0026-895X/97/040567-09$3.00/0

MOLECULAR PHARMACOLOGY, **51**:567–575 (1997).

Probing Interactions between Viral DNA and Human Immunodeficiency Virus Type 1 Integrase Using Dinucleotides

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Received November 8, 1996; Accepted January 13, 1997

SUMMARY

Retroviral integrases are essential for viral replication and represent an attractive chemotherapeutic target. In the current study, we demonstrated the activity of micromolar concentrations of dinucleotides against human immunodeficiency virus type 1 (HIV-1), HIV type 2 (HIV-2), simian immunodeficiency virus, and feline immunodeficiency virus integrases. The structure-activity relationship indicates that 5′-phosphorylation enhances potency and that phosphodiester and sugar modifications affect the inhibition of HIV-1 integrase. Base sequence selectivity was observed: pAC, pAT, and pCT were the most potent inhibitors, whereas pAA, pGA, and pGC showed low activity at 100 μM. The inhibition by pAC is consistent with the interaction of the enzyme with the 5′ end of the noncleaved strand (5′-AC-3′). The linear and cyclic dinucleotides released by the 3′-processing reaction did not affect enzymatic activity at physiological concentrations. An increase in the length to trinucleotides or tetranucleotides enhanced potency by only 2–3-fold, suggesting that two neighboring bases may be sufficient for significant interactions. Inhibition of a truncated (50–212) integrase mutant and global inhibition of all nucleophiles in the 3′-processing reaction suggest that dinucleotides bind in the catalytic core. All of the active dinucleotides inhibited enzyme/DNA binding in their respective IC_{50} range. Although the dinucleotides tested showed no antiviral activity, these observations demonstrate the usefulness of dinucleotides in elucidating enzyme mechanisms and as potential ligands for cocrystallization and as lead structures for development of antivirals.

Several key enzymes in the replication cycle of the HIV can be targeted for chemotherapeutic intervention, most notably, reverse transcriptase and protease (1). Research is now in progress to develop clinically active agents against other proteins in the viral life cycle. Toward this goal, several laboratories have investigated the pharmacological activity of various drugs as inhibitors of HIV integrase (2–4).

Retroviruses encode the integrase protein at the 3′ end of the *pol* gene (5). This enzyme, a proteolytic cleavage product of a *gag-pol* fusion protein precursor, is contained in the virus particle and is required for viral replication. It integrates a double-stranded DNA copy of the RNA genome, synthesized by reverse transcriptase, into a host chromosome. During viral infection, integrase catalyzes the excision of the last two nucleotides from each 3′ end of the linear viral DNA, leaving the terminal dinucleotide CA-3′-OH at these recessed 3′ ends. This activity is referred to as the 3′-processing or dinucleotide cleavage. After transport to the nucleus within the preintegration complex, integrase catalyzes a DNA strand transfer reaction involving the nucleophilic attack of these ends on the host chromosome [for recent reviews, see Katz and Skalka (6) and Rice *et al.* (7)].

We previously demonstrated that AZT nucleotides inhibit HIV-1 integrase with an IC_{50} value of ~100 μM (8) and recently showed that the *gyr*B inhibitor, coumermycin A1, and several mononucleotide analogs that are currently undergoing clinical trials as inhibitors of HIV-1 replication are more potent inhibitors of HIV-1 integrase than are AZT nucleotides (2).

Non-nucleotide inhibitors of HIV-1 integrase have also been described (9–12) that have the potential, as shown by molecular modeling, to stack their aromatic rings and poten-

This project was supported by a grant from the National Institutes of Health Intramural AIDS Targeted Antiviral Program and by the Delaware Research Foundation (H.K.C).

ABBREVIATIONS: HIV, human immunodeficiency virus; HIV-1, human immunodeficiency virus type 1; HIV-2, human immunodeficiency virus type 2; LTR, long terminal repeat; STP, strand transfer products; SIV, simian immunodeficiency virus; FIV, feline immunodeficiency virus; PNA, peptide-nucleic acid; AZT, 3′-azido-2′,3′-dideoxythymidine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography; Rp and Sp, chiral phosphorus with an R or S configuration, respectively.

tially mimic a dinucleotide (11, 12). Because of the structural similarities between RNase H (from either *Escherichia coli* or HIV-1) and retroviral integrase (from either HIV-1 or ASV) with respect to both the overall topology of the active sites and location of conserved acidic amino acids (7) and because of the recent finding that dinucleotides can inhibit RNase H (13), we theorized that dinucleotides may inhibit integrase. In the present study, we investigated the effect of dinucleotides (including those released physiologically during 3′-processing) on enzyme activity and the inhibition of HIV-1 integrase by a wide variety of dinucleotides having differing base sequences or modifications to the backbone or sugar moiety. These data can be used to explore the enzyme mechanisms, develop strategies for the design of integrase-inhibitor co-crystals, and develop novel integrase inhibitors.

Materials and Methods

Dinucleotides and analogs. All dinucleotides and trinucleotides were purchased from Midland Certified Reagent (Midland, TX) with the exception of the following. The 2′,5′-oligoadenylates were a gift from Dr. Robert J. Suhadolnik (Temple University, Philadelphia, PA). Nonphosphorylated dinucleotides incorporating modified nucleoside analogs were obtained from Dr. Jean-Pierre Sommadossi (University of Alabama-Birmingham, Birmingham, Alabama). The PNA dinucleotide was supplied by Dr. Peter Nielsen (University of Copenhagen, Copenhagen, Denmark). The cyclic dinucleotides were from the laboratories of Dr. Roger Jones and Dr. H. Keith Chenault.

The methylphosphonate dCpdA and phosphorothioates dCpdA and dCpdT were synthesized by phosphoramidite coupling, separated into their protected Rp and Sp diastereomers by normal-phase HPLC, chemically phosphorylated, deprotected, and purified by reverse-phase HPLC to homogeneous peaks. Each diastereomer displayed the expected mass spectra and ^{31}P NMR peaks.

Preparation of radiolabeled DNA substrates. The following oligodeoxynucleotides were HPLC purified by and purchased from Midland Certified Reagent: AE117, 5′-ACTGCTAGAGATTTTCCACAC-3′; AE118, 5′-GTGTGGAAAATCTCTAGCAGT-3′; AE157, 5′-GAAAGCGACCGCGCC-3′; AE146, 5′-GGACGCCATAGCCCCGGCGCGGTCGCTTTC-3′; AE156, 5′-GTGTGGAAAATCTCTAGCAGGGGCTATGGCGTCC-3′; AE118S, 5′-GTGTGGAAAATCTCTAGCA-3′; and RM22M, 5′-TACTGCTAGAGATTTTCCACAC-3′. AE117, AE118, and the first 19 nucleotides of AE156 correspond to the U5 end of the HIV-1 LTR.

To analyze the extents of 3′-processing and strand transfer using 5′ end-labeled substrates, AE118 was 5′ end labeled using T_4 polynucleotide kinase (GIBCO BRL, Baltimore, MD) and [γ-^{32}P]ATP (DuPont-New England Nuclear). The kinase was heat inactivated, and AE117 was added to the same final concentration. The mixture was heated at 95°, allowed to cool slowly to room temperature, and run on a G-25 Sephadex quick-spin column (Boehringer-Mannheim Biochemicals, Indianapolis, IN) to separate annealed double-stranded oligonucleotide (Fig. 1C) from unincorporated label.

To analyze the choice of nucleophile for the 3′-processing reaction (14, 15), AE118 was 3′ end-labeled using [α-^{32}P]cordycepin triphosphate (DuPont-New England Nuclear) and terminal transferase (Boehringer-Mannheim Biochemicals). The transferase was heat inactivated, and RM22M was added to the same final concentration. The mixture was heated at 95°, allowed to cool slowly to room temperature, and run on a G-25 spin column as before. To analyze the extent of strand transfer using the "precleaved" substrate, AE118S was 5′ end-labeled, annealed to AE117, and column purified as described above. To determine the extent of 30-mer target strand generation during disintegration (the reverse of the strand transfer reaction) (16), AE157 was 5′ end labeled; annealed to AE156, AE146, and AE117; annealed; and column purified as described above.

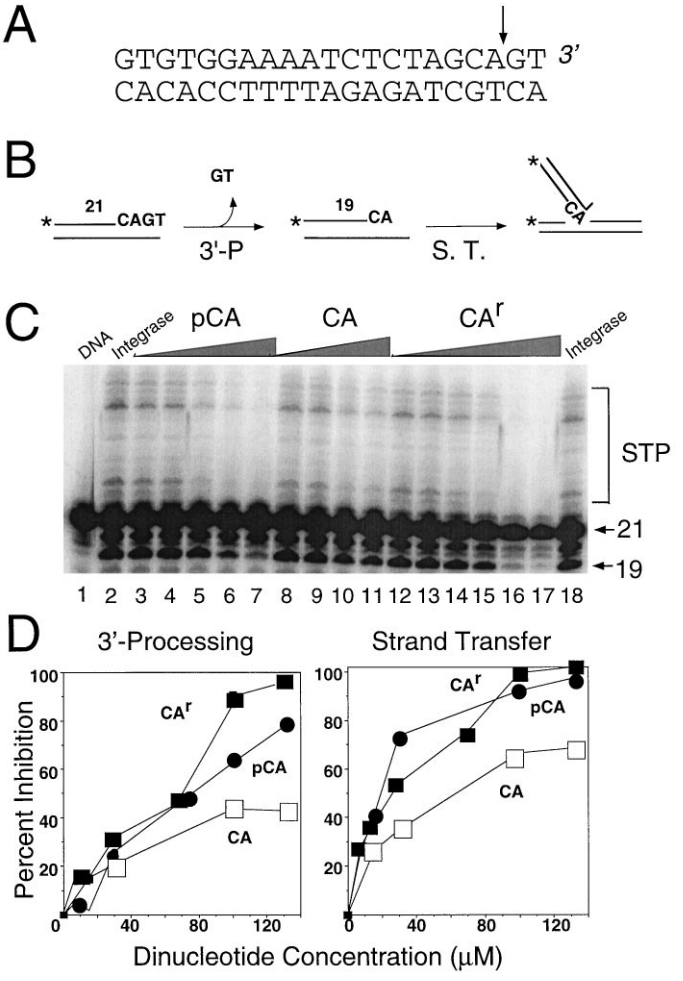

Fig. 1. Inhibition of HIV-1 integrase by CA dinucleotides. A, Oligonucleotide used in the 3′-processing and strand transfer reactions and corresponding to the last 21 bp of the U5 end of the HIV-1 LTR. *Arrow*, dinucleotide cleavage site in the 3′-processing reaction. B, The two catalytic activities of HIV-1 integrase: 3′-processing (*3′-P*) and strand transfer (*S. T.*). C, PhosphorImager picture of a representative experiment with CA, pCA, and CA^r. The DNA substrate and 3′-processing product are shown as 21 and 19 mer, respectively. *STP*, strand transfer products. *Lane 1*, DNA alone. *Lanes 2 and 18*, DNA plus integrase without dinucleotide. *Lanes 3–7*, DNA plus integrase in the presence of pCA at 7.5, 15, 30, 100, and 132 μM, respectively. *Lanes 8–11*, DNA plus integrase in the presence of CA at 15, 30, 100, and 132 μM, respectively. *Lanes 12–17*, DNA plus integrase in the presence of ribo-CA at 7.5, 15, 30, 70, 100, and 132 μM, respectively. D, Quantification of C. ■, CA^r; ●, pCA; □, CA.

Integrase proteins. Purified recombinant wild-type HIV-1 integrase (14) and deletion mutant IN$^{50–212}$ (17) were generous gifts of Drs. R. Craigie and A. Engelman (Laboratory of Molecular Biology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD). Dr. Craigie also provided the expression system for the wild-type HIV-1 integrase. A plasmid encoding the HIV-2 integrase was generously provided by Dr. R. H. A. Plasterk (Netherlands Cancer Institute, Amsterdam, The Netherlands). Purified recombinant wild-type FIV and SIV integrases were generous gifts of Drs. S. Chow (University of California, Los Angeles) and R. Craigie (National Institute of Diabetes and Digestive and Kidney Diseases), respectively.

3′-Processing, strand transfer, and disintegration assays. Integrase was preincubated at a final concentration of 200 nM (for HIV-1 and HIV-2) or 600 nM (for FIV and SIV) with inhibitor in reaction buffer [50 mM NaCl, 1 mM HEPES, pH 7.5, 50 μM EDTA, 50

μM dithiothreitol, 10% glycerol (w/v), 7.5 mM $MnCl_2$, 0.1 μg/ml bovine serum albumin, 10 mM 2-mercaptoethanol, 10% dimethylsulfoxide, and 25 mM 3-(*N*-morpholino)propanesulfonic acid, pH 7.2] at 30° for 30 min. Preincubation for 30 min of the enzyme with inhibitor was performed to optimize the inhibitory activity in the 3′-processing reaction (18). Then, 20 nM of the 5′ end ^{32}P-labeled linear oligonucleotide substrate was added, and incubation was continued for an additional hour. The final reaction volume was 16 μl.

Disintegration reactions (16) were performed as above except that the Y oligonucleotide (i.e., the branched substrate in which the U5 end was "integrated" into target DNA) was used.

Electrophoresis and quantification. Reactions were quenched by the addition of an equal volume (16 μl) of Maxam-Gilbert loading dye (98% deionized formamide, 10 mM EDTA, 0.025% xylene cyanol, 0.025% bromphenol blue). An aliquot (5 μl) was electrophoresed on a denaturing 20% polyacrylamide gel (0.09 M Tris-borate, pH 8.3, 2 mM EDTA, 20% acrylamide, 8 M urea). Gels were dried, exposed in a Molecular Dynamics PhosphorImager cassette (Sunnyvale, CA), and analyzed using a Molecular Dynamics PhosphorImager. Percent inhibition was calculated using the equation $100 \times [1 - (D - C)/(N - C)]$, where C, N, and D are the fractions of 21-mer substrate converted to 19-mer (3′-processing product) or strand transfer products for DNA alone, DNA plus integrase, and integrase plus drug, respectively. The IC_{50} value was determined by plotting the drug concentration versus percent inhibition and determining the concentration that produced 50% inhibition.

UV cross-linking experiments. We used the method of Yoshinaga *et al.* (19). Briefly, integrase was incubated with substrate in reaction buffer as described above for 5 min at 30°. Reactions were then irradiated with an UV transilluminator (254-nm wavelength) from 3 cm above (2.4 mW/cm^2) at room temperature for 10 min. An equal volume (16 μl) of 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis buffer (100 mM Tris, pH 6.8, 4% 2-mercaptoethanol, 4% sodium dodecyl sulfate, 0.2% bromphenol blue, 20% glycerol) was added to each reaction, and the reaction was heated at 95° for 3 min before loading of a 20-μl aliquot onto a 12% sodium dodecyl sulfate-polyacrylamide gel. The gel was run at 120 V for 1.5 hr, dried, and exposed in a PhosphorImager cassette. For inhibition of DNA binding experiments, integrase (200 nM) was preincubated with the dinucleotide (at the indicated concentration) for 30 min at 30° before the subsequent addition of the radiolabeled viral DNA substrate (20 nM).

HIV cytopathic effect assay. HIV cytopathic effect assays were performed essentially as previously described (20). ATH8 cells were used as the host cell line and were grown in cell culture in the absence or presence of 300 median tissue culture infective dose of HIV-1_{LAI} strain and in the absence or presence of dinucleotides.

Results

Inhibition of HIV-1 integrase by dinucleotides and trinucleotides. The first dinucleotide tested was 5′-CA as an inhibitor of HIV-1 integrase because this sequence is conserved at the 3′ end of all retroviral LTR sequences and immediately 5′ from the dinucleotide cleavage site (Fig. 1A) (5) and is critical for optimum enzymatic activity (21, 22). To be as concise as possible, from this point on, the nonphosphorylated CA dinucleotide will be represented as CA and its 5′-phosphorylated version will be represented as pCA. Fig. 1A shows the oligonucleotide used in the 3′-processing and strand transfer reactions (Fig. 1B). Fig. 1, C and D, shows that CA was only minimally active against strand transfer (IC_{50} ~ 60 μM) and even less active against 3′-processing (<50% inhibition at 132 μM). Similar results were obtained for a CT dinucleotide (data not shown). Our previous observation that modification of the deoxyribose of a physiological deoxynucleotide can confer inhibitory activity against integrase (2) led us to test whether incorporation of modified nucleoside analogs (e.g., 3′-azido-3′-deoxythymidine or 2′,3′-dideoxyinosine) into a dinucleotide would result in increased potency. No increase in potency was observed (data not shown).

Our previous observation that 5′-phosphorylation of nucleoside analogs conferred activity against integrase (2, 8) led us to test the phosphorylated version of CA and other dinucleotides. 5′-Phosphorylation of CA (generating pCA) enhanced the inhibitory activity (Fig. 1, C and D). This pCA dinucleotide also inhibited strand transfer using a precleaved oligonucleotide (which mimics the product of the 3′-processing reaction) consisting of sequences from the U3 end of the LTR in the same concentration range (data not shown). Interestingly, when the nonphosphorylated CA dinucleotide in which a ribose was substituted for the deoxyribose was tested, significant inhibition of both strand transfer and 3′-processing was observed at 100 μM (Fig. 1, C and D). Therefore, substitution of the deoxyribose by a ribose also enhanced the potency of the nonphosphorylated CA dinucleotide.

We then tested all 16 possible sequence combinations of 5′-phosphorylated deoxydinucleotides against HIV-1 integrase to determine whether base sequence selectivity existed (Table 1). The inhibitory activity observed with pAC, pAT, and pCT is in contrast with the low level of activity observed with the pTN family (N = A, C, T, or G).

The correlation between potency and oligonucleotide length was tested further using several trinucleotides and tetranucleotides. In addition, the effect of 5′-phosphorylation on trinucleotide potency was examined (Table 2). The nonphosphorylated trinucleotides CAG and CTA inhibited 3′-processing at ~60 μM (Table 2), whereas nonphosphorylated dinucleotides were inactive in this range (Fig. 1). The phosphorylated CTA trinucleotide showed a further enhancement of potency by ~2–3-fold (Table 2), which is consistent with results obtained from dinucleotides. Two 5′-phosphorylated tetranucleotides corresponding to the HIV LTR end were also tested (Table 2), and they were found to not be better inhibitors than the corresponding trinucleotides or dinucleotides (Tables 1 and 2).

Changes in the DNA backbone. Substitution of the phosphodiester backbone of pCA by an anionic phosphorothiodiester did not abolish potency (Fig. 2). For example, both the Rp (*lanes 8–12*) and Sp (*lanes 13–17*) diastereomers of pCA inhibited strand transfer, although at higher concentrations than did pCA (*lanes 3–7*).

TABLE 1

Inhibition of HIV-1 integrase by 5′-phosphorylated deoxydinucleotides

For each dinucleotide combination, the first line represents the IC_{50} for 3′-processing and the lower line the IC_{50} for strand transfer (in micromolar).

5′	3′ A	3′ C	3′ G	3′ T
A	>100	6 ± 2	15 ± 7	7 ± 2
	100 ± 30	3 ± 1	9 ± 4	7 ± 3
C	105 ± 20	15 ± 0.5	71 ± 11	8 ± 0.5
	13 ± 5	12 ± 5	12.4 ± 1.7	6 ± 1
G	>100	>100	54 ± 34	22 ± 1
	100 ± 40	100 ± 40	11.5 ± 3.5	7.5 ± 0.5
T	85 ± 21	35 ± 17	65 ± 35	73 ± 27
	18 ± 10	37 ± 9	31 ± 4	53 ± 4

TABLE 2
IC_{50} values for inhibition of HIV-1 integrase by trinucleotides and tetranucleotides

5′-Sequence-3′	3′-Processing	Strand transfer
	μM	
CAG	57 ± 12	53 ± 11
CTA	65 ± 15	25 ± 5
pCTA	27 ± 6	6 ± 2
pCTT	>100	13 ± 4
pCAT	>100	20 ± 4
pCAC	93 ± 19	27 ± 6
pCAA	42 ± 9	22 ± 5
pCAG	57 ± 11	15 ± 4
pGTC	40 ± 5	9 ± 1
pGTCA	32 ± 4	5 ± 1
pCAGT	34 ± 4	7 ± 1

The importance of the ionic character of the dinucleotide backbone in conferring potency was examined by its replacement with neutral, hydrophobic internucleotidic linkages. We examined replacement of the phosphodiester backbone in the pCA dinucleotide with a methylphosphonodiester linkage to preserve the basic phosphorus-linked backbone. Both the Rp and Sp diastereomers of the methylphosphonodiester pCA were inactive for both 3′-processing and strand transfer at 500 μM (Fig. 2, *lanes 19–24*), suggesting that the ionic character of the backbone is important. More radical alterations were tested by using a CA dinucleotide analogue with a PNA backbone (23) in the CA dinucleotide. Like the phosphodiester version, the CA with the PNA backbone exhibited IC_{50} values for both 3′-processing and strand transfer at ~100 μM (Fig. 2, *lanes 25–29*). We conclude that the anionic character of the backbone, although important for activity (Fig. 2, compare *lanes 3–7* with *lanes 19–24*), can be substituted by an amide backbone, which may exhibit a partial dipole character, simulating the anionic character of the phosphodiester.

The results of sugar and backbone substitution on the CA dinucleotide are summarized in Fig. 3. Modification of both the sugar and the internucleotidic linkage had a significant impact on potency. Taken together with the data in Table 1, interactions between HIV-1 integrase and the base, sugar, and phosphodiester linkages all appear to play critical roles in potency (and, presumably, binding affinity).

Other backbone modifications that retained the anionic character of the backbone but altered its conformation were also tested. For example, nine available cyclic dinucleotides were assayed. Such cyclic dinucleotides can be generated by HIV-1 integrase during the 3′-processing reaction *in vitro* by the attack of the 3′-hydroxyl of the viral DNA on the scissile bond (14, 15). Cyclic dinucleotides also exhibited a sequence-dependent activity, although they were active at >100 μM (Fig. 4). For example, the cyclic CC dinucleotide (*lanes 25–27*) was the most potent of the nine tested, whereas the cyclic CT dinucleotide was inactive at 1 mM (*lanes 6–8*). Interestingly, a cyclic dinucleotide with abasic sites in place of the bases (DD) also exhibited inhibitory activity (*lanes 13–15*). These results are in contrast to the significant inhibition of both 3′-processing and strand transfer obtained with the linear, 5′-phosphorylated dinucleotides (Table 2). We conclude that the cyclic structure reduces potency relative to the linear structure. In addition, 2′–5′-oligoadenylates, recently shown to be active against HIV-1 replication due to inhibition of reverse transcriptase (24), were found to be inactive at 300 μM (data not shown). These results are not unexpected given the lack of inhibition of integrase observed with the pAA (Table 1). Therefore, the 2′–5′ phosphodiester backbone does not seem to enhance potency relative to the 3′–5′ linkage.

Site and mechanism of action. The binding site of these deoxyribodinucleotides was first examined by testing the dinucleotide pCT with an integrase deletion mutant containing only amino acids 50–212 (catalytic core of integrase). Because deletion mutants of integrase are inactive in both 3′-processing and strand transfer (17) but can catalyze the disintegration reaction (16), the branched Y oligonucleotide was used as the substrate. Our finding that the integrase deletion mutant missing both the amino-terminal zinc finger and the carboxyl-terminal DNA-binding domains could be inhibited by pCT (Fig. 5A) suggests that dinucleotides bind in the catalytic core.

The second question we asked was whether dinucleotides affected the choice of nucleophile in the 3′-processing reaction. An oligonucleotide substrate labeled at the 3′ end was used for these experiments (14, 15). As seen in Fig. 5B, all of the dinucleotides tested inhibited glycerolysis, hydrolysis, and circular nucleotide formation to the same extent. Thus, dinucleotides exert a global inhibition by blocking the use of the three nucleophiles (glycerol, water, or the hydroxyl group of the viral DNA terminus) in the 3′-processing reaction. These results are consistent with the binding of dinucleotides to the central core domain of the HIV-1 integrase.

We next wanted to determine whether dinucleotides inhibited binding of the substrate DNA, which presumably interacts with the catalytic acidic acid residues Asp64, Asp116, and Glu152 of the core enzyme domain (6, 25). Dinucleotides inhibited binding of the enzyme to its substrate DNA (Fig. 6). For example, pTA showed strong inhibition of binding only at 132 μM (*lane 16*), whereas pAC (*lane 13*) and pGT (*lane 23*) exhibited the same level of inhibition at 3.2 and 8 μM, respectively. Thus, IC_{50} values for DNA binding inhibition correlated with IC_{50} values for inhibition of HIV-1 integrase catalytic activities (Table 1). These results suggest that dinucleotides affect the binding site of the enzyme for its DNA substrate.

Inhibition of related lentiviral integrases. The pCA dinucleotide was tested for inhibition of the related retroviral integrases from HIV-2, SIV, and FIV. As seen in Fig. 7, when HIV-1 DNA was used in all reactions, pCA inhibited both 3′-processing and strand transfer catalyzed by HIV-1 integrase in the expected concentration range. The IC_{50} values were 88 and 15 μM for 3′-processing and strand transfer, respectively. Similarly, HIV-2 integrase was inhibited with IC_{50} values of 125 and 10 μM for 3′-processing and strand transfer, respectively. As in the case of HIV-1 integrase, pCA was ~12-fold more selective in inhibiting the strand transfer reaction than the 3′-processing reaction with HIV-2 integrase. Although strand transfer catalyzed by FIV integrase was inhibited in the same concentration range (IC_{50} = 46 μM) as that for the HIV-1 and -2 integrases, 3′-processing catalyzed by this integrase was inhibited with an IC_{50} value of ~1000 μM. The differential inhibition of the two activities catalyzed by FIV and SIV integrases was ~20–40-fold. Therefore, the pCA dinucleotide appeared to inhibit HIV-1 and -2 integrases more efficiently than the FIV and SIV integrases.

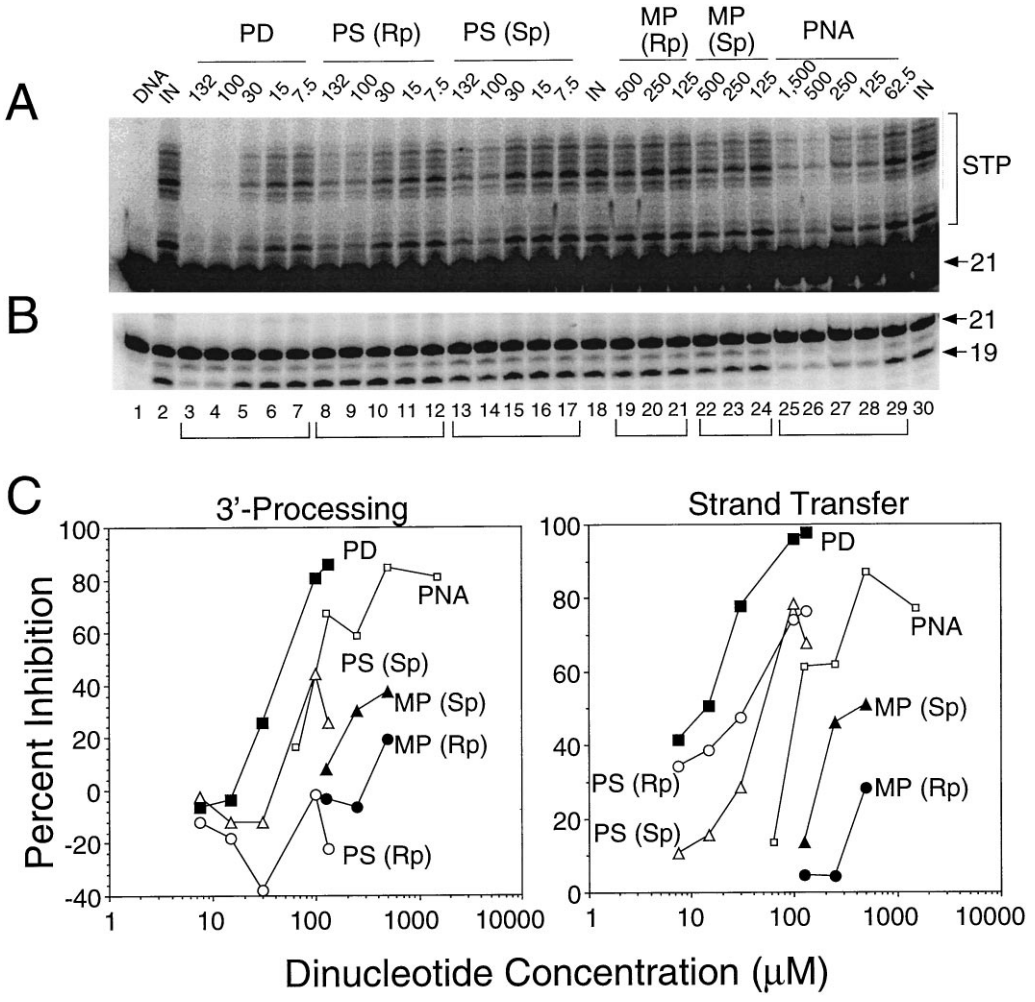

Fig. 2. Effect of phosphodiester substitution on the inhibition of 3′-processing and strand transfer by the 5′-phosphorylated deoxyribodinucleotides. Darker (A) and lighter (B) exposures of the gel are shown for inhibition of the strand transfer and 3′-processing activities, respectively. Dinucleotide concentrations in micromolar are indicated *above each lane*. *STP*, DNA strand transfer products. *19* (mer), 3′-processing product. *21* (mer), DNA substrate. *Lane 1*, DNA alone. *Lanes 2, 18,* and *30*, DNA plus integrase (*IN*) without dinucleotide. *Lanes 3–7*, DNA plus integrase in the presence of pCA, which has a phosphodiester backbone (*PD*). *Lanes 8–12*, DNA plus integrase in the presence of pCA, which has a phosphorothiodiester backbone (Rp diastereomer) (*PS Rp*). *Lanes 13–17*, DNA plus integrase in the presence of pCA, which has a phosphorothiodiester backbone (Sp diastereomer) (*PS Sp*). *Lanes 19–21*, DNA plus integrase in the presence of pCA, which has a methylphosphonodiester backbone (Rp diastereomer) (*MP Rp*). *Lanes 22–24*, DNA plus integrase in the presence of pCA, which has methylphosphonodiester backbone (Sp diastereomer) (*MP Sp*). *Lanes 25–29*, DNA plus integrase in the presence of CA, which has a PNA backbone (*PNA*). C, Quantification of A and B.

Discussion

Sequence selectivity in the inhibition of HIV-1 integrase. In the present study, several dinucleotides were found to be potent inhibitors of both 3′-processing and strand transfer catalyzed by HIV-1 integrase. Some sequence selectivity was evident, with pAC, pAT, and pCT being the most potent and pAA, pGA, and pGC showing weak activity at 100 μM. The lack of a more unambiguous sequence selectivity was consistent with the nonspecific DNA binding capability of integrase (26) and with interactions with other parts of the dinucleotide (e.g, the phosphodiester backbone or the sugar moiety) being critical (Figs. 3 and 4).

The sequence selectivity observed in the inhibition by dinucleotides is, however, consistent with the catalytic mechanism for 3′-processing. For example, before the reaction, integrase presumably binds near the scissile bond (i.e., the bond between the A and G nucleotides on the plus strand) (Fig. 1A). The dinucleotides immediately upstream of the scissile bond are a 5′-CA-3′ and 5′-TG-3′ on the plus and minus strand, respectively (Fig. 1A). Binding of integrase to these dinucleotides in the recognition sequence should therefore not inhibit the subsequent endonuclease reaction or catalysis would not occur. Accordingly, neither of these dinucleotides exhibited potency against 3′-processing at <100 μM (Table 1). These dinucleotides could therefore provide a binding site on the viral DNA substrate, which would allow the 3′-processing reaction to proceed unhindered. These results are consistent with earlier reports that mutation of the 5′-GCA-3′ trinucleotide at the-processing site on the scissile strand resulted in inhibition of the 3′-processing reaction (21, 27, 28), further supporting the contention that the residues immediately upstream of the dinucleotide cleavage site in the 3′-processing reaction may provide a critical recognition/binding site for the integrase. The relative lack of potency observed with the 5′-GT-3′ dinucleotide (Table 1) suggests that this reaction product (Fig. 1C), which results from hydrolysis of the scissile phosphodiester bond in the 3′-processing reaction, may not serve to regulate this reaction.

The most potent dinucleotide that exhibited IC_{50} values of ≤5 μM was pAC. This finding is consistent with the observation that interactions between integrase and the ultimate and penultimate bases at the 5′ end of the noncleaved strand (5′-AC-3′) (Fig. 1A) could be the basis for stable complex formation before the strand transfer reaction (29). Therefore, the potent inhibitory activity of pAC is presumably a manifestation of the binding affinity that integrase may have for this dinucleotide sequence.

Effects of 5′-phosphorylation and trinucleotides. 5′-Phosphorylation of both dinucleotides and trinucleotides enhanced potency compared with the corresponding nonphosphorylated form, which is consistent with the earlier reported effects of 5′-phosphorylation on mononucleosides (8)

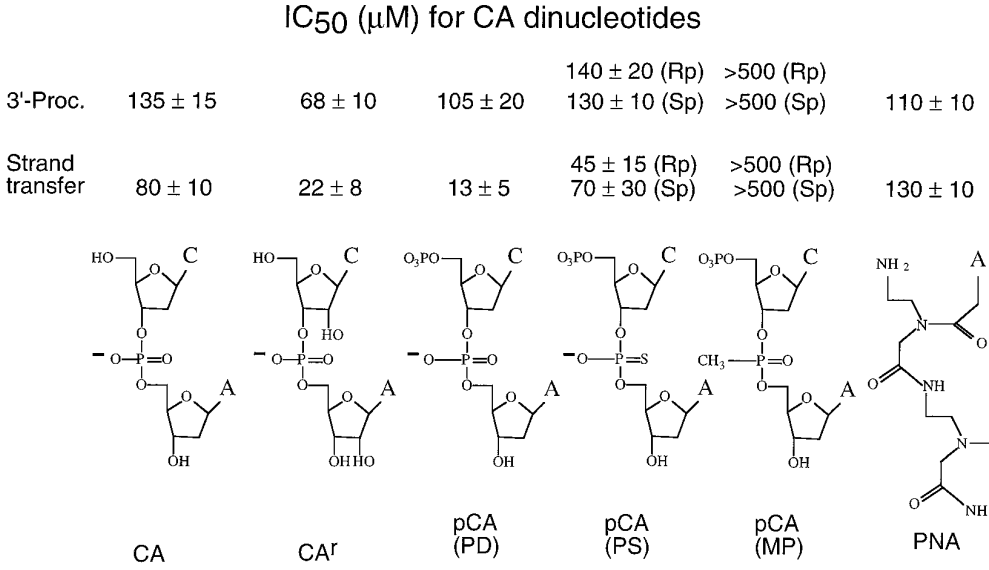

Fig. 3. Activities and structures of the CA dinucleotides and PNA analog with the sugar and backbone modifications used in this study. Micromolar IC_{50} values for each activity are shown *above each structure*.

and with the nonspecific binding of integrase to and inhibition by nucleic acids (26) and polyanions (30). Inhibition with 5′-phosphorylated dinucleotides was observed at 10–20-fold lower concentrations than with mononucleotides such as AZT (8) and D4T monophosphates (2). However, lengthening of the oligonucleotide further did not increase potency as significantly. These data suggest that two neighboring bases may be sufficient to provide the essential interactions when integrase recognizes its viral DNA substrate.

Interaction of HIV-1 integrase with the phosphodiester backbone. Previous reports using duplex oligonucleotide substrates containing ethylated phosphodiester (31) or methylphosphonodiester (32) backbones have shown that these substitutions dramatically lower the extent of both 3′-processing and strand transfer reactions. These data suggested the importance of interactions with the phosphodiester backbone. This hypothesis was probed by investigating the effect of neutralizing the negative charge in the backbone of various CA dinucleotides and examining the resulting extent of inhibition. Elimination of the anionic character of the backbone by substitution with a methylphosphonodiester backbone was sufficient to abolish potency. Therefore, the

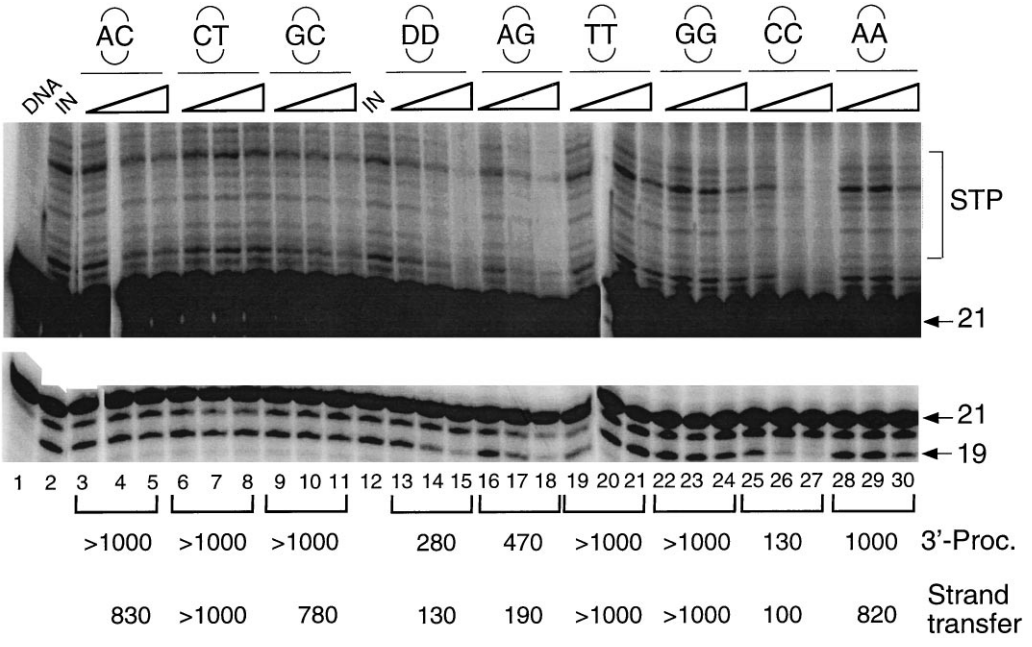

Fig. 4. Effects of cyclic dinucleotides on HIV-1 integrase activity. Darker (*top*) and lighter (*bottom*) exposures of the gel are shown for inhibition of the strand transfer and 3′-processing activities, respectively. *STP*, DNA strand transfer products. *19* (mer), 3′-processing product. *21* (mer), DNA substrate. Dinucleotide concentrations are indicated *above each lane*. *Lane 1*, DNA alone. *Lanes 2 and 12*, DNA plus integrase without dinucleotide. *Lanes 3–5*, DNA plus integrase in the presence of cyclic AC. *Lanes 6–8*, DNA plus integrase in the presence of cyclic CT. *Lanes 9–11*, DNA plus integrase in the presence of cyclic GC. *Lanes 13–15*, DNA plus integrase in the presence of cyclic DD (where D is a tetrahydrofuran moiety representing a synthetic version of an abasic site). *Lanes 16–18*, DNA plus integrase in the presence of cyclic AG. *Lanes 19–21*, DNA plus integrase in the presence of cyclic TT. *Lanes 22–24*, DNA plus integrase in the presence of cyclic GG. *Lanes 25–27*, DNA plus integrase in the presence of cyclic CC. *Lanes 28–30*, DNA plus integrase in the presence of cyclic AA. Micromolar IC_{50} values for each activity are shown *below each dinucleotide.*

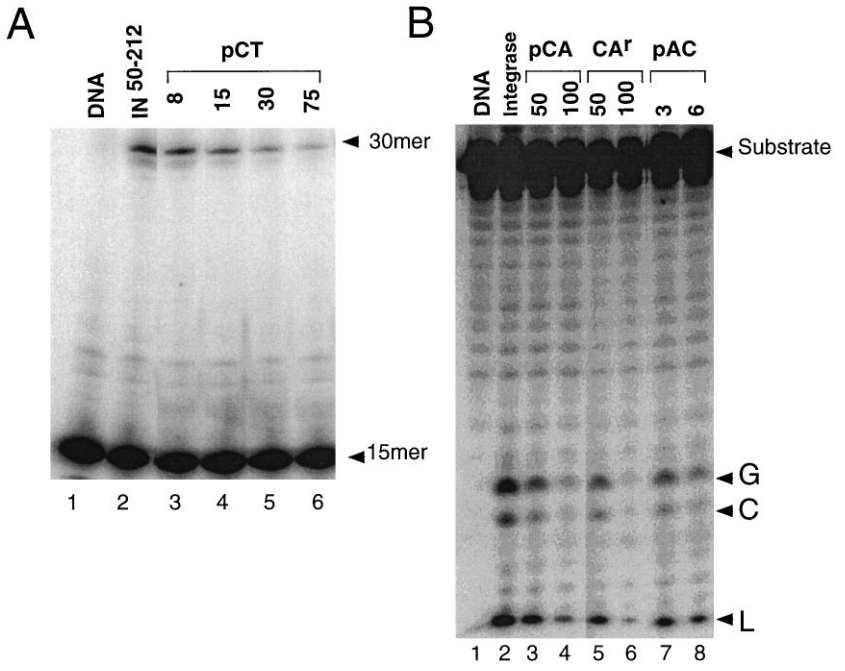

Fig. 5. Analysis of the dinucleotide binding site and effect on choice of nucleophile in the 3′-processing reaction. A, Inhibition of disintegration catalyzed by the integrase catalytic domain (IN^{50-212}) in the presence of pCT. *Lane 1*, DNA alone. *Lane 2*, DNA plus integrase. *Lanes 3–6*, DNA plus integrase in the presence of the indicated concentration of pCA. *15mer*, DNA substrate. *30mer*, DNA product. B, Inhibition of the 3′-processing reaction using a 3′ end-labeled substrate. *G*, Products of the glycerolysis reactions. *C*, Products of the circular nucleotide formation reactions. *L*, Products of the hydrolysis reactions. *Lane 1*, DNA alone. *Lane 2*, DNA plus wild-type integrase. *Lanes 3–4, 5–6*, and *7–8*, DNA plus integrase in the presence of the indicated micromolar concentrations of pCA, CA^r, and pAC, respectively.

negative charge in these dinucleotides may serve to position the inhibitor for critical interactions with the divalent metal ion (29) or basic amino acids in the active site (33) so that high affinity binding can occur.

Two predictions would be consistent with this hypothesis. First, an increase in anionic character could serve to optimize interactions within the active site. This prediction is supported by the observation that inhibition with 5′-phosphorylated dinucleotides was observed at 10–20-fold lower concentrations than with nonphosphorylated dinucleotides. The same trend was observed with mononucleotides of AZT (8) and D4T (2). Second, correct positioning of the 5′-phosphorylated dinucleotide may require proximity of the anionic charge from both the phosphodiester backbone and the 5′-phosphate to appropriate counter ions. This prediction is supported by the observation that the cyclic structure abolished potency relative to the linear structure. In this case, either the decrease in anionic charge from three (for the linear, phosphorylated dinucleotide) to two (for the cyclic dinucleotide) or the movement of the 5′-phosphate might be responsible for the lack of inhibition. The lack of inhibition by, and presumably low affinity for, the cyclic dinucleotides may also be a manifestation of the requirement for product dissociation after the 3′-processing reaction (if the viral DNA hydroxyl has been used as a nucleophile in this endonuclease reaction to generate a cyclic dinucleotide) and before the strand transfer reaction. The low affinity for the cyclic dinucleotides also suggests that these compounds, like their

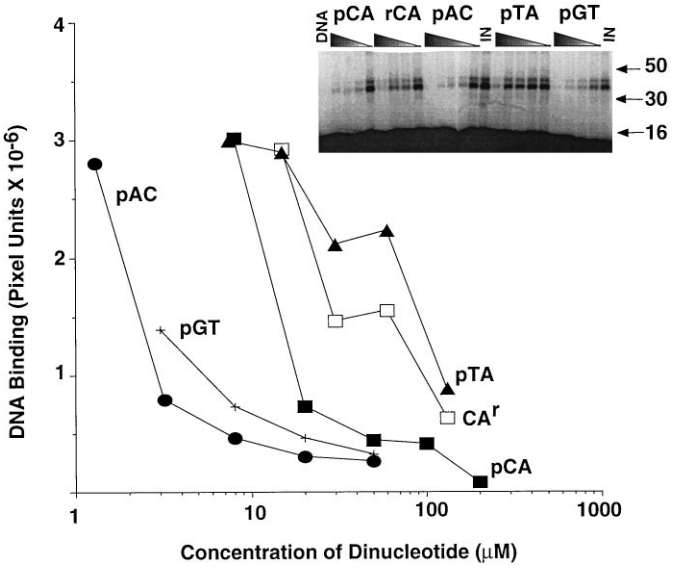

Fig. 6. Inhibition of the DNA-binding activities of HIV-1 integrase in the presence of dinucleotides. *Inset*, PhosphorImager picture showing UV cross-linking of wild-type integrase with duplex U5 oligonucleotide substrates in the presence of increasing concentrations of dinucleotides. *Right*, migrations of proteins of known molecular mass. *DNA*, DNA alone. *IN*, DNA plus integrase without dinucleotide. *pCA*, DNA plus integrase in the presence of 100, 50, 20, and 8 μM pCA, respectively. *rCA*, DNA plus integrase in the presence of 132, 60, 30, and 15 μM rCA, respectively. *pAC*, DNA plus integrase in the presence of 50, 20, 8, 3.2, and 1.3 μM pAC. *pTA* , DNA plus integrase in the presence of 132, 60, 30, 15, and 7.5 μM pTA, respectively. *pGT*, DNA plus integrase in the presence of 50, 20, 8, and 3.2 μM pGT, respectively.

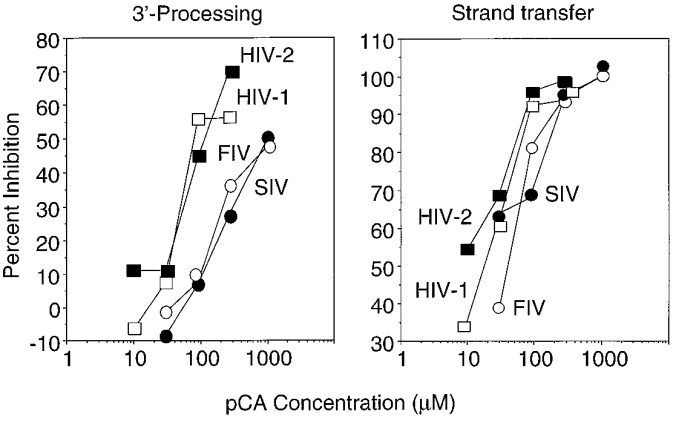

Fig. 7. Inhibition of the related retroviral integrases from HIV-1, HIV-2, FIV, and SIV by the pCA dinucleotide.

linear counterparts, do not inhibit the enzymatic activities of integrase through a feedback loop.

Interaction of HIV-1 integrase with the sugar moiety. Previous reports with mononucleotides have suggested that critical interactions between integrase and the sugar moeity may contribute to the binding affinity of and inhibition by these compounds (2, 33). For example, deoxyribose modifications such as substitution of a β-L-enantiomer for its β-D-counterpart, unsaturation between the 2′ and 3′ carbons, or substitution by an azido or a fluoro substituent at the 3′ position conferred inhibitory activity against HIV-1 integrase compared with the unsubstituted nucleotide (2). Furthermore, oxidation of ATP, generating the 2′,3′-dialdehyde, resulted in a nucleotide analog that was >30-fold as potent as an integrase inhibitor than ATP (33).

In this study, the possible effect of sugar pucker in integrase inhibition was examined by substitution of the deoxyribose (C2′-*endo*) by a ribose (C3′-*endo*) in a CA dinucleotide. Substitution of the deoxyribose enhanced potency of the nonphosphorylated CA dinucleotide to approximately the same extent as did 5′-phosphorylation. These data also suggest that interactions with the sugar may play as critical a role as those with the 5′-phosphomonoester.

Dinucleotides as pharmacological agents. In addition to being 10–100-fold more potent than their corresponding mononucleotides, 5′-phosphorylated dinucleotides also exhibit potency in the same range as other recently described integrase inhibitors (10, 12, 34). Furthermore, cellular uptake of 5′-phosphorylated dinucleotides may not require specialized delivery vehicles. Other anionic mononucleotides and oligonucleotides have been found to cross the cell membrane and act as inhibitors of intracellular targets. For example, phosphonylmethoxypropyladenine (35) and a guanosine quartet-forming oligonucleotide (36), both of which are currently under preclinical investigation as anti-HIV agents, have been shown to accumulate inside cells. None of the dinucleotides tested in that study, however, exhibited inhibition of HIV replication or cytotoxicity at concentrations of ≤30 μM.

We investigated whether some of the most potent dinucleotides in this report had antiviral activity by using the cell protection assay against the cytopathic effects of HIV-1_{LAI} strain. None of the dinucleotides tested [pCA, rCA, CA, pAC, cDD, cCC, pCA(PS), and pCA(MP); see Figs. 3 and 4 for structures] had significant antiviral activity. They also exhibited no cytotoxicity by themselves at concentrations of ≤200 μM. At this time, it is not clear whether this lack of activity/cytotoxicity is due to cellular uptake, stability, or both. These data suggest that additional modifications may be required to achieve antiviral effects. Along these lines, it is interesting to note that a guanosine quartet oligonucleotide with potent antiviral activity is also among the most potent known inhibitors of HIV integrases (37). Another application of dinucleotides involves the possible use of these water-soluble inhibitors as lead compounds for further antiviral development if cocrystals could be obtained.

Acknowledgments

We thank Drs. Robert Craigie and Alan Engelman (Laboratory of Molecular Biology, National Institute of Diabetes and Digestive and Kidney Diseases) for generously providing us with purified HIV-1 integrase; Dr. Kurt Kohn for his support during the course of these experiments; and Drs. Jean-Pierre Sommadossi (University of Alabama-Birmingham), Peter Nielsen (University of Copenhagen, Copenhagen, Denmark), and Robert J. Suhadolnik (Temple University, Philadelphia, Pennsylvania) for providing nonphosphorylated dinucleotides containing modified nucleoside analogs, the PNA dinucleotide, and 2′,5′-oligoadenylates, respectively.

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