pauwels et al., 1988). All compounds were inactive in this assay except for the NSC 18806, which showed moderate protection of infected cells from HIV-induced cytopathic effect with an estimated IC_{50} of approximately 12 μM (Fig. 7). The IC_{50} could only be estimated because of the presence of toxicity at concentrations at and above 12 μM. Note, that the cytoprotective concentration for this compound is comparable with the IC_{50} for integrase inhibition in vitro in the presence of Mg^{2+}.

Discussion

Tropolone derivatives have a range of antimicrobial activities. They are antifungal (Baya et al., 2001), antibacterial (Morita et al., 2004), and insecticidal (Morita et al., 2003). They also exhibit antioxidant properties (Doulias et al., 2005). Specific inhibition of the RNaseH domain of HIV-1 reverse transcriptase by 7-hydroxytropolone derivatives (α-hydroxytropolones) have been reported (Budihas et al., 2005). These various biological activities of tropolone derivatives have been linked with their ability to chelate metals (Matsumura et al., 2001; Budihas et al., 2005; Doulias et al., 2005). Tropolone interacts with Mg^{2+} by forming of a 1:1 complex (Andreu and Timasheff, 1982). The current study suggests that α-hydroxytropolones may also inhibit HIV-1 integrase by chelation of one or two Mg^{2+} or Mn^{2+} in the enzyme active site (Fig. 8).

According to the two cross-linking assays, α-hydroxytropolones interfere with the protruding 5′ end of the LTR and the integrase amino group glutamine 148 in the integrase flexible loop (inhibition of disulfide cross-linking) without affecting the overall binding of integrase to the viral DNA end (no inhibition of Schiff-base cross-linking). It has been suggested that the interaction between the cytosine in the 5′ overhang of the viral DNA and the Q148 occurs after a conformational change of the integrase-viral (donor) DNA complex, which is necessary for triggering ST (Johnson et al., 2006). Thus, the cross-linking results coupled with the ability of α-hydroxytropolones to preferentially inhibit ST (full-length or preprocessed substrate) compared with 3′-P in the presence of Mg^{2+} suggest preferential binding of the α-hydroxytropolones to the integrase-DNA complex after 3′-P.

The lack of inhibition of the disintegration reaction by α-hydroxytropolones in the presence of Mg^{2+} compared with effective inhibition of the ST reaction is noticeable because disintegration corresponds to the reverse reaction of strand transfer (Chow et al., 1992). The same selectivity for strand transfer versus disintegration was shown for the diketo acid derivative L-731,988 and led to the interpretation that diketo acids bind to the target DNA site (Espeseth et al., 2000). Competition with target (acceptor) DNA could explain why NSC 18806 has a lower affinity for binding to the integrase catalytic active site if this site is already occupied by the donor and acceptor DNA,

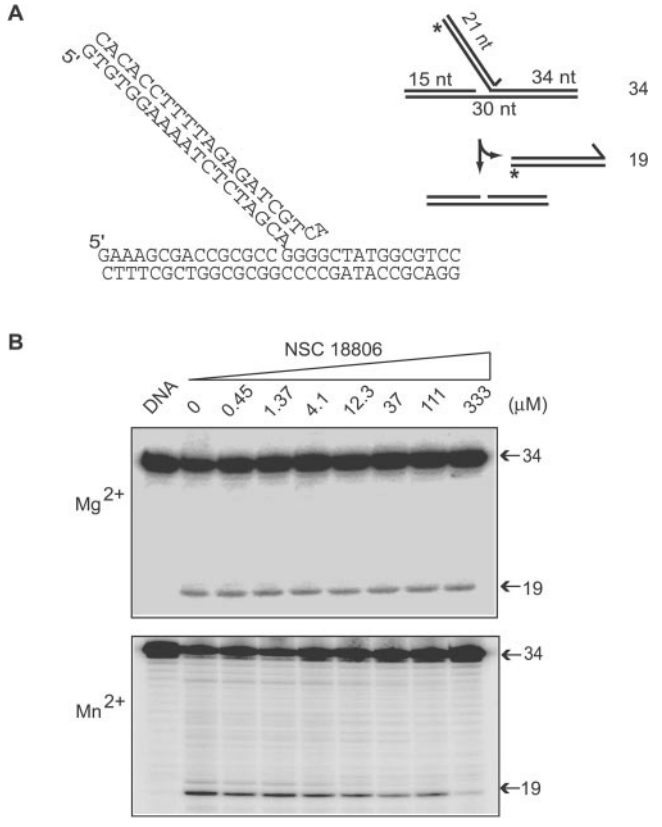

Fig. 3. Lack of inhibition of HIV-1 integrase-mediated disintegration by NSC 18806 in the presence of Mg^{2+}. A, sequence of the oligonucleotides used as substrate for disintegration (Y-oligomer). The disintegration product results from cleavage of the 34-mer oligonucleotide and can be detected as a radiolabeled 19-bp oligonucleotide. Asterisks represent the 5′ ^{32}P-label. B, PAGE analysis of the HIV-1 integrase-mediated disintegration reactions in the presence of NSC 18806 and Mg^{2+} or Mn^{2+}. Drug concentrations are shown above each lane.

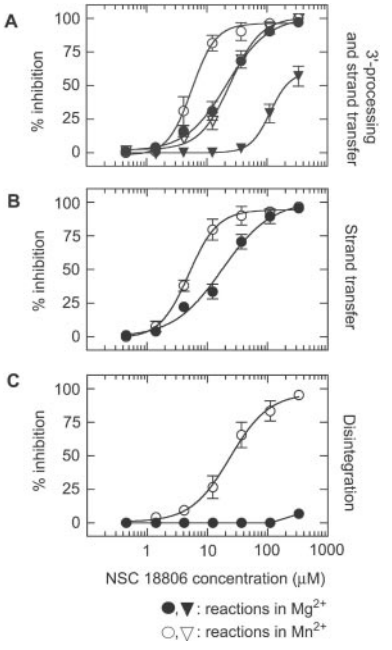

Fig. 4. Summary and quantitative comparison of inhibition of the HIV-1 integrase reactions by NSC 18806 in the presence of Mg^{2+} (filled symbols) or Mn^{2+} (open symbols). A, reactions with the 21-bp duplex substrate (see Fig. 1A): 3′-P, triangles; and ST, circles. B, strand transfer assays with the preprocessed DNA substrate (see Fig. 2A). C, disintegration assays with the Y-substrate (see Fig. 3A). Data represent mean ± S.D. for at least three independent experiments.

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which would be the case for the disintegration substrate. Hence, α-hydroxytropolones might act as interfacial inhibitors (Pommier and Cherfils, 2005; Pommier and Marchand, 2005) for HIV-1 integrase-divalent metal-DNA complexes and block the binding of the acceptor (genomic) DNA.

We find that α-hydroxytropolones are more potent but less selective for ST in the presence of Mn^{2+} than in the presence of Mg^{2+}. Such metal-dependent inhibition might be because of a different folding of the integrase active site in the presence of Mg^{2+} or Mn^{2+}. Mn^{2+} is geometrically wider than Mg^{2+} (Huang et al., 1997; Bock et al., 1999); therefore, the catalytic site of integrase could be more "open" in the presence of Mn^{2+}, which might allow the α-hydroxytropolones to enter various conformations of this site. The lack of integrase inhibition using preprocessed substrate by NSC 310618 in the presence of Mg^{2+} might be indicative of different integrase configurations when ST proceeds from a precleaved (preprocessed) substrate versus a blunt end substrate.

NSC 18806 shows weak cytoprotective activity on HIV-infected cells, which seems limited by the cytotoxicity of the drug. The cytoprotection against virus could be because of

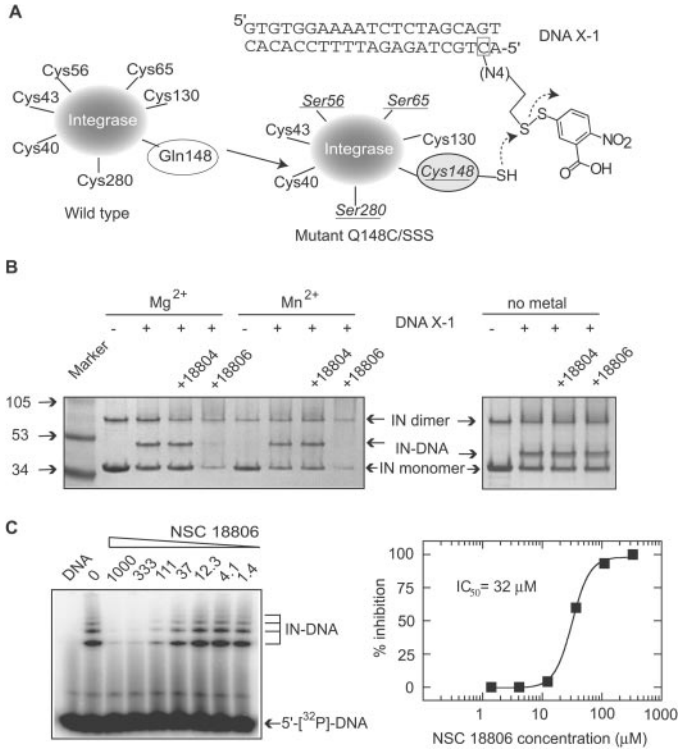

Fig. 5. The tropolone 7-hydroxy group is crucial for inhibition of disulfide cross-linking between of HIV-1 integrase Q148 and the 5′ C of the DNA substrate. A, integrase-DNA cross-linking strategy. Left, schematic representation of the HIV-1 integrase cysteine (Cys) residues. Bottom right, schematic representation of the mutant integrase used for cross-linking; residue 148 on the flexible loop is mutated Q → C; cysteines 56, 65, and 280 are mutated to serine to eliminate nonspecific cross-linking. Top right, modified oligonucleotide used for cross-linking (DNA X-1) with a thioalkyl modification (Johnson et al., 2006). The cross-linked complex forms between the cysteine residue 148 and the 5′ C from the DNA X-1 substrate. B, SDS-PAGE analysis of the cross-linking reaction showing metal-dependent inhibition by NSC 18804 and NSC 18806. Molecular mass markers (in kilodaltons) are shown on the left. Drug concentrations in the reaction mixture are 1 mM. C, concentration-dependent inhibition of disulfide cross-linking by NSC 18806 in the presence of Mg^{2+} using the DNA X-1 substrate labeled with ^{32}P at the 5′ end of the top strand. Left, representative gel with drug concentration indicated at the top. Right, quantitation of integrase-DNA cross-linking by NSC 18806.

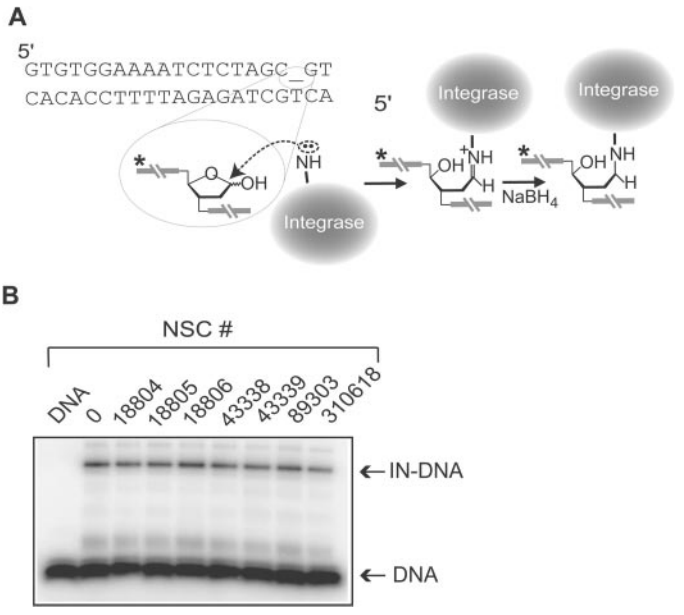

Fig. 6. NSC 18806 does not interfere with HIV-1 integrase overall binding to viral DNA end in the Schiff-base cross-linking assay. A, principle of the cross-linking assay. An abasic site is introduced by uracil DNA glycosylase in the DNA substrate containing uracil at the position corresponding to the adenine in the conserved CA-dinucleotide. The asterisks indicate the 5′ ^{32}P label. An integrase nitrogen nucleophile (probably lysine) attacks the C1′ carbon of the abasic site (Mazumder and Pommier, 1995). Rearrangement of the initial enzyme-DNA complex leads to the formation of a Schiff-base intermediate that can be stabilized by $NaBH_4$. B, SDS-PAGE analysis showing no inhibition by the various tropolone derivatives at 1 mM concentration on the cross-linking reactions between integrase and DNA in the presence of Mg^{2+}.

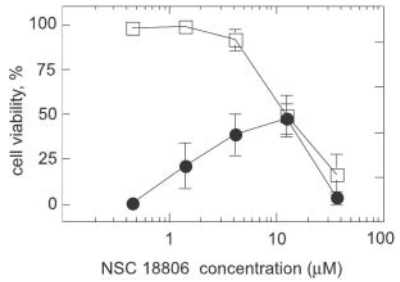

Fig. 7. Moderate activity of NSC 18806 against the cytopathic action of HIV-1_{IIIB} on lymphoid MT-2 cells. Effects of NSC 18806 on HIV-infected (●) and mock-infected cells (□).

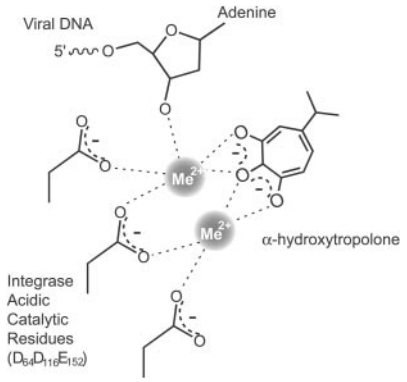

Fig. 8. Proposed model of action α-hydroxytropolones against HIV-1 integrase. NSC 18806 is shown bound at the integrase-DNA complex chelating the divalent metals (Mg^{2+} or Mn^{2+}) in the integrase active site. Me^{2+}, divalent cation.

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drug action against other steps beside HIV replication. However, we can exclude HIV protease as NSC 18806 failed to inhibit HIV-1 protease at concentration up to 25 μM in a standard fluorescent-based HIV-1 protease assay (data not shown). It has been reported that the α-hydroxytropolones inhibit the RNaseH domain of HIV-1 reverse transcriptase (Budihas et al., 2005). The topological similarity between the catalytic domain of HIV integrase and the HIV reverse transcriptase RNaseH domain could explain a common mechanism of metal chelation in the enzyme catalytic site(s) (Dyda et al., 1994; Yang and Steitz, 1995). While our study was under review, it was reported that 3,7-dihydroxytropolones also inhibit HIV integrase but at the same time block preferentially the polymerase activity of HIV reverse transcriptase over its RNaseH activity (Didierjean et al., 2005). The synthesis of more active compounds based on the 7-hydroxytropolone core is needed for developing therapeutically relevant compounds and for investigating which step of the retroviral infection is inhibited by α-hydroxytropolones.

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