Effects of Tyrphostins, Protein Kinase Inhibitors, on Human Immunodeficiency Virus Type 1 Integrase†

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ABSTRACT: Efficient replication of HIV-1 requires establishment of the proviral state, i.e., the integration of a DNA copy of the viral genome, synthesized by reverse transcriptase, into a chromosome of the host cell. Integration is catalyzed by the viral integrase protein. We have previously reported that phenolic moieties in compounds such as napthoquinones, flavones, caffeic acid phenethyl ester (CAPE), and curcumin confer inhibitory activity against HIV-1 integrase. We have extended these findings by examining the effects of tyrphostins, tyrosine kinase inhibitors. The catalytic activities of HIV-1 integrase and the formation of enzyme−DNA complexes using photocross-linking were examined. Both steps of the integration reaction, 3′-processing and strand transfer, were inhibited by tyrphostins at micromolar concentrations. The DNA binding activity of integrase was inhibited at higher concentrations of tyrphostins. Disintegration, an apparent reversal of the strand transfer reaction, catalyzed by an integrase mutant lacking the N-terminal zinc finger and C-terminal DNA binding domains is also inhibited by tyrphostins, indicating that the binding site for these compounds resides in the central catalytic core of HIV-1 integrase. Binding of tyrphostins at or near the integrase catalytic site was also suggested by experiments showing a global inhibition of the choice of attacking nucleophile in the 3′-processing reaction. None of the tyrphostins tested inhibited eukaryotic topoisomerase I, even at 100 μM, suggesting selectivity for integrase inhibition. Molecular-modeling studies have revealed that, after energy minimization, several tyrphostins may adopt folded conformations. The similarity of the tyrphostin family to other families of inhibitors is discussed. Tyrphostins may provide lead compounds for development of novel antiviral agents for the treatment of acquired immunodeficiency syndrome based upon inhibition of HIV-1 integrase.

The three viral enzymes encoded by the *pol* gene of human immunodeficiency virus (HIV) play key roles in the virus replication cycle. Two of these enzymes, reverse transcriptase and protease, are the focus of intense research as targets for chemotherapeutic intervention (Johnston & Hoth, 1993). Research to develop clinically active agents against other proteins in the viral life cycle is now in progress. Toward this goal, several laboratories have investigated the pharmacological activity of various drugs as inhibitors of HIV integrase (Carteau et al., 1993a−c; Cushman & Sherman, 1992; Fesen et al., 1993, 1994; Mazumder et al., 1994c, 1995a,b; LaFemina et al., 1995). These drug candidates span several families such as DNA groove binders and intercalators, polyhydroxylated aromatic compounds, polysulfonated compounds, and nucleotides.

Retroviruses encode the integrase protein at the 3′-end of the *pol* gene (Varmus & Brown, 1989). 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 (Wiskerchen & Muesing, 1995; Engelman et al., 1995; Shin et al., 1994; LaFemina et al., 1992). 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 (Katz et al., 1990; Craigie et al., 1990). This activity is referred to as the 3′-processing or dinucleotide cleavage. After transport to the nucleus as a nucleoprotein 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 (1994), Vink and Plasterk (1993), and Goff (1992).]

We have previously reported that phenolic moieties in compounds such as napthoquinones, flavones, caffeic acid phenethyl ester (CAPE), and curcumin inhibit HIV-1 integrase *in vitro* (Fesen et al., 1993, 1994; Mazumder et al., 1995a). In the present report, we have extended these findings by examining the effects of tyrphostins (Table 1) on the DNA binding and catalytic activities of HIV-1 integrase.

Tyrphostins are synthetic compounds initially designed as analogs of erbstatin, a natural product known to inhibit tyrosine kinases (Yaish et al., 1988). Tyrphostins are potent

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tyrosine kinase inhibitors and, through this mechanism, manifest antiproliferative effects (Yaish et al., 1988; Levitzki, 1990, 1992; Levitzki & Gazit, 1995). Certain tyrphostins can discriminate in their inhibition between receptor and nonreceptor tyrosine kinases. For example, tyrphostin AG957 has been shown to block the autokinase activity of the nonreceptor tyrosine kinase $p210^{bcr\text{-}abl}$ (Kaur et al., 1994), while another tyrphostin, AG879, inhibited the tyrosine kinase activity of the nerve growth factor receptor (Ohmichi et al., 1993). These results suggest the possibility for selective inhibition via modulation of the drug structure.

These compounds were selected for several reasons. First, their structural similarity to the flavones, CAPE, and curcumin suggested that they would exhibit some level of activity against integrase. Second, the presence of ortho phenolic hydroxyls would allow further confirmation that this catechol structure may represent a pharmacophore for HIV-1 integrase inhibitors. Third, tyrphostins have recently been shown to inhibit replication of Moloney murine leukemia virus (Aflalo et al., 1994b), a retrovirus related to HIV-1, and topoisomerase I (Aflalo et al., 1994a), an enzyme which, like HIV-1 integrase (Engelman et al., 1991), catalyzes a transesterification reaction (Pommier et al., 1994). Fourth, the low cytotoxicity of tyrphostins in cell culture (Aflalo et al., 1994b) makes these agents attractive as potential antiretroviral agents.

MATERIALS AND METHODS

Preparation of Radiolabeled DNA Substrates

The following oligonucleotides were high-performance liquid chromatography (HPLC) purified by and purchased from Midland Certified Reagent Co. (Midland, TX): 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′. The AE117, AE118, and the first 19 nucleotides of AE156 correspond to the U5 end of the HIV-1 long terminal repeat (LTR).

To analyze the extent of 3′-processing and strand transfer using 5′-end-labeled substrates (Bushman & Craigie, 1991), AE118 was 5′-end labeled using T_4 polynucleotide kinase (Gibco BRL) and γ-[^{32}P]ATP (Dupont-NEN). The kinase was heat-inactivated, and AE117 was added to the same final concentration. The mixture was heated at 95 °C, allowed to cool slowly to room temperature, and run on a G-25 Sephadex quick spin column (Boehringer Mannheim) to separate annealed double-stranded oligonucleotide from unincorporated label.

To analyze the extent of 3′-processing and strand transfer using 3′-end-labeled substrates, AE118 was 3′-end labeled using α-[^{32}P]cordycepin triphosphate (Dupont-NEN) and terminal transferase (Boehringer Mannheim). The transferase was heat-inactivated, and RM22M was added to the same final concentration. The mixture was heated at 95 °C, allowed to cool slowly to room temperature, and run on a G-25 spin column as before.

To determine the extent of 30mer target strand generation during disintegration, AE157 was 5′-end labeled, annealed to AE156, AE146, and AE117, and column purified as above.

Integrase Proteins

Purified recombinant wild-type HIV-1 integrase (Engelman et al., 1991; Bushman et al., 1990) and deletion mutant IN^{50-212} (Bushman et al., 1993) were generous gifts of Drs. R. Craigie and A. Engelman, Laboratory of Molecular Biology, NIDDK, NIH, Bethesda, MD.

3′-Processing, Strand Transfer, and Disintegration Assays

Integrase was incubated at a final concentration of 200 nM with 20 nM 5′-end-^{32}P-labeled oligonucleotide substrate 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 mg/mL bovine serum albumin, 10 mM 2-mercaptoethanol, 10% dimethyl sulfoxide, and 25 mM MOPS (pH 7.2) at 30 °C for 60 min]. The final reaction volume was 16 μL.

Disintegration reactions (Chow et al., 1992) 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 Quantitation

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, and 0.025% bromophenol 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, and 8 M urea]. Gels were dried and exposed in a Molecular Dynamics phosphorimager cassette. Gels were analyzed using a Molecular Dynamics phosphorimager (Sunnyvale, CA).

UV Cross-Linking Experiments

The method used was described by Yoshinaga et al. (1994). Briefly, integrase was incubated with substrate in reaction buffer as above for 5 min at 30 °C. Reaction mixtures were then irradiated with a 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× SDS−PAGE buffer [100 mM Tris (pH 6.8), 4% 2-mercaptoethanol, 4% sodium dodecyl sulfate (SDS), 0.2% bromophenol blue, and 20% glycerol] was added to each reaction mixture, and the reaction mixture was heated at 95 °C for 3 min prior to loading a 20 μL aliquot on a 12% SDS−polyacrylamide gel. The gel was run at 120 V for 1.5 h, dried, and exposed in a phosphorimager cassette.

Topoisomerase Reactions

Reactions were performed in 10 μL of reaction buffer [0.01 M Tris-HCl (pH 7.5), 150 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, and 15 μg/mL bovine serum albumin] with the following duplex oligonucleotide substrate labeled with α-[^{32}P]cordycepin at the 3′-end of the upper strand (asterisk):

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5'-GATCTAAAAGACTT^GGAAAAATTTTTAAAAAA*
        ATTTTCTGAA-CCTTTTTAAAAATTTTTTCTAG-5'
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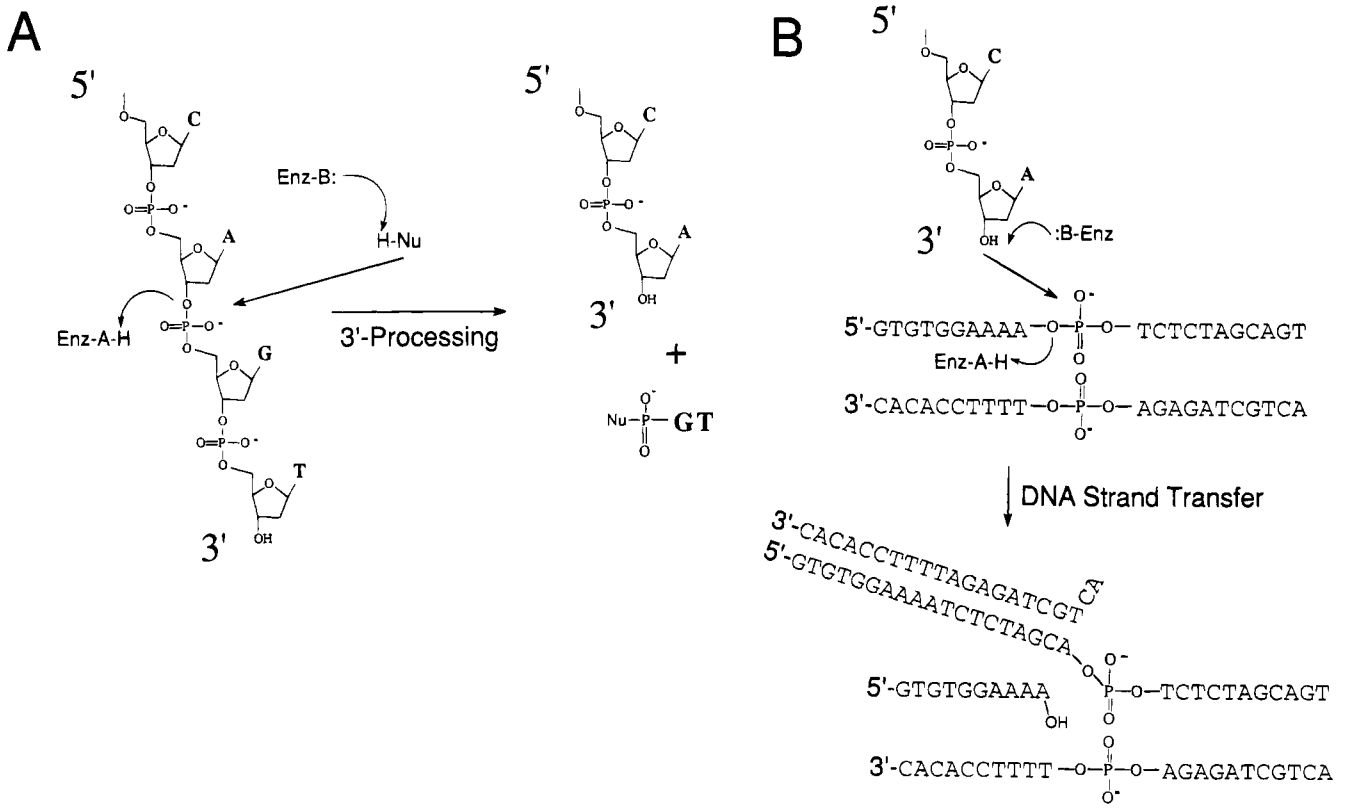
This oligonucleotide contains a single topoisomerase I

Scheme 1: (A) Reaction Mechanism for the 3′-Processing Reaction Catalyzed by HIV-1 Integrase and (B) Reaction Mechanism for the DNA Strand Transfer Reaction Catalyzed by HIV-1 Integrase[a]



[a] In part A, the nucleophile *in vitro* can be either water, glycerol, or the hydroxyl end of the viral DNA. In part B, the attack on the phosphodiester bond shown is only to demonstrate the chemical mechanism and is not meant to imply any sequence specificity for that site.

cleavage site (caret on the upper strand) (Fujimori et al., 1995). Approximately 50 fmol of oligonucleotide per reaction was incubated with 10 units of calf thymus DNA topisomerase I (Gibco BRL, Gaithersburg, Maryland). Reactions were stopped by addition of sodium dodecyl sulfate (0.5% as final concentrations). Proteinase K (ICN Biochemicals, Cleveland, OH) (0.5 mg/mL final concentration) was then added to the reaction mixtures, and proteolysis was carried out for an additional 60 min at 50 °C. Proteolysis was halted by the addition of 36 μL 2.5× loading buffer (98% formamide, 0.01 M EDTA, 1 mg/mL xylene cyanol, and 1 mg/mL bromophenol blue).

Tyrphostin Compounds

Tyrphostin tyrosine kinase inhibitors AG82, AG490, AG538, AG555, and AG55 were prepared as described previously (Gazit et al., 1991). The synthesis of the dimeric tyrphostins was done in a manner analogous to that of AG1717. Full details will be given in pending publications.

AG526. 1,3-Propanediamine (2.2 g, 30 mM) and methyl arano acetate (6.3 g, 64 mM) were stirred for 1 h at room temperature. The semisolid was recrystallized from ethanol to give 4.6 g: 74% yield; white solid; mp 148 °C; NMR (acetone-d_6) 3.58 (4H, S), 3.28 (4H, J = 6.7 Hz), 1.70 (2H, quintet, J = 6.7 Hz); MS *m/e* 208 (M + 22), 140 (M − $COCH_2CN$, 10), 125 (M − $NHCOCH_2CN$), 98 (57), 72 (20).

AG1717. AG526 (175 mg, 0.84 mM) and 3,4,5-trihydroxybenzaldehyde hydrate (300 mg, 1.74 mM) and β-alanine (20 mg) in 15 mL of ethanol were refluxed for 2 h. Cooling and filtering gave 338 mg: 88% yield; light orange solid; mp >300; NMR (DMSO-d_6) 7.82 (2H, S vinyl), 7.01 (4H, S), 3.20 (4H, ar T, J = 6.4 Hz), 1,70 (2H, quintet, J = 6.4 Hz).

RESULTS

Inhibition of HIV-1 Integrase by Tyrphostins

The 3′-processing reaction liberates the 3′-terminal dinucleotide, producing a 19mer oligonucleotide from a 21mer duplex substrate (Scheme 1A). Presumably a general base (Enz-B:) at the enzyme active site could deprotonate an incoming nucleophile (H-Nu) for attack on the scissile phosphodiester, while a general acid (Enz-A-H) could assist by protonation of the anionic 3′-hydroxyl leaving group. The strand transfer reaction (Scheme 1B) is an isoenergetic transesterification reaction resulting in the insertion of one 3′-processed oligonucleotide into another oligonucleotide, yielding higher molecular weight species which migrate more slowly than the 21mer substrate. Presumably a general base at the enzyme active site could deprotonate the DNA 3′-hydroxyl group for nucleophilic attack on a phosphodiester in the target DNA, while a general acid could assist by protonation of the anionic 3′-hydroxyl leaving group at the site of attack.

Tyrphostin derivatives studied are shown in Table 1, and their effects on HIV-1 integrase are summarized in Table 2. The effects of the six most potent tyrphostins on 3′-processing and strand transfer are shown in Figure 1. The tyrphostins AG575 and -1075 showed significant inhibition of both 3′-processing and strand transfer at concentrations as low as 0.56 μM (Figure 1A). Even more potent were the tyrphostins AG1717 and -1718 which inhibited with detectable activity at 0.18 μM (Figure 1B). In the assay shown in

Table 1: Structures of Tyrphostins

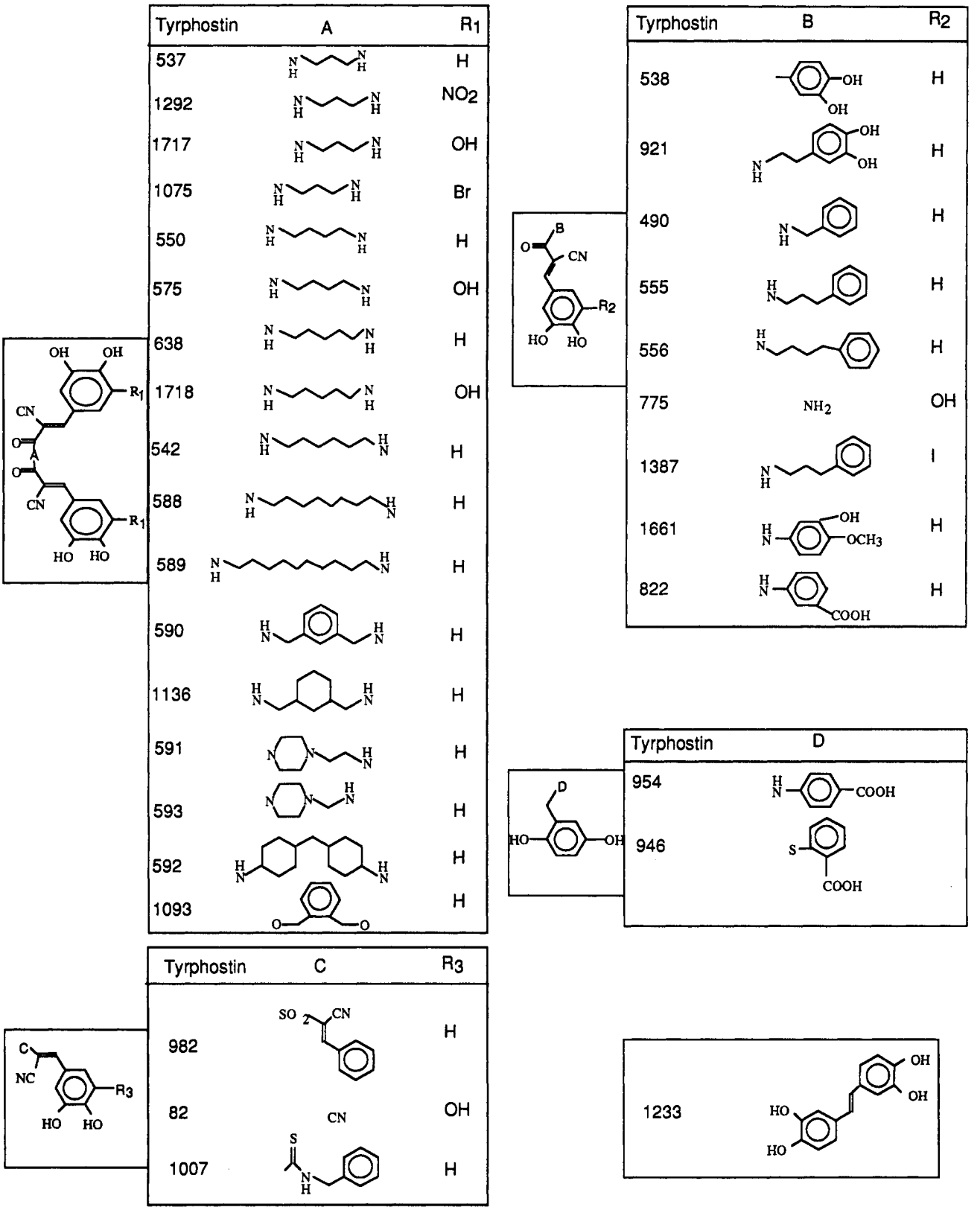


Figure 1, inhibition of 3′-processing alone would also result in reduction of the strand transfer products because the substrate for the strand transfer reaction (i.e., the product from the 3′-processing reaction) is not generated. In order to determine whether the strand transfer reaction was truly being inhibited or whether the inhibition of the 3′-processing reaction caused the decrease in the subsequent strand transfer products, a precleaved oligonucleotide substrate was used. The six most active tyrphostins shown in Figure 2A exhibited significant inhibition of the strand transfer activity at drug concentrations below 1 μM (Figure 2A). Quantitation of the data (Figure 2B) demonstrates a good agreement between the IC_{50} values derived using either the blunt-ended or precleaved substrates. These data suggest that tyrphostins

Table 2: In Vitro and in Vivo Inhibition by Tyrphostins

	IC_{50} (μM)[a]			antiviral data	
drug	3′-processing	strand transfer	disinteg	IC_{50} (μM)	EC_{50} (μM)[b]
AG537	1.9	0.8	8.6	40.9 ± 0.8	NR
AG542	1.35 ± 0.6	1.1 ± 0.5	3	>200	NR
AG575	0.66 ± 0.5	0.57 ± 0.1	0.4	49.8 ± 6.4	NR
AG1075	0.8 ± 0.4	0.4 ± 0.3	0.8	113 ± 1	NR
AG550	+	+		28.6 ± 6	NR
AG638	+	+		13 ± 2.5	NR
AG588	+	+		13.4 ± 0.1	NR
AG589	+	+		2.7 ± 0.5	NR
AG590	−/+	+		10.6 ± 0.1	NR
AG1136	−/+	+		11.8 ± 1.8	NR
AG591	+	+		23.1 ± 1.4	NR
AG593	−/+	+		16.8 ± 0.2	NR
AG592	+	3	>12		
AG1093	3	0.85	5.6	8.5 ± 0.3	NR
AG1292	3.3	1.9	>12	72.2 ± 15.3	NR
AG1717	0.4 ± 0.1	0.16	2	17.3 ± 2.5	15.6
AG1718	0.45 ± 0.1	0.17	2	38.3 ± 1.5	NR
AG538	1.0 ± 0.5	0.5 ± 0.4	4.2	36.2 ± 0.2	NR
AG921	+	2.7		29.8 ± 4	NR
AG490	−/+	−/+		33.7 ± 1.3	NR
AG555	−/+	−/+		24.3 ± 0.4	NR
AG556	−/+	−/+		11.8 ± 1	NR
AG775	−/+	+			
AG1387	−/+	−/+		9.6 ± 0.3	NR
AG1661	−/+	−/+		10.9 ± 1.9	NR
AG822	−/+	+		36.5 ± 1.2	NR
AG982	4.7 ± 1.1	1.2		25.7 ± 1.2	NR
AG82	+	+		31.8 ± 2.3	NR
AG1007	−/+	−/+		31.7	NR
AG954	−/+	−/+			
AG946	−/+	+		33.3 ± 1.5	NR
AG1233	+	1		16.8 ± 4.1	NR

[a] −/+ means that the IC_{50} value is between 10 and 100 μM. + means that the IC_{50} value is between 5 and 10 μM. [b] NR means that the EC_{50} value was never reached due to the cytotoxicity.

are able to inhibit both the 3′-processing and strand transfer reactions.

Mechanism and Site of Inhibition

UV Cross-linking of 5′-Labeled Substrate DNA in the Absence or Presence of Tyrphostins. In order to ascertain whether DNA binding was affected by the tyrphostins, UV cross-linking of integrase−DNA reaction mixtures was performed. Cross-linking of substrate DNA to integrase followed by electrophoresis results in a product having a molecular mass of approximately 39 kDa (Yoshinaga et al., 1994; Engelman et al., 1994). As seen in Figure 3, tyrphostin AG1717 can inhibit binding of integrase to the substrate DNA. Significant inhibition of binding is seen in the presence of 2 μM AG1717 (lane 3). This concentration is higher than the IC_{50} value for 3′-processing and strand transfer. However, at a concentration closer to the IC_{50} values for 3′-processing and strand transfer, no significant inhibition of DNA binding was observed (lane 1). Thus, inhibition of integrase activity by tyrphostins is detectable at lower concentrations than inhibition of DNA binding.

Disintegration Reactions with HIV-1 Integrase Deletion Mutants. Integrase can catalyze *in vitro* an apparent reversal of the DNA strand transfer reaction (Scheme 1B), called disintegration (Chow et al., 1992; Sherman et al., 1992) (Scheme 2). In this intramolecular reverse reaction, a double-stranded oligonucleotide corresponding to either the U5 or U3 end of the retroviral long terminal repeat (LTR) is spliced out from a Y (i.e., branched) oligonucleotide in which the viral DNA is joined to a target double-stranded oligonucleotide. Presumably a general base at the enzyme active site could deprotonate the DNA 3′-hydroxyl terminus for nucleophilic attack on the phosphodiester 3′ to the CA dinucleotide, while a general acid could assist by protonation of the anionic 3′-hydroxyl leaving group of the CA. An intermolecular disintegration reaction can also be catalyzed by HIV-1 integrase in which the branched substrate has an X structure consisting of both the U5 and U3 ends of the LTR joined to target DNA (Mazumder et al., 1994a; Chow & Brown, 1994; van Den Ent et al., 1994). In contrast to the 3′-processing and strand transfer reactions, intra- and intermolecular disintegration do not require either the N-terminal zinc-finger region or the C-terminal DNA binding domain of integrase (Bushman et al., 1993; Mazumder et al., 1994a). For this reason, an integrase deletion mutant IN^{50-212} lacking both of these domains can be used in the intramolecular disintegration assay where the level of activity is decreased only 4−5-fold relative to wild-type enzyme (Bushman et al., 1993).

In an attempt to define the tyrphostin binding site on HIV-1 integrase in more detail, tyrphostins AG1717 and -1718 were studied in this assay (Figure 4). Significant inhibition of disintegration catalyzed by the core deletion mutant (IN^{50-212}) was observed at 3 μM and higher concentrations (Figure 4A, lanes 4 and 9, and Figure 4B). The concentration of tyrphostin required for inhibition of disintegration was higher than that required for inhibition of either 3′-processing or strand transfer. These results are consistent with those observed with other inhibitors (Mazumder et al., 1995a). The finding that the tyrphostins are active against the IN^{50-212} mutant implies that the binding of these tyrphostins to the integrase core region is responsible for integrase inhibition.

Global Inhibition of the Choice of Nucleophile in the 3′-Processing Reaction. The mechanism of the inhibition of the 3′-processing reaction by tyrphostins was probed by labeling the substrate DNA at the 3′-end. Retroviral integrases can use different nucleophiles in the 3′-processing reaction (Engelman et al., 1991; Vink et al., 1991); use of glycerol, water, or the hydroxyl group of the viral DNA terminus yields a linear trinucleotide with a glycerol esterified to the 5′-phosphate (G), a linear trinucleotide with a 5′-phosphate (L), or a circular trinucleotide (C), respectively. *In vitro* HIV-1 integrase prefers water or glycerol as the nucleophile when a di- or trinucleotide is released (van Gent et al., 1993; Mazumder et al., 1994b). Water is presumably the nucleophile *in vivo*. All of the tyrphostins tested inhibited glycerolysis, hydrolysis, and circular nucleotide formation to the same extent (Figure 5). These data suggest that there is a global block of nucleophilic attack in the 3′-processing reaction whether the nucleophile is derived from solvent (water or glycerol) or from the DNA (deoxyribose hydroxyl). This global inhibition of nucleophilic attack is suggestive of tyrphostins binding at the HIV-1 integrase active site.

Kinetic Mechanism. The mechanism of inhibition was probed by incubating tyrphostin AG1075 with integrase and increasing concentrations of substrate DNA. Inhibition by AG1075 was reversed by higher substrate DNA concentrations, and Lineweaver−Burke analysis suggested a competitive mode of inhibition with respect to the DNA substrate (data not shown).

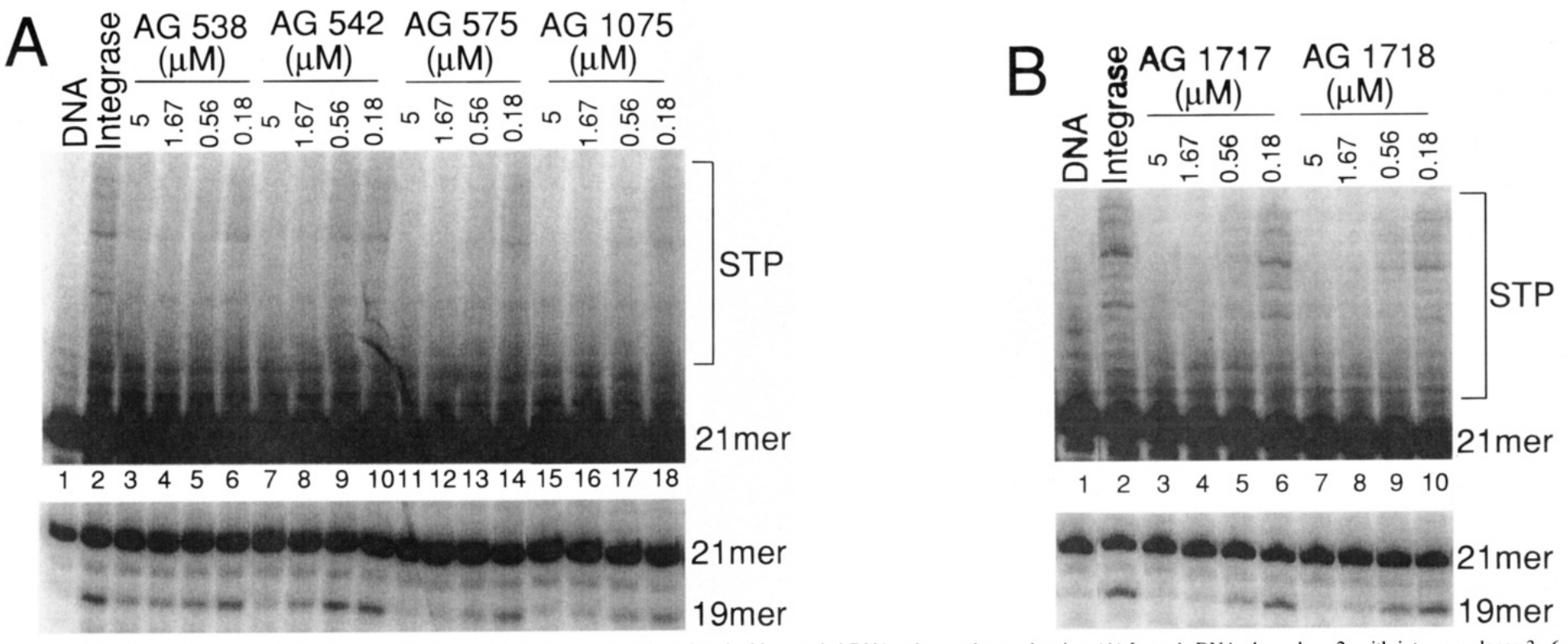


FIGURE 1: Inhibition of HIV-1 integrase-catalyzed 3′-processing and strand transfer using the blunt-ended DNA substrate by tyrphostins. (A) Lane 1, DNA alone; lane 2, with integrase; lanes 3−6, in the presence of the indicated concentrations of AG538; lanes 7−10, in the presence of the indicated concentrations of AG542; lanes 11−14, in the presence of the indicated concentrations of AG575; lanes 15−18, in the presence of the indicated concentrations of AG1075. The lower panel is a lighter exposure to show the 3′-processing product, and the upper panel is a darker exposure to show the strand transfer products. (B) Lane 1, DNA alone; lane 2, with integrase; lanes 3−6, in the presence of the indicated concentrations of AG1717; lanes 7−10, in the presence of the indicated concentrations of AG1718.

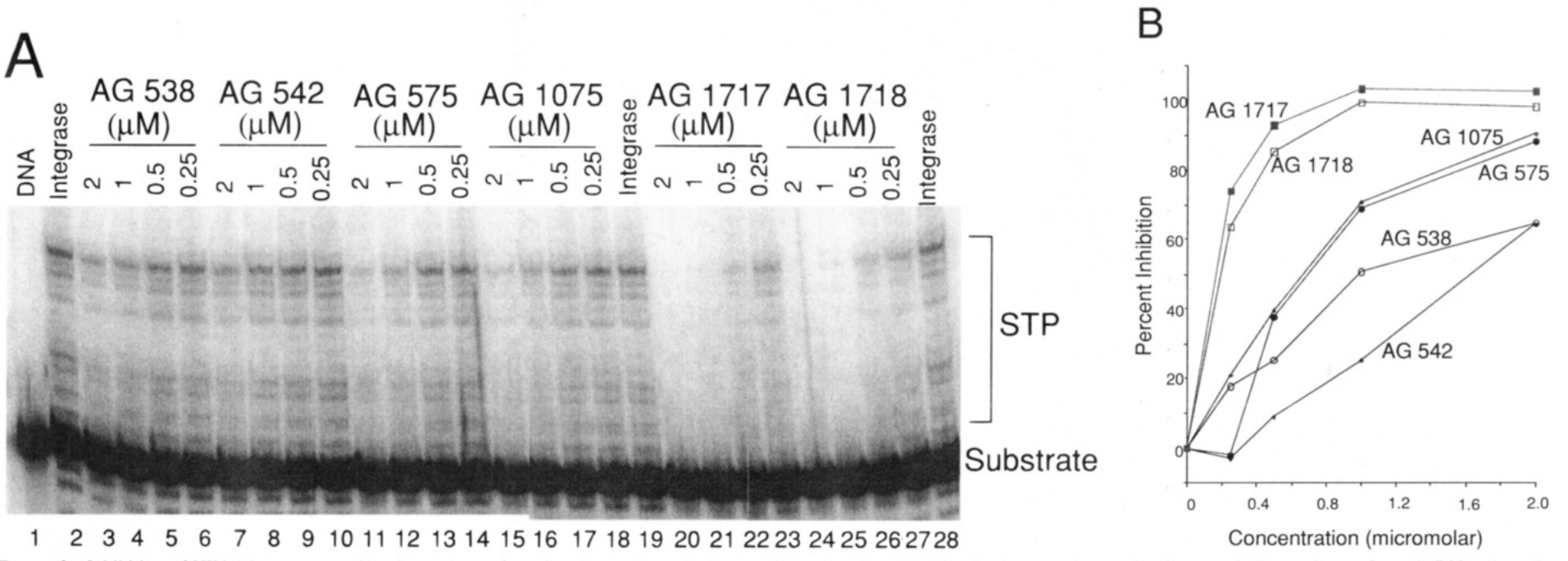


FIGURE 2: Inhibition of HIV-1 integrase-catalyzed strand transfer using the precleaved substrate by tyrphostins. (A) Phosphorimager picture showing a typical experiment. Lane 1, DNA alone; lanes 2, 19, and 28, with integrase; lanes 3−6, in the presence of the indicated concentrations of AG538; lanes 7−10, in the presence of the indicated concentrations of AG542; lanes 11−14, in the presence of the indicated concentrations of AG575; lanes 15−18, in the presence of the indicated concentrations of AG1075; lanes 20−23, in the presence of the indicated concentrations of AG1717; lanes 24−27, in the presence of the indicated concentrations of AG1718. (B) Graph showing the quantitation of the results presented in panel (A). The dose−response curves obtained in the presence of AG1717, -1718, -575, -538, -542, and -1075 are depicted by the filled squares, open squares, filled circles, open circles, filled diamonds, and open diamonds, respectively.

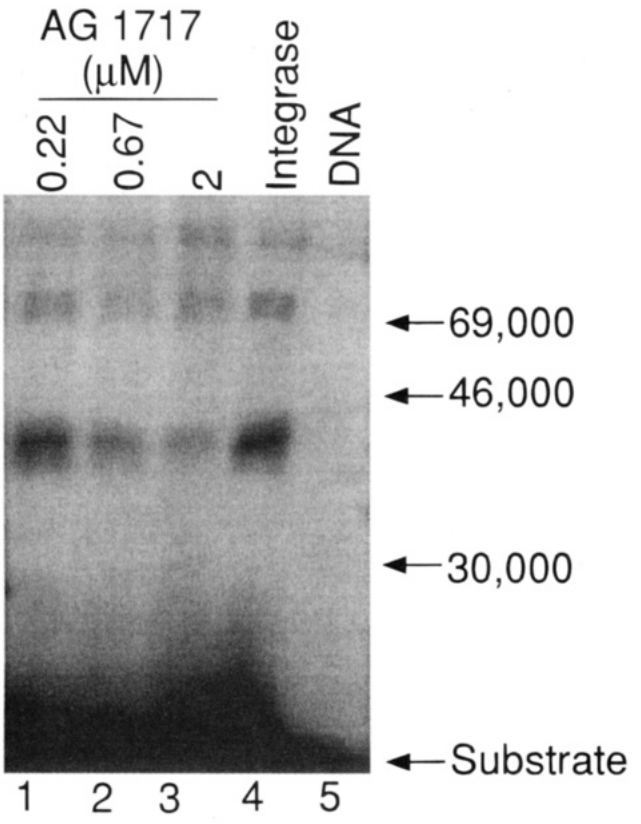


FIGURE 3: Inhibition of the DNA binding activity of HIV-1 integrase in the presence of tyrphostin AG1717. Lane 5, DNA alone; lane 4, with integrase; lanes 1−3, in the presence of the indicated concentrations of AG1717. The migrations of proteins of known molecular weight are shown to the right of the gel.

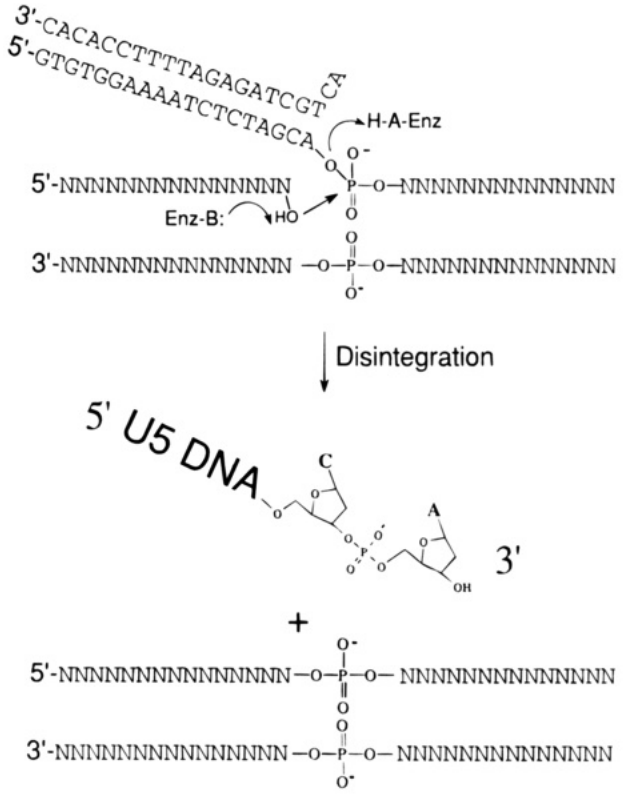
Selectivity of Inhibition by Tyrphostins

In light of the recent finding that some tyrphostins can inhibit topoisomerase I (although only at concentrations approaching 100 μM) (Aflalo et al., 1994a), AG1717 and -1718, the two most potent inhibitors of HIV-1 integrase, and AG775, which exhibited a low level of inhibition (Table 2), were tested with eukaryotic topoisomerase I. An oligonucleotide bearing a unique strong topoisomerase I cleavage site in its center was used. The ability of each of these tyrphostins to either induce cleavable complex formation or inhibit the formation of a cleavable complex induced by camptothecin was assayed. None of the tyrphostins tested induced cleavable complex formation (Figure 6A) or inhibited the ability of topoisomerase to generate a camptothecin-mediated cleavable complex (Figure 6B), even at 100 μM drug concentrations. These results suggest that the tyrphostins tested may not inhibit all DNA-binding enzymes and also do not bind DNA with a high affinity. Furthermore, an inherent selectivity for integrase inhibition may be present just as the selective inhibition of various protein tyrosine kinases (Levitzki & Gazit, 1995) by tyrphostins implies that selectivity can be achieved by modulation of the chemical structure.

Antiretroviral and Cytotoxic Effects of Tyrphostins

The tyrphostins were tested in the National Cancer Institute's Anti-HIV Screen to determine their efficacy in infected CEM cells. Of all the tyrphostins tested, only AG1717 showed moderate activity (Table 2). An EC_{50} value was not attainable with all other tyrphostins possibly due to their cytotoxicity.

Scheme 2: Reaction Mechanism for the Disintegration Reaction Catalyzed by HIV-1 Integrase[a]



[a] The sequence of the host DNA is not important and is designated by the letter N, while the sequence of the U5 viral DNA end is shown.

DISCUSSION

The benzylidene moiety of erbstatin and other arylidene compounds was incorporated into a new class of protein kinase inhibitors called tyrphostins (to indicate tyrosine phosphorylation inhibitors). Like erbstatin, tyrphostins possess the hydroxylated phenyl ring, but unlike erbstatin, the nitrile which is *cis* to the ring rather than the *trans* formylamino group may play a critical role in potency. First generation tyrphostins were found to inhibit the epidermal growth factor receptor (EGFR) *in vitro* between 0.3 and 1 μM, whereas the insulin receptor was inhibited in the millimolar range (Yaish et al., 1988). Recently, tyrphostin dimers, in which two amide derivatives are linked by several methylene groups, were found to inhibit EGFR in the 0.1−1 μM IC_{50} range (A. Gazit, unpublished). Over the years, tyrphostins have been developed which inhibit the various protein kinases involved in a number of diseases such as breast and ovarian cancer, psoriasis, atherosclerosis, gliomas, chronic myelogenous leukemia, and sepsis (Levitzki & Gazit, 1995). Most tyrphostins are 100−10000-fold more potent in inhibiting protein tyrosine kinases than PKA, PKC, or Ca^{2+}/calmodulin-dependent kinases (Levitzki, 1992). This selectivity is derived partly from their initial design as competitors for the protein substrate site and not for the ATP binding site on the kinase (Yaish et al., 1988).

Structure−Activity Relationships in the Inhibition of HIV-1 Integrase by Tyrphostins. This study demonstrated the inhibition of HIV-1 integrase by several tyrphostins. The data suggest that structures having a catechol at each end meet the minimum requirements for activity. For example, all of the compounds in family A (Table 1) contained this minimum requirement and were all active (Table 2). Two compounds (AG538 and AG921) from family B and AG1233

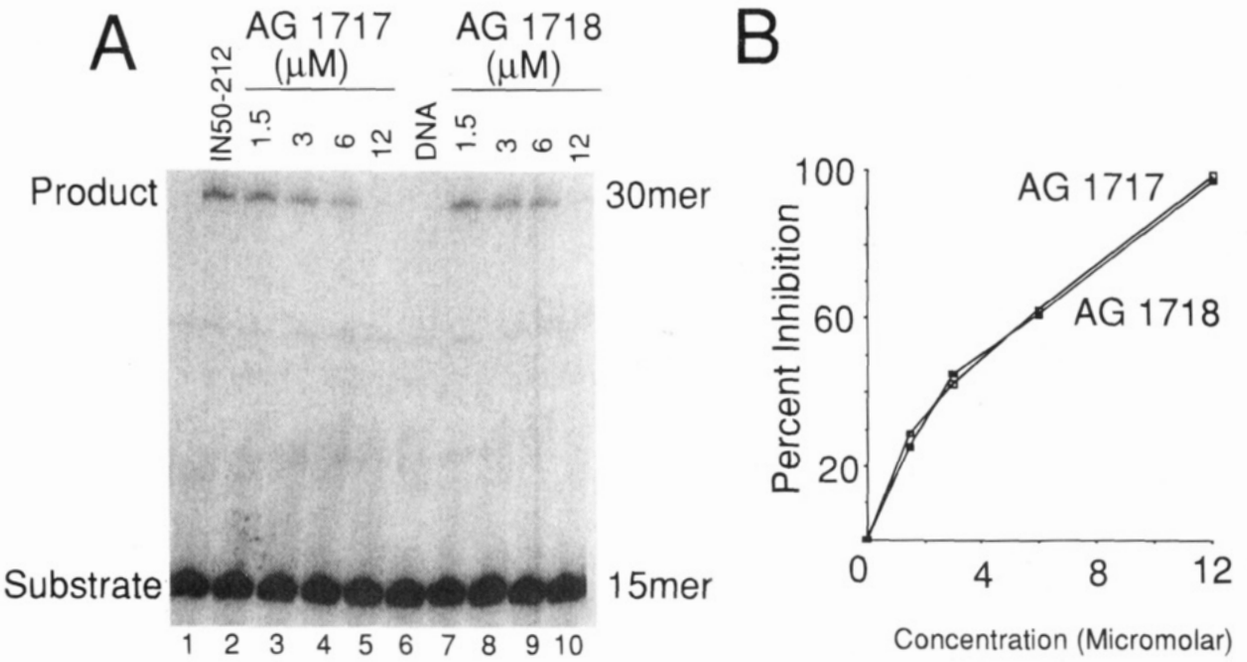


FIGURE 4: Inhibition of disintegration catalyzed by the deletion mutant IN^{50-212} in the presence of tyrphostins. (A) Phosphorimager picture showing a typical experiment. Lane 1, with IN^{50-212}; lanes 2–5, in the presence of the indicated concentrations of AG1717; lane 6, DNA alone; lanes 7–10, in the presence of the indicated concentrations of AG1718. (B) Graph showing the quantitation of the results presented in panel A. The dose–response curves obtained in the presence of AG1717 and -1718 are depicted by the open and filled squares, respectively.

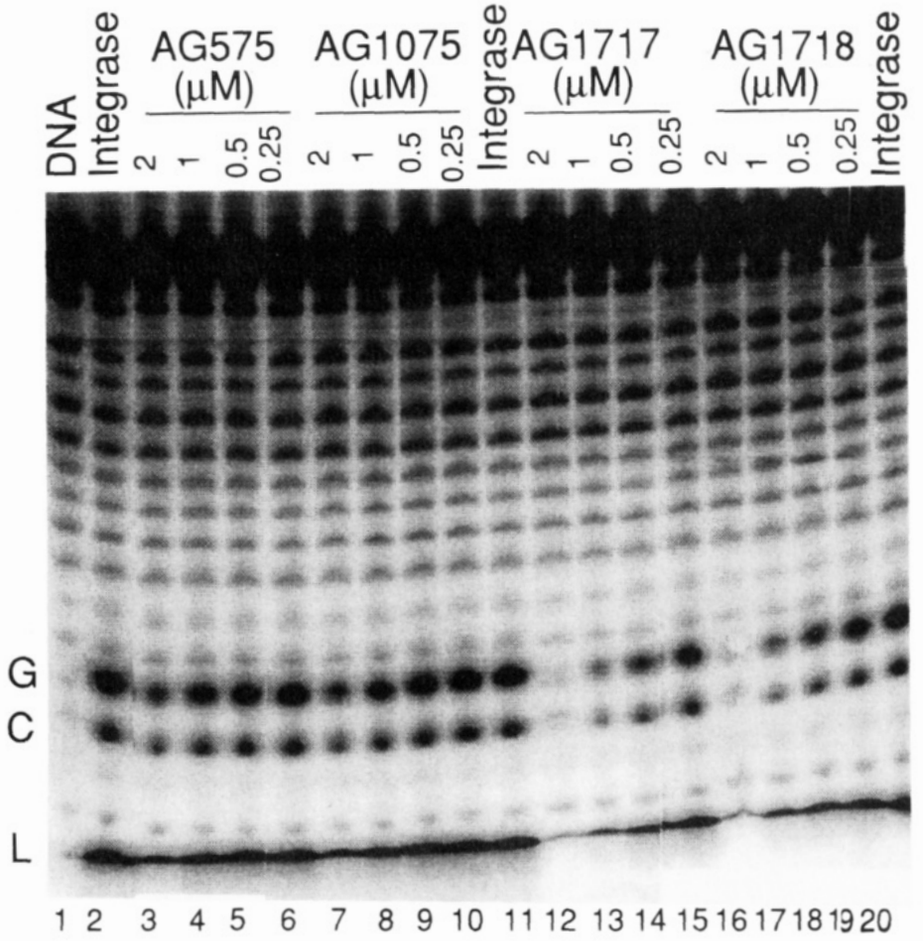


FIGURE 5: Inhibition of HIV-1 integrase-catalyzed 3′-processing using the 3′-end-labeled substrate by tyrphostins. Phosphorimager picture showing a typical experiment. Lane 1, DNA alone; lanes 2, 11, and 20, with integrase; lanes 3–6, in the presence of the indicated concentrations of AG575; lanes 7–10, in the presence of the indicated concentrations of AG1075; lanes 12–15, in the presence of the indicated concentrations of AG1717; lanes 16–19, in the presence of the indicated concentrations of AG1718.

also contained two catechols and were also active (Table 2). In contrast, other members of family B which had a catechol at one end and only the aromatic phenyl at the other end (e.g., AG490, AG555, AG556, and AG1387) were not

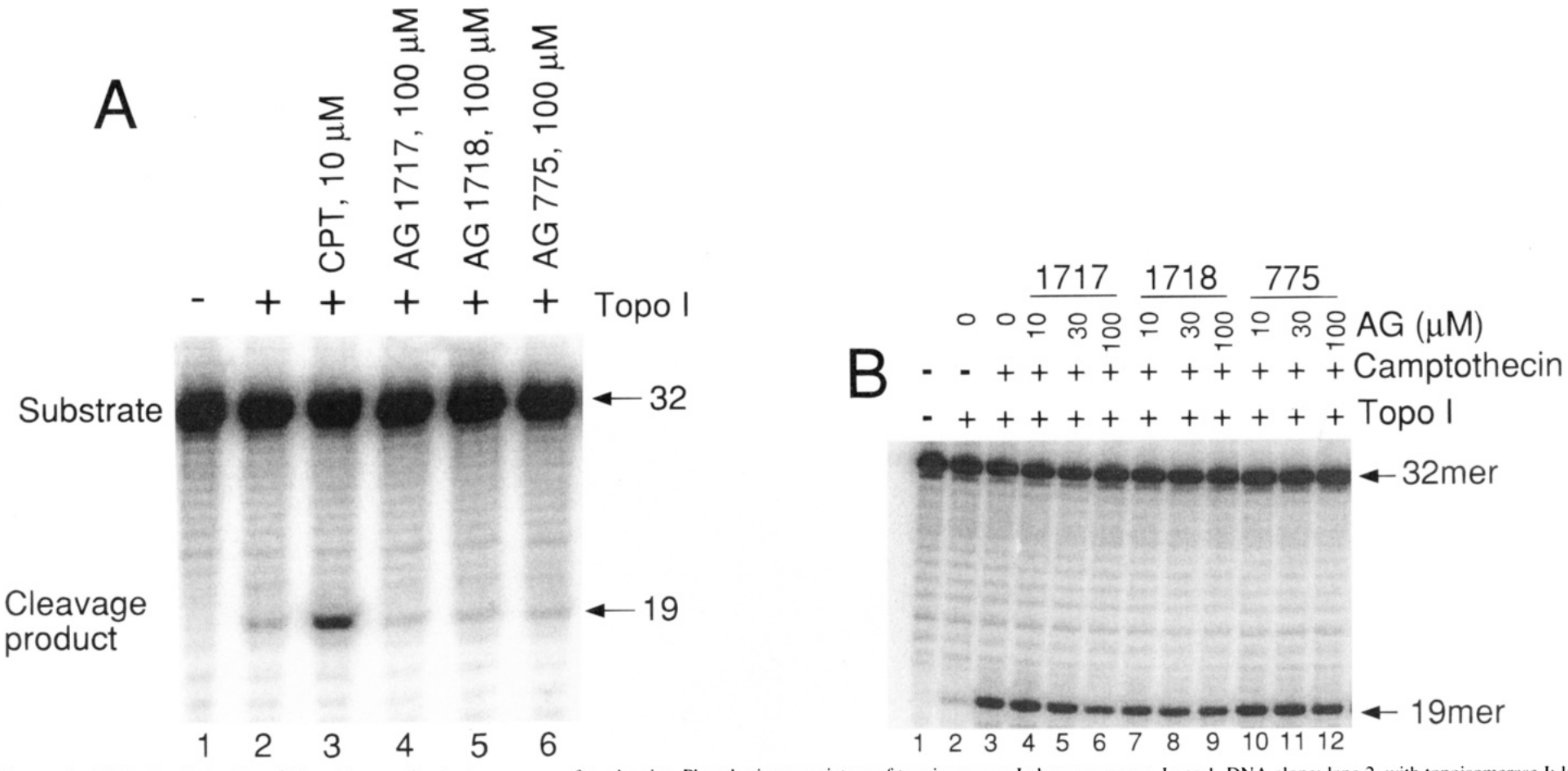


FIGURE 6: (A) Lack of induction of cleavable complex in the presence of tyrphostins. Phosphorimager picture of topoisomerase I cleavage assays. Lane 1, DNA alone; lane 2, with topoisomerase I; lane 3, in the presence of camptothecin; lane 4, in the presence of AG1717; lane 5, in the presence of AG1718; lane 6, in the presence of 10 μM AG775. (B) Lack of inhibition of cleavable complex formation in the presence of tyrphostins. Lane 1, DNA alone; lane 2, with topoisomerase I; lane 3, with topoisomerase I and camptothecin; lanes 4−6, in the presence of the indicated concentrations of AG1717; lanes 7−9, in the presence of the indicated concentrations of AG1718; lanes 10−12, in the presence of the indicated concentrations of AG775.

as active (Table 2). These results are consistent with those obtained using caffeic acid phenethyl ester (CAPE) and analogs (T. Burke, A. Mazumder, and Y. Pommier, manuscript in press). For example, methyl and ethyl cinnamate, which contain only one phenyl ring, were found to be inactive, while those which contained a catechol at one end and only a phenyl ring at the other end were moderately active.

In addition, potency was enhanced by certain changes in the aromatic ring or linker region. For example, the addition of an electron-donating hydroxyl group to the ring substantially altered potency. AG575 and AG550 each have four methylene groups between the amides. However, AG575, which has three hydroxyl groups on each aromatic ring, was about 5 times more potent than AG550 which only has two hydroxyls (Table 2). A similar increase in potency was observed when AG537 and AG1717 or AG638 and AG1718 were compared with each other (Table 2). A prediction of this increase in potency is that the addition of amino groups to the ring may also increase potency. Substitution of the 1,3-bis(aminomethyl)phenyl linker in AG590 by the 1,3-diethylphenyl linker in AG1093 increased potency about 5-fold. Therefore, the amide linkage is not required for activity.

Interestingly, addition of an electron-withdrawing group to the ring did not have as significant an impact as addition of an electron-donating hydroxyl group. AG537 and AG1075 each have three methylene groups between the amides. However, AG1075, which has an additional bromine on each ring, was not significantly more potent than AG537 (Table 2). Similarly, AG537 and AG1292 differ only in the fact that the latter has an additional nitro group on each ring. Only a 2-fold decrease in potency was observed after the addition of this nitro group (Table 2). Another comparison can be made between AG555 and AG1387. These two compounds differ only in the fact that the latter has an iodine on the catechol ring. Neither compound, however, exhibits much activity against integrase (Table 2). These data suggest that various ring substituents may be added with the intent of increasing lipophilicity and intracellular concentration without compromising potency.

Other changes in the linker region also did not have a significant impact on potency. The length of the linker between the amides (i.e., the number of methylene groups) had a minimal, if any, effect on potency. For example, increasing the number of methylene groups in the series AG537 (three), AG550 (four), AG638 (five), AG542 (six), and AG588 (eight) did not have any significant effect on potency (Table 2). Furthermore, the nature of the linker (i.e., methylene groups, aromatic ring, cyclohexyl, or piperazinyl) did not dramatically alter potency. These data suggest that the linker region is not part of the basic pharmacophore.

Could the length or nature of the linker region allow the drug to adopt novel structural conformations? To address this question, molecular modeling was performed on AG1075, AG537, and AG575. All three of these compounds exhibited IC_{50} values for strand transfer below 1 μM (Table 2). Energy minimization showed that both AG1075 and AG537 were able to adopt folded conformations such that the phenyl rings are able to stack with each other (data not shown). However, AG575 adopted a conformation in which the phenyl rings were perpendicular to each other (data not shown). These results imply that, although stacking may play a role in potency, it is not the sole determinant. As mentioned previously, the number of ortho electron-donating groups (in this case, hydroxyls) is also critical in the mechanism of inhibition.

Taken together, these structure−activity data suggest that several features of the tyrphostin structure may be altered in an effort to generate a drug which will be more selective for integrase and more membrane-permeable. For example, the moderate level of anti-HIV activity observed with AG1717 (Table 2) may be improved by altering the structure to make it less cytotoxic (so that higher concentrations may be administered) or to make it more lipophilic (so that a higher intracellular concentration may be reached).

Similarities to Other Known Integrase Inhibitors. We have recently demonstrated that compounds such as flavones, dihydroxynaphthoquinone, lignans, and caffeic acid phenethyl ester derivatives which have ortho hydroxyl groups present on phenyl rings are potent inhibitors of HIV-1 integrase (Fesen et al., 1993, 1994; Eich et al., 1993). In addition, a recent report from another laboratory has also demonstrated that the bis-catechol structure present in, for example, β-connindendrol confers a high level of potency against HIV-1 integrase in both 3′-processing and strand transfer reactions (LaFemina et al., 1995). This structure is also present in lignans, a family of compounds shown to have anti-HIV and anti-integrase activity (Pfeifer et al., 1992). Thus, the basic catechol structure may provide the necessary sites of interaction with integrase and contribute to the binding affinity and the subsequent inhibition.

The results in this study suggest that the binding site of tyrphostins may reside in the same domain of HIV-1 integrase as the binding site for other inhibitors. For example, tyrphostin binding to the catalytic domain is responsible for integrase inhibition, consistent with results obtained previously using the structurally related flavones and curcumin (Fesen et al., 1994; Mazumder et al., 1995a).

How could stacking interactions in AG1075 and AG537 contribute to potency? The analogy of stacked drug structures to the stacking of base pairs in DNA (the substrate for HIV-1 integrase) suggests that such folded conformations adopted by tyrphostins may mimic the structure of the natural substrate more closely, providing a higher binding affinity to the enzyme. The finding that a common structural feature may exist between the DNA substrate and an integrase inhibitor can be supported by examination of other integrase inhibitors. For example, suramin (Carteau et al., 1993c) and aurintricarboxylic acid (Cushman & Sherman, 1992) have previously been demonstrated to be potent HIV-1 integrase inhibitors *in vitro*. These compounds share with DNA a polyanionic nature, possibly allowing them to bind HIV-1 integrase with high affinity due to their mimicking of the negative charge of the DNA phosphodiester backbone.

Relevance to the Enzymatic Reaction Mechanism. Assuming that stacking interactions (see above) and hydrogen bonding (as inferred from the potency associated with multiple, ortho hydroxyl groups on the phenyl ring) allow tyrphostins to bind with high affinity to the catalytic domain (as suggested by the inhibition of an integrase deletion mutant), how does the subsequent inhibition of catalytic activity occur?

Site-directed mutagenesis and sequence alignment have identified three amino acid residues in the catalytic core which are conserved among all retroviral integrases (Kulkosky et al., 1992) and are critical for activity (van Gent et al., 1992; Engelman & Craigie, 1992). These are Asp64,

Asp116, and Glu152. Such acidic residues are known to be essential for the 3′−5′ exonuclease activity of the Klenow fragment of DNA polymerase I due to their coordination to metal ions and the phosphodiester backbone at the active site (Beese & Steitz, 1991).

Previous work has hypothesized possible coordination of the hydroxyl groups of the catechol structure to the acidic amino acid side chains of integrase (Fesen et al., 1994; Mazumder et al., 1995a). This hypothesis is consistent with the results from this study. For example, coordination of the hydroxyl groups of AG1075 either to the metal ion or to the carboxylate groups present in the integrase active site could inhibit binding of the DNA phosphodiester backbone such that an appropriately positioned transition state could not be generated. This hypothesis awaits further *in vitro* testing and analysis via cocrystal structures of integrase bound to drug candidates.

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