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Mode of Interaction of G-Quartets with the Integrase of Human Immunodeficiency Virus Type 1

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SUMMARY

Oligonucleotides that can form a highly stable intramolecular four-stranded DNA structure containing two stacked guanosine-quartets (G-quartets) have been reported to inhibit the replication of the human immunodeficiency virus type 1 (HIV-1) in cell culture. Two possible mechanisms for the observed antiviral activity have been proposed: interference with virus adsorption to the cell and/or inhibition of HIV-1 integrase. We investigated the molecular interaction of G-quartet-containing oligonucleotides with HIV-1 integrase in comparison with random oligonucleotides and dextran sulfate. The prototypical G-quartet-containing oligonucleotide, T30177 (Zintevir), inhibited the overall integration reaction with an IC_{50} value of 80 nM. A random oligonucleotide was 10-fold less potent, but dextran sulfate was more potent, with an IC_{50} value of 7 nM. We developed novel kinetic assays to dissect the overall integration reaction in three steps: the formation of the initial stable complex (ISC), the 3′-processing reaction, and the DNA strand-transfer step. We then analyzed the kinetics of the ISC formation and 3′-processing. The rate constant determined for the conversion of ISC into the cleaved product was 0.08 ± 0.01 min^{-1}. T30177 did not inhibit 3′-processing or DNA strand transfer, whereas dextran sulfate inhibited DNA strand transfer to some extent. Binding studies using surface plasmon resonance technology revealed that both T30177 and dextran sulfate were capable of preventing the binding of integrase to specific DNA. We propose a model in which the interaction of HIV-1 integrase with G-quartets results in the inhibition of the formation of the ISC between integrase and substrate DNA. Finally, we selected for an HIV-1 strain that was resistant to T30177 in cell culture. DNA sequence analysis revealed mutations in the envelope glycoprotein gp120 but not in the integrase gene. Although gp120 seems to be the main target for the antiviral activity in cell culture of G-quartets, the study of their specific inhibition of HIV-1 integrase may lead to the development of effective integrase inhibitors.

For the treatment of infection with HIV-1, a number of inhibitors of both the reverse transcriptase and HIV protease have been formally approved (1). Targeting the third viral enzyme IN with effective inhibitors remains an elusive goal. The IN enzyme inserts the viral cDNA copy into the host cell chromosome; this step is essential for the replication of the virus (2, 3). Because no human counterpart of the enzyme is known, there is considerable interest in developing effective and selective inhibitors of the HIV integration process. The recent establishment of high-throughput microtiter plate assays (4, 5), on the one hand, and the elucidation of the three-dimensional structure of the catalytic domain of HIV-1 IN, on the other hand (6), will likely boost the antiviral screening of chemical libraries as well as the structure-based design of enzyme inhibitors.

The only enzyme required for HIV-1 integration is IN, a protein of 32 kDa encoded at the 3′-end of the *pol* gene (for a review, see Ref. 7). The enzyme is produced by protease-mediated cleavage of the gag-pol precursor during virion maturation. Integrase recognizes specific sequences in the LTR elements of the viral cDNA. The terminal 15 bp of the LTR are necessary and sufficient for site-specific cleavage and integration. A highly conserved dinucleotide CA repeat immediately upstream of the cleavage site is critical for enzymatic activity. In the first step of the integration reaction, termed 3′-end processing, two nucleotides are removed from each 3′-end to produce new 3′-hydroxyl ends (CA-3′OH). This reaction occurs in the cytoplasm, within a large viral nucle-

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ABBREVIATIONS: HIV-1, human immunodeficiency virus type 1; IN, integrase; ss, single-stranded; ds, double-stranded; G-quartet, guanosine-quartet; PCR, polymerase chain reaction; LTR, long terminal repeat; ISC, initial stable complex; PSC, processed stable complex; DS, dextran sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

oprotein complex. After entering the nucleus, the processed viral dsDNA is joined to host target DNA. The joining reaction includes a coupled 4–6-bp staggered cleavage of the target host DNA and the ligation of processed CA-3′OH viral DNA ends to the 5′ phosphate ends of the target DNA. Repair of the remaining gaps, although not understood at this time, is probably accomplished by host-cell DNA repair enzymes. Oligonucleotide-based assays have been designed to mimic both processing and joining reactions *in vitro* (8).

HIV IN is composed of three functional domains (7). The amino-terminal region (residues 1–50) is characterized by an HHCC zinc finger-like sequence whose exact function remains unknown. The central region (residues 60–160) is characterized by three highly conserved amino acid residues D, D (35)E and encodes the catalytic domain for both 3′-processing and DNA strand-transfer activities. The central core domain alone can carry out an apparent reversal of the DNA strand-transfer reaction *in vitro*, the so-called disintegration reaction (9); however, both the amino- and carboxyl-terminal domains are necessary for catalysis of 3′-processing and strand transfer. A single amino acid substitution (F185K) within the catalytic core domain generates a soluble protein that has enabled the core domain to be crystallized and the structure to be solved (6). The carboxyl-terminal domain (200–270) is involved in nonspecific DNA binding. NMR structures of a truncated carboxyl-terminal domain have been determined (10, 11). Complementation studies with IN proteins mutated in the different domains suggest that the active form of IN is an oligomer, although the exact stoichiometry remains unknown (12, 13).

Different approaches to interfering with HIV-1 integration have been reported: (i) triple helix-mediated inhibition, (ii) inhibition by peptides derived from combinatorial peptide libraries (14), (iii) screening of chemical libraries and natural compounds (15), and (iv) inhibition by G-quartet-forming oligonucleotides (16). The IN-binding site located in the U3 LTR contains a purine motif, 5′-GGAAGGG-3′, that can be selectively targeted by oligonucleotide/intercalator conjugates (oligopurines-oxazolopyridocarbazole) (17). Under neutral pH and at physiological temperature, these conjugates readily form a stable complex with the viral DNA that involves a short DNA triplex. It has been shown that triple-helix formation at this site can prevent the catalytic functions of IN *in vitro.* This elegant approach is complicated by the issue of intracellular delivery of the conjugates and may be jeopardized inherently by the high mutation rate of HIV that may result in base substitutions in the LTRs. Screening of synthetic peptide combinatorial libraries in an oligonucleotide-based microtiter plate assay has been used recently to identify the hexapeptide HCKFWW as an inhibitor of IN activity with an IC_{50} value of 2 μM (14). A number of inhibitors that show activity in this sort of oligonucleotide-based *in vitro* assays have been reported; they belong to three main categories: DNA-binding agents, polyhydroxylated aromatic compounds, and nucleotides. Many DNA-binding agents were found to inhibit HIV-1 IN, probably due to a nonspecific interaction with the DNA-binding domain of the enzyme. Intercalators and DNA groove binding compounds belong to this category (18). Polyhydroxylated aromatic compounds are believed to interact with the catalytic domain, possibly by interfering with the coordination of the metal ions, required for the phosphoryl transfer reactions (15). From structure-activity relationships with flavones, lignans, and caffeic acid phenethylester derivatives, the ortho hydroxyl configuration (catechol) seems to be required for inhibitory activity. However, the antiviral potency of catechol-type inhibitors in cell culture is difficult to estimate due to cellular toxicity of the compounds. A possible exception may be formed by the recently reported dicaffeoyl derivatives, which inhibit IN and HIV-1 replication in cell culture (19). Nucleotides such as 3′-azido-2′,3′-dideoxy-TMP interfere with the enzymatic activity of IN, possibly by binding to the polynucleotide binding site (20). Structure-activity studies of various nucleotide analogues have been reported recently (21). Although the concentration required for inhibition is high (IC_{50} = 150 μM), it is not excluded that IN inhibition may contribute to the antiviral effect of AZT because concentrations of $\leq$1 mM 3′-azido-2′,3′-dideoxy-TMP can accumulate *in vivo* (22).

Recently, two classes of oligonucleotides composed of deoxyguanosine and thymidine were reported to inhibit HIV-1 replication in cell culture (16, 23). The first class of oligonucleotides consists of 16- or 17-mers that contain single phosphorothioate internucleoside linkages at their 5′- and 3′-ends. In the presence of potassium, these oligonucleotides can fold upon themselves to form a highly stable four-stranded DNA structure containing two stacked G-quartets (24, 25). Two possible mechanisms for the observed antiviral activity have been proposed: inhibition of viral entry into the cell and/or inhibition of HIV-1 IN (16). The phosphorothioate oligonucleotide TTGGGGTT, which adopts a parallel-stranded tetrameric G-quartet structure (ISIS 5320), is also a potent inhibitor of HIV replication in cell culture. This compound exerts its antiviral effect through potent and specific inhibition of HIV envelope-mediated virus binding and cell fusion (26).

To gain further insight into the mechanism of action of the G-quartet-containing oligonucleotides at the HIV-1 IN level, we investigated the interaction of HIV-1 IN with the intramolecular G-quartet-forming oligonucleotides and compared their activity with that of aspecific oligonucleotides, the intermolecular G-quartet-forming ISIS 5320 compound, and the polyanionic compound DS (DS5000). Because few efficient IN inhibitors have been developed, we used the G-quartets as tools to explore potential mechanisms of IN inhibition. We developed some novel kinetic assays to dissect the IN reaction. In parallel, we initiated studies to elucidate the mechanism by which G-quartets interfere with HIV-1 replication in cell culture. We selected for an HIV-1 strain that is resistant to the G-quartet T30177 (Zintevir) and sequenced both the IN and the envelope glycoprotein genes.

Experimental Procedures

Enzyme. The plasmid pRP1012 encoding HIV-1 IN under the control of the T7 promoter was a generous gift from Dr. R. H. A. Plasterk (Netherlands Cancer Institute, Amsterdam, The Netherlands). The plasmid also encodes for six histidine residues (a so-called His-tag) at the amino terminus of the enzyme to facilitate purification on a nickel-chelating column. The protein was expressed in the *Escherichia coli* BL21(DE3) strain. Cells were grown in 1.5 l of LB medium to an absorbance measured at 600 nm of 0.85, when isopropylthiogalactopyranoside was added to a final concentration of 0.4 mM and cells were grown for an additional 3 hr. Bacteria were

resuspended in cold 10 mM Tris·HCl, pH 7.6, 1.1 M NaCl ,1 mM β-mercaptoethanol, and 0.05 mM EDTA and lysed by passage through an X-press five times. The resulting suspension was subjected to a Dounce homogenizer and centrifuged at 30,000 × *g* for 25 min. The supernatant containing IN was loaded onto 4 ml of Ni-NTA (nitrilotriacetic acid)-chelating resin (Qiagen, Hilden, Germany) in the presence of 30 mM imidazole. The column was washed extensively with buffer A (10 mM Tris·HCl, pH 7.6, 1 mM β-mercaptoethanol, 1 M NaCl, 0.05 mM EDTA, 10% glycerol, 10 mM 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate) containing 70 mM imidazole, and the protein was eluted in buffer A with a gradient of imidazole of 70–150 mM. Fractions were analyzed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and IN was detected by silver staining or immunoblotting with monoclonal antibodies to HIV-1 IN (Intracell, Cambridge, MA). Fractions containing IN were pooled, diluted 3-fold with buffer A, and loaded onto a 5-ml HiTrap heparin column (Pharmacia Biotech, Uppsala, Sweden). The bound protein was eluted with a linear gradient of NaCl of 300 mM to 1 M in buffer A. IN eluted from the column at a salt concentration of ~0.59 M. All fractions containing IN were pooled, concentrated by ultrafiltration using Centricon-10 concentrators (Amicon, Beverly, MA) to 1 mg/ml, and stored at −80°. The purity of the enzyme as assayed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and silver staining was 85–90%.

Substrate and target DNA. The following high performance liquid chromatography-purified deoxyoligonucleotides were purchased from Pharmacia Biotech: INT1, 5′-TGTGGAAAATCTCTAGCAGT; INT2, 5′-ACTGCTAGAGATTTTCCACA; T35, 5′-ACTATACCAGACAATAATTGTCTGGCCTGTACCGT; and SK70, 5′-ACGGTACAGGCCAGACAATTATTGTCTGGTATAGT.

The oligonucleotides INT1 and INT2 correspond to the U5 end of the HIV-1 LTR. The oligonucleotide INT1 was purified by electrophoresis through a 20% polyacrylamide denaturing gel and was 5′-end labeled using polynucleotide T4 kinase and [γ-^{32}P]ATP. Unincorporated ATP was removed by gel filtration. The DNA substrate for both the 3′-processing and strand-transfer reactions was made by annealing the oligonucleotides INT1 and INT2. An equimolar mixture of the two oligonucleotides in the presence of 100 mM NaCl was heated shortly at 95° and allowed to cool slowly to room temperature. Likewise, annealing of SK70 and T35 resulted in a 35-bp dsDNA molecule that was used as a target DNA molecule (T35/SK70). Efficiency of annealing was 95% as determined by native polyacrylamide gel electrophoresis.

Inhibitors. The oligonucleotides T30177, T30677, and T30695 were provided by Dr. R. Rando (Aronex Pharmaceuticals, The Woodlands, TX). These are 17-mers (or a 16-mer in the case of T30695) composed of deoxyguanosine and thymidine that when incubated in the presence of potassium fold into two G-quartets (Fig. 1). Synthesis and purification were described previously (24). The three oligonucleotides contain single phosphorothioate internucleoside linkages at their 5′- and 3′-ends for stability. The control oligonucleotides (from Pharmacia) used were the ss 17-mer KS (5′-TCGAGGTCGACGGTATC) and a ds 20-mer T3/T3rev (5′-AATTAACCCTCACTAAAGGG/5′-CCCTTTAGTGAGGGTTAATT). The ISIS 5320 compound, a phosphorothioate oligonucleotide with the sequence TTGGGGTT (23), was synthesized by Pharmacia. Tetrameric ISIS 5320 was prepared through annealing of a 1 mM solution of compound at 4° for 1 week in 25 mM Tris·HCl, pH 7.8, 100 mM KCl, and 50 mM NaCl. A solution of denatured compound was made by heating a 1 mM solution of ISIS 5320 in 25 mM Tris·HCl, pH 7.8, at 95° for 5 min immediately before the addition to the reaction mixture. DS5000 was obtained from Sigma Chemical (St. Louis, MO).

The 3′-processing and DNA strand-transfer assays. The final reaction mixture for the 3′-processing assay contained 20 mM HEPES, pH 7.5, 5 mM dithiothreitol, 10 mM $MgCl_2$, 75 mM NaCl, 15% (v/v) dimethylsulfoxide, 5% (v/v) polyethylene glycol 8000, 0.3 pmol (30 nM) of the oligonucleotide substrate, and 100 ng (330 nM) of the His-tag IN in a total volume of 10 μl. Reactions were started by the addition of the enzyme. Inhibitors were incubated shortly with the reaction components before the addition of IN. Reactions were allowed to proceed at 37° for 7–40 min and stopped by the addition of formamide dye.

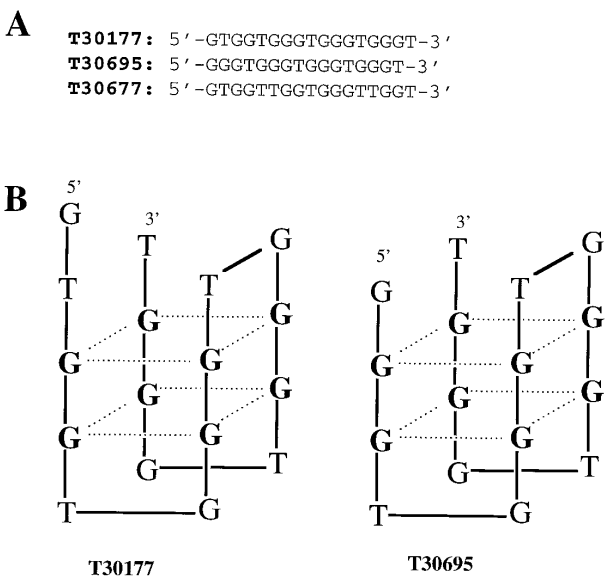

Fig. 1. Sequence and structure of G-quartet-forming oligonucleotides. A, Sequences of T30177, T30695, and T30677. These oligonucleotides contain single phosphorothioate internucleoside linkage at the 3′- and 5′-ends. B and C, Proposed folding pattern (in greater detail) for a T30177 monomer with the formation of two G-quartets. The guanine bases are indicated.

The strand-transfer activity was assayed in the following way: 20 nM DNA substrate was preincubated with 330 nM IN at 37° for 3 min to allow the cleavage reaction to occur. The composition of the reaction mixture was identical to that in the cleavage assay. After 3 min, 1 μl of excess target DNA (final concentration, 133 nM) with or without inhibitor was added, and the samples were incubated at 37° for 1 hr. This excess target DNA blocks competitively further binding of IN to the viral DNA substrate.

Reactions were stopped by the addition of formamide dye, and products were separated in a 17% polyacrylamide/urea gel. Autoradiography was performed by exposing the wet gel to X-ray film (CURIX RP1; Agfa). Quantification of the results was performed using the PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

HIV-1 IN-binding experiments. Binding experiments were carried out using biosensor technology on BIAcore2000 (Pharmacia Biotech) and sensor chips SA with preimmobilized streptavidin. The binding experiment was carried out at 37° according to the manufacturer's instructions. Binding buffer B (20 mM HEPES, pH 7.5) contained 50 mM NaCl, 10 mM $MgCl_2$, and 5 mM dithiothreitol. First, the 3′-biotinylated oligonucleotide 5′-ACTGCTAGAGATTTTCCACACTGACTAAAAGGGTCAAAA-3′ was bound to the sensor chip. Subsequently, the complementary 35-mer 5′-GACCCTTTTAGTCAGTGTGGAAAATCTCTAGCAGT-3′ was hybridized to the captured oligonucleotide, resulting in an IN recognition sequence at the free end. Then, 100 μl of HIV-1 IN (33 μM) diluted 10-fold in dilution buffer (10 mM Tris·HCl, pH 7.5, 750 mM NaCl, 10% glycerol, 1 mM β-mercaptoethanol) and a further 10-fold in buffer B was injected at a final concentration of 330 nM with a flow rate of 2 μl/min and passed both a blank channel and the channel with the specific oligonucleotide. Aspecific adsorption in the blank channel was negligible. Identical experiments were done in the presence of 1 μM concentration of the inhibitors T30177 and DS5000.

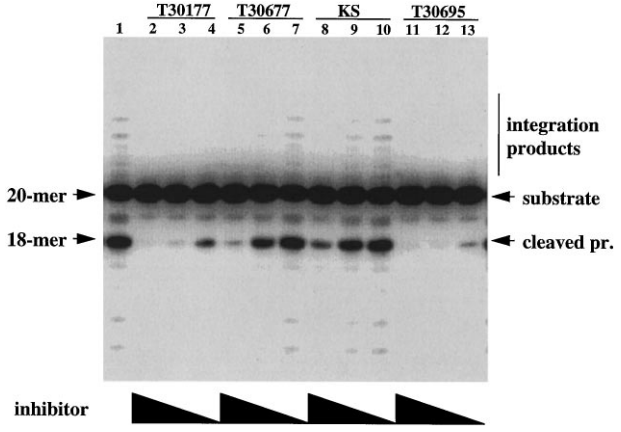

Fig. 2. Inhibition of HIV-1 IN activity by oligonucleotides. Integrase reactions were performed for 40 min in the absence (*lane 1*) or presence of the inhibitors T30177 (*lanes 2–4*), T30677 (*lanes 5–7*), the 17-mer KS (*lanes 8–10*), or T30695 (*lanes 11–13*). The concentrations of the inhibitors were 1000 nM (*lanes 2, 5, 8, and 11*), 333 nM (*lanes 3, 6, 9, and 12*), and 111 nM (*lanes 4, 7, 10, and 13*).

PNL43 HIV-1 isolates and PCR amplification of the IN gene. DNA from the wild-type and T30177-resistant PNL43 HIV-1 virus was provided by Esté *et al.*[1] Full-length IN gene was amplified by PCR using Ampli-*Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT) and the primers HP4149, 5′-CATGGGTACCAGCACACAAAGG, and INPCRC, 5′-CCCAAATGCCAGTCTCTTTCTCCTG.

Sequencing analysis. The sequence of both strands of the 1136 bp PCR product was determined using the Dye-Terminator Sequencing Kit and ABI Automatic Sequenator (Perkin-Elmer). The primers for sequencing were INPCRA, 5′-GGAGGAAATGAACAAGTAGAT; INSEQ3, 5′-GGATATATAGAAGCAGAAGTAA; INSEQ4, 5′-GAACATCTTAAGACAGCAGTA; INSEQ5, 5′-AAGCTCCTC TGGAAAGGTGAA; INPCRB, 5′-CCTTGAAATATACATATGGTG; A2, 5′-TA CTGCCCCTTCACCTTTCCAG; INSEQ1, 5′-TTAAGATGTTCAGCCTGATCT; and INSEQ2, 5′-CATTGTCTGTATGTACTGTTT.

Results

Inhibition of HIV-1 IN activity *in vitro* by G-quartet-forming oligonucleotides. Recently, oligonucleotides composed of deoxyguanosine and thymidine were reported to inhibit HIV-1 replication in cell culture (16). In the presence of potassium, these oligonucleotides fold upon themselves to form a highly stable intramolecular four-stranded DNA structure containing two stacked G-quartets (Fig. 1) (24, 25). The compounds were reported to inhibit HIV-1 integration in an *in vitro* HIV-1 IN system (16).

There are two distinct catalytic steps during retroviral integration: the first is processing of the viral dsDNA ends, in which two nucleotides are removed from the 3′-ends, and the second is integration of the processed viral DNA ends into target DNA. If the products of the reaction are separated in a denaturing acrylamide gel, the first step of the reaction gives rise to a clearly visible band two bases shorter than the substrate, whereas DNA strand transfer produces a DNA ladder of variable length. The effect of the addition of various concentrations of the prototypical G-quartet-forming oligonucleotide T30177 to the classic IN reaction is shown in Fig. 2. The addition of T30177 results in a concentration-dependent inhibition of the 3′-processing reaction and the formation of strand-transfer products.

Oligonucleotide and polyanionic inhibitors. Next, we compared the inhibition of HIV-1 integration by G-quartet-forming oligonucleotides with the effect of the addition of other polyanionic compounds to the reaction mixture. In Fig. 2, the inhibitory effect on the IN activity is shown for three G-quartet-forming oligonucleotides (T30177, T30677, and T30695). An oligonucleotide of random sequence but with the same length as T30177 (17-mer) was included as a control (KS). The compound T30695 was more active than T30177, whereas both T30677 and KS were much less inhibitory. When the reaction is allowed to proceed for only a short period of time (e.g., 7 min), the amounts of integration products are negligible. Under these conditions, it is possible to measure specifically the 3′-processing activity of the enzyme and evaluate the potency of IN inhibitors on this reaction step. The IC_{50} values for inhibition of 3′-processing were determined for the three G-quartet-forming oligonucleotides T30177, T30695, and T30677; ssDNA KS; dsDNA oligonucleotide T3/T3-rev (20-mer); polyanionic compound DS5000; and ISIS 5320 compound (Table 1).

T30177 inhibits the formation of the cleaved product with an IC_{50} value of 80 nM; T30695 is somewhat more active, whereas T30677 is ~7-fold less active. The ss KS is 10-fold less active. On the other hand, both DS5000 and the ds 20-mer T3/T3-rev are highly potent inhibitors of 3′-processing, with IC_{50} values of 7 and 60 nM, respectively. The ISIS 5320 compound inhibits the 3′-processing reaction with an IC_{50} value of 150 nM. After heat-denaturation, T30177 retains its inhibitory potency, whereas the IC_{50} value for ISIS 5320 increases 20-fold (data not shown). To verify whether T30177 is merely competing with the specific DNA substrate for binding to IN, we repeated the reaction with a 6-fold-higher concentration of substrate. The inhibition by T30177 was not affected by the increase in substrate concentration, whereas the dsDNA 20-mer was 2-fold less active. Increasing

TABLE 1
Inhibition of integrase activities by different compounds

Inhibitor	S[b]	IN[b]	IC_{50}[a]	
			3′-processing	Strand-transfer
	nM		nM	
T30177	20	330	80 ± 13	> 3000
	120	330	85 ± 15	
	20	660	250 ± 50	
T30695	20	330	65 ± 10	> 3000
T30677	20	330	550 ± 90	> 3000
ss 17-mer KS	20	330	850 ± 150	
ds 20-mer T3/T3-rev	20	330	60 ± 6	
	120	330	120 ± 15	
DS5000[c]	20	330	7 ± 2	50 ± 10
	120	330	7 ± 2	
	20	660	30 ± 5	

[a] Concentration that inhibits HIV-1 integrase reaction by 50%. Results presented are mean values ± standard deviation for three or four separate experiments.
[b] Concentration of substrate (S) or integrase (IN) in the reaction.
[c] The average molecular weight of DS5000 is approximately 10,000 g/mol.

[1] J. A. Esté, C. Cabrera, D. Schols, P. Cherepanov, M. Witvrouw, C. Pannecouque, Z. Debyser, R. F. Rando, B. Clotet, J. Desmyter, E. De Clercq. Human immunodeficiency virus glycoprotein GPI2O as the primary target for the antiviral action of AR177 (Zintevir). Manuscript in preparation.

the enzyme concentration 2-fold, on the other hand, resulted in a 2–3-fold increase in the IC_{50} value for T30177. These results suggest a direct interaction of T30177 with the enzyme.

Kinetics of the formation of the ISC. Theoretically, IN inhibitors may be directed against the 3′-processing or the DNA strand-transfer catalytic activities of the enzyme; however, the first step in the course of retroviral integration is the interaction of HIV-1 IN with viral cDNA. In integration-competent nucleoprotein complexes isolated from retrovirus-infected cells, IN maintains a very stable interaction with processed viral DNA (27). Likewise, the initial complex of purified recombinant IN with a viral DNA end sequence was shown to be highly stable (28). After 3′-cleavage of the viral DNA, the PSC seems to be resistant to the addition of EDTA, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate, 200 mM NaCl, or excess competitor DNA (29). Knowledge of the kinetics of the initial interaction between HIV IN and the DNA substrate might be crucial for understanding of the mechanism by which G-quartet-forming oligonucleotides interfere with the enzymatic activity of HIV IN. Therefore, we investigated the kinetics of ISC formation by using a modified integration assay similar to the pulse chase assay described by Ellison and Brown (29).

IN was incubated with the specific oligonucleotide substrate for different periods of time (from 30 sec to 25 min), after which an excess of DS was added. As a polyanion, DS prevents the formation of new DNA/IN complexes by trapping free IN. Indeed, when present before the addition of IN, an identical concentration of DS inhibits the reaction completely (Fig. 3B, *lane M*). Immediately after the addition of DS, half of the reaction mixture was denatured with formamide loading buffer, whereas the second half was incubated for an additional 30 min to allow the cleavage reaction to proceed in the preformed ISCs (Fig. 3B). The amounts of the ISC were determined by subtracting the amount of the processed DNA product in both samples. The time course of the accumulation of ISC is linear during the first 3 min of the reaction (Fig. 3A).

Kinetics of the cleavage reaction. To measure the cleavage reaction directly, we repeated the same pulse chase experiment but used several time points after the addition of DS; the cleavage process is very slow and takes minutes to proceed (Fig. 4, A and B). We determined the rate constant for the conversion of ISC into the cleaved product to be 0.08 ± 0.01 min^{-1}. This is in good agreement with the reported k_{cat} values for HIV-1 (0.069 min^{-1}) and RSV (0.18 min^{-1}) INs (30, 31). However, a much lower value for the processing rate (0.24 hr^{-1}) was measured for HIV-1 IN using fluorescence resonance energy transfer (32). To examine the inhibitory effect of G-quartets on 3′-processing, we repeated the same experiment in the presence of T30177. No inhibition of 3′-processing was observed (Fig. 4A). Increasing the concentration of the challenger DS5000 by 5-fold also did not inhibit cleavage. Although the absolute amounts of cleaved product were slightly different, the k_{cat} values were very similar for the three conditions. The measured k_{cat} value was independent of IN, substrate, or ISC concentration in the reaction mixture.

Inhibition of DNA strand transfer. To test the effect of the various inhibitors on the DNA strand-transfer activity of HIV-1 IN, we set up the following experiment; 20 nM DNA substrate and 330 nM IN were preincubated for a brief period of time to allow the cleavage reaction to occur. Then, an excess of nonspecific target DNA was added with or without inhibitor. The excess of target DNA will trap free IN and prevent the formation of new processing complexes. Thus, under these conditions, the enzymatic activity will be restricted to the strand-transfer reaction, although some processing in the preformed complexes may still occur. Fig. 5A represents results of the strand-transfer assay performed in the presence of T30177, T30695, and DS5000. The IC_{50} values are given in Table 1. The final concentration of target DNA (T35/SK70) was 133 nM, whereas the inhibitors were used at a concentration of 677 nM. Only marginal inhibition of the DNA strand-transfer activity was observed at micromolar concentrations of T30177. In contrast, DS5000 inhibited DNA strand transfer with an IC_{50} value only 7-fold higher than in the processing reaction. In a modification of the experiment, a preprocessed DNA substrate was used to exclude all processing activity. Again, no inhibition of DNA

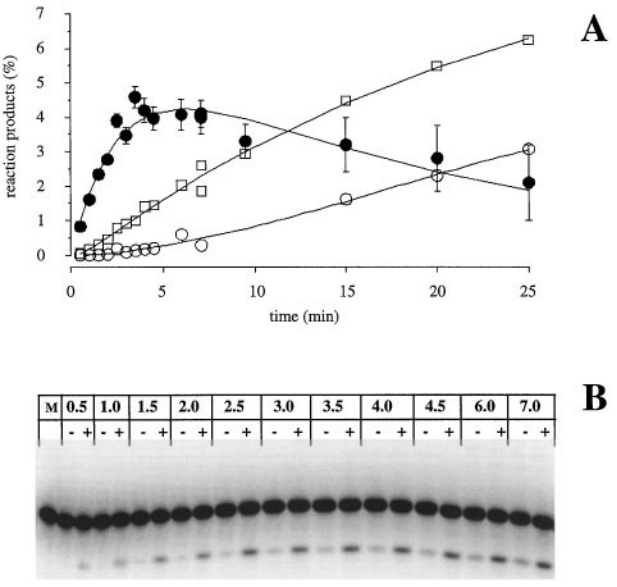

Fig. 3. Formation of the ISC. The kinetics of ISC formation were analyzed using a modified integration assay. Integrase was preincubated with the specific oligonucleotide substrate (INT1/INT2) for different time periods, after which 1 μM DS was added as a challenger. Immediately after the addition of DS, half of the reaction mixture was denatured with formamide loading buffer, whereas the second half was incubated for an additional 30 min to allow the cleavage reaction in the preformed ISCs to proceed. A, Quantification of the experiment. B, Autoradiogram of the original gel for the first 11 time points. Lane M, control in which DS was added before the addition of IN, followed by incubation at 37° for 30 min. *Other lanes*, different time points (0.5–7 min) of the preincubations before the addition of DS, stopped immediately after the addition of DS (−), or incubated for an additional 30 min (+). The amounts of the ISC accumulated after each time interval were determined by subtracting the amount of the reaction products in both − and + lanes. Time courses are shown for the accumulation of ISC (●), cleaved product (○), and products of strand transfer (□). The amounts of reaction products are given relative to the starting amount of the DNA substrate. Error bars, standard errors estimated for each time point on the assumption that the relative error for quantification of the cleaved and integrated products in the gel is 10%. Curve fitting for ISC accumulation was performed using the SigmaPlot 5.0 fitting software and was based on the equation: $A * (e^{-k_1 t} - e^{-k_2 t})$.

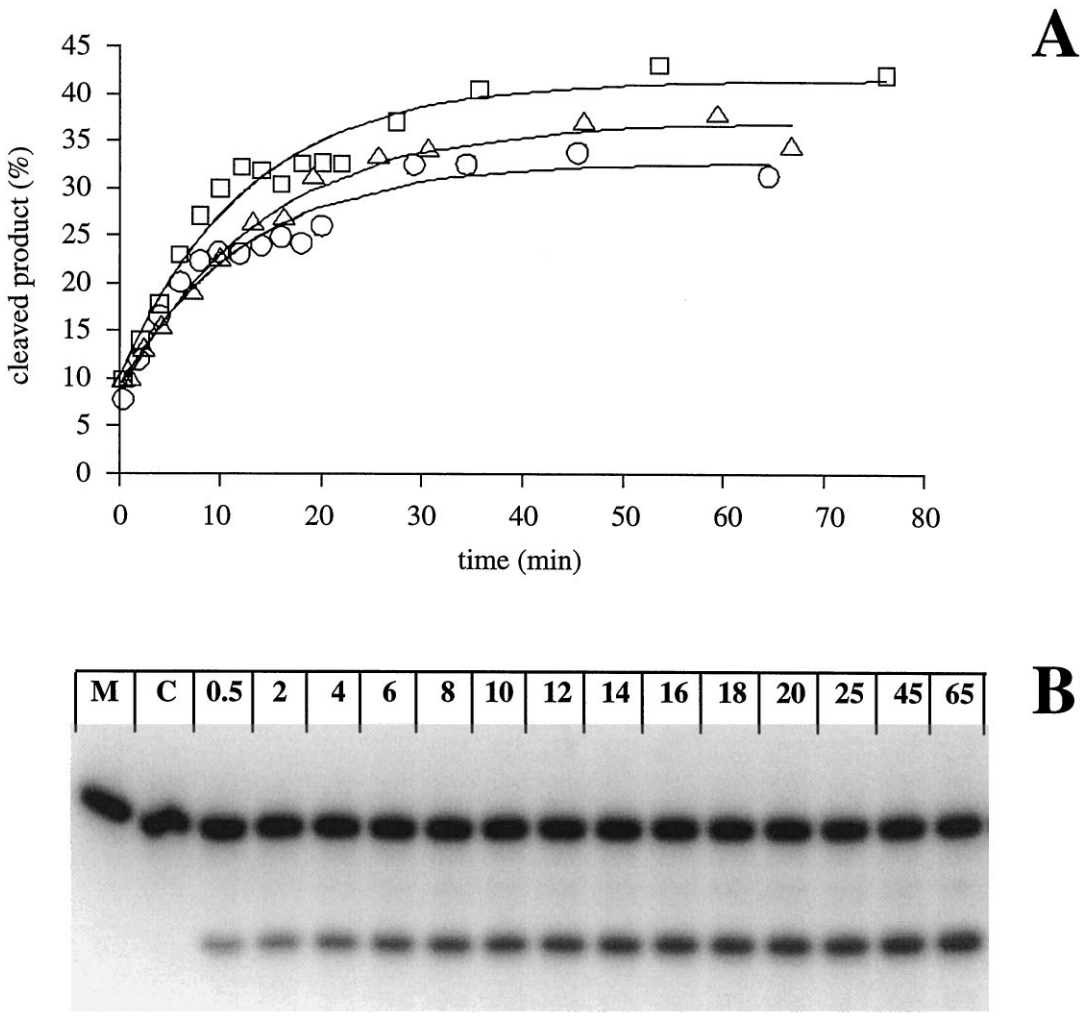

Fig. 4. Cleavage reaction in the presence of DS and T30177. The rate of the cleavage reaction was assayed in the following way: IN plus substrate reaction mixture (70 μl in total) was preincubated for a short period of time (3 min) at 37° to allow a maximum amount of initial complexes to form, after which DS or a mixture of DS and T30177 was added. After different periods of time, 5-μl aliquots were taken and denatured in an equal volume of formamide loading buffer. Reaction products were separated on denaturing polyacrylamide gels and quantified. B, DS at a final concentration of 0.3 μM was used as a quencher of the IN-substrate association. *Lane M*, mock sample, without enzyme added. *Lane C*, control sample with DS present in the reaction mixture before the addition of IN. Both samples were incubated for 30 min at 37°. *Other lanes*, different time points of the cleavage reaction after the addition of DS (0.5–65 min). Quantitative results shown for three different experiments in which 0.3 μM of DS (○), 1.5 μM of DS (□), or 0.3 μM of DS plus 1 μM of T30177 (△) was added. The amounts of the cleaved product are given relative to the starting amount of the oligonucleotide substrate. The curve fitting was performed using the SigmaPlot 5.0 curve fitter and the equation: $A - B * e^{-kt}$. The following k_{cat} values were calculated: 0.08 ± 0.01 min^{-1} (0.3 μM DS5000), 0.07 ± 0.006 min^{-1} (1.5 μM DS5000), and 0.08 ± 0.01 min^{-1} (0.3 μM DS5000 plus 1 μM T30177).

strand transfer by the G-quartet-forming oligonucleotides was observed (data not shown).

Binding of HIV-1 IN to DNA substrate. Although G-quartet-forming oligonucleotides do inhibit the enzymatic activity of HIV-1 IN, we have shown that they do not interfere with either the 3′-processing or DNA strand-transfer reactions as such; therefore, it could be deduced that they might interfere with the formation of the ISC. To show this inhibition of complex formation directly, we performed binding experiments using surface plasmon resonance technology (BIAcore2000). A biotinylated ssDNA oligonucleotide was bound to a streptavidin sensor chip. A DNA substrate with the IN recognition sequence at the free end was created by annealing of the complementary oligonucleotide to the bound biotinylated oligonucleotide. HIV-1 IN bound to this annealed dsDNA substrate. In the presence of 1 μM T30177, the binding of IN to the specific substrate was reduced by 88%; in the presence of 1 μM DS5000, it was even reduced by 100% (Fig. 6). In a separate experiment, HIV-1 IN was bound to a duplex oligonucleotide without recognition sequence. The addition of 1 μM T30177 inhibited the binding of IN to the aspecific duplex DNA as well (data not shown).

Sequence of the IN open reading frame from a T30177-resistant HIV-1 strain. In the first report on the antiviral effect of T30177 in cell culture, it was postulated that G-quartets could interfere with the viral replication cycle in two ways: inhibition of the internalization of the virus and/or viral integration (16). To clarify the antiviral mechanism in cell culture, a T30177-resistant virus strain was selected by passaging HIV-1 strain PNL43 in the pres-

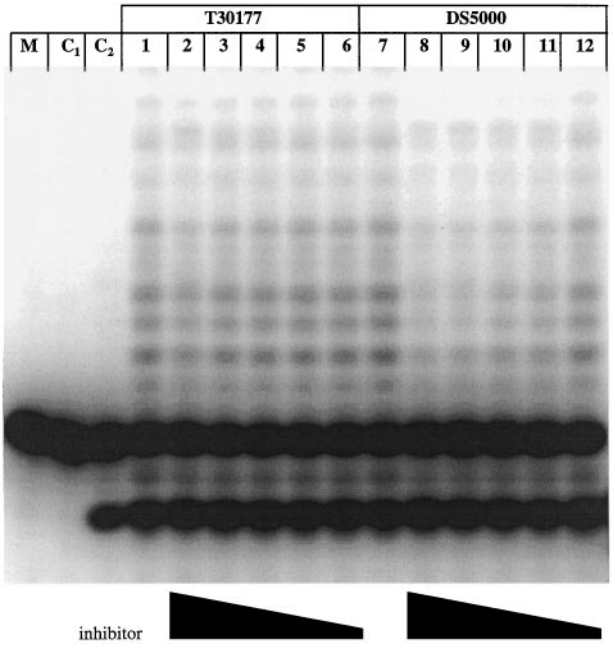

Fig. 5. Strand-transfer reaction in the presence of DS and T30177. To determine the inhibitory effect of DS and T30177 on the strand-transfer reaction of HIV-1 IN, we set up the following experiment. Integrase was preincubated in the substrate reaction mixture for 8 min at 37° to allow the IN/substrate association to occur. Then, an excess of target dsDNA (T35/SK70 ds oligonucleotide at a final concentration of 150 nM) was added with or without inhibitor, and the reaction samples were incubated for an additional 50 min. The excess of target DNA will trap free IN and prevent the formation of new processing complexes. Under these conditions, the measured enzymatic activity will be restricted to the strand-transfer reaction (although some cleavage in the preformed complexes may still occur). *Lanes 2–6*, strand transfer assay performed in the presence of T30177. *Lanes 8–12*, strand transfer assay performed in the presence of DS. *Lanes 1 and 7*, independent reference lanes that contain no inhibitor. *Lane M*, control lane (mock) without IN but incubated for 60 min at 37°. *Lane* C_1, control lane with target DNA added before the addition of IN and incubated for 60 min at 37°. *Lane* C_2, control lane in which the reaction was stopped by mixing with an equal volume of the formamide loading buffer immediately after the addition of target DNA. The final concentrations of the inhibitors are 4, 2, 1, 0.5, and 0.25 μM for T30177 (*lanes 2–6*, respectively) and 450, 250, 125, 62.5, and 31.25 nM for DS (*lanes 8–12*, respectively). The most prominent bands of the integration products were quantified.

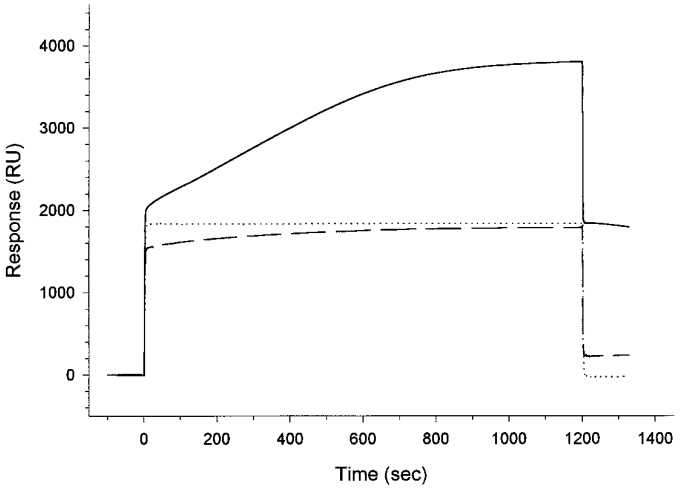

Fig. 6. DNA substrate binding of IN in the presence of T30177 or DS. Binding experiments were carried out using surface plasmon resonance technology on BIAcore2000 (Pharmacia Biotech) and sensor chips SA with preimmobilized streptavidin. The binding experiment was carried out at 37° according to the manufacturer's instructions. A specific biotinylated duplex oligonucleotide was captured by the streptavidin-coated chip. Then, 100 μl of HIV-1 IN was injected at a final concentration of 330 nM with a flow rate of 2 μl/min and passed both a blank channel and the channel with the specific oligonucleotide. Injection of IN started at time 0 sec; after 1200 sec, buffer was run through the channels. The response (*RU*) at 1200 sec is corrected for background binding to a blank SA sensor chip (not shown) and is a measure of the amount of IN bound to the duplex DNA. The binding of HIV-1 IN to the specific oligonucleotide (*solid line*) and inhibition of binding in the presence of 1 μM T30177 (*dashed line*) or DS5000 (*dotted line*) are shown.

ence of T30177.[1] Although the replication in cell culture of wild-type PNL43 is inhibited with an IC_{50} value of 0.2 μg/ml, the resistant virus strain is capable of growing in the presence of 125 μg/ml T30177. The full open reading frames of the IN gene from the wild-type and T30177-resistant strains were sequenced. Only two differences were found, with both being silent mutations. In the triplet coding for Gly193, we found a GGG-to-GGA substitution, and the triplet coding for Ile266 changed from ATC to ATT; these mutations in the HIV-1 IN cannot explain why HIV-1 became resistant to the inhibitory action of T30177 in cell culture.

Discussion

The G-quartet-forming oligonucleotides studied are potent inhibitors of the replication of HIV-1 in cell culture at submicromolar concentrations (16). Based on time of addition experiments, an inhibition of the binding (or fusion) of the virus with its target cell was put forward, not unlike that for other known polyanionic inhibitors of HIV replication, such as DS5000 (for a review, see Ref. 1). On the other hand, the potent inhibition of IN activity *in vitro* by G-quartet-forming oligonucleotides may indicate an alternative mechanism of action. We reasoned that the characterization of the specific interaction of G-quartets with HIV-1 IN, regardless of the relevance of the observed inhibition for the antiviral effect in cell culture, may lead to the development of effective IN inhibitors. Moreover, we used the G-quartet-forming oligonucleotide T30177 as a tool to understand the enzymology of HIV-1 IN and the formation of the ISC of the enzyme with its target DNA.

With the aim of using IN as a target for antiviral therapy, we may envisage inhibition of either the formation of the enzyme/LTR complex or the subsequent strand-transfer reaction. It may prove difficult to inhibit the cleavage reaction itself because it is a monomolecular reaction proceeding in a stable complex, yet compounds that inactivate the active center but do not prevent substrate binding may exist theoretically. The compounds that prevent binding of the enzyme to the LTR may not always inhibit the strand-transfer reaction because the interactions that result in the formation of the enzyme/LTR complex must be different from the nucleoprotein/target DNA complex. Many HIV-1 IN inhibitors have been evaluated for their inhibition of strand transfer in an assay using a precleaved LTR substrate, which does not need to be processed and is ready for

the strand-transfer reaction (15, 33). However, if the cleaved LTR substrate is added with the inhibitor, this method does not permit one to distinguish between an LTR-binding inhibitor and a strand-transfer inhibitor.

In this report, we describe a novel DNA strand-transfer assay. After a short preincubation of the LTR substrate with the enzyme, an excess of nonspecific ds target DNA is added, which prevents further specific LTR/IN association. When an inhibitor causes a decrease in integration activity in this assay, the inhibitor interferes with the strand-transfer. Under these conditions, the use of a precleaved substrate molecule further improves the specificity of the assay because it abolishes the limited 3′-processing activity in the preformed complexes. In our DNA strand-transfer assay, T30177 is considerably less active ($IC_{50} > 3$ μM) than in the 3′-processing assay, whereas the polyanion DS5000 interferes with DNA strand-transfer ($IC_{50} = 50$ nM), albeit at a 7-fold higher concentration than in the cleavage assay. This result points to a difference in the inhibitory effects of DS and T30177.

The cleavage reaction in the ISC as such is inhibited by neither polyanions nor G-quartets (for DS5000, see Fig. 3; for T30177, see Fig. 4). Therefore, in both cases, the reaction is blocked at a stage before the formation of functional ISC. In fact, inhibition of the formation of the IN/LTR complex was demonstrated in a direct way using biosensor technology (Fig. 6), although this experiment did not permit us to distinguish between functional and aspecific complexes that might arise in this *in vitro* system. The IC_{50} values for inhibition of IN activity are dependent on the IN concentration but not on the concentration of the DNA substrate (Table 1). These results clearly prove that T30177 interacts with IN and not with the substrate DNA. Although the binding of the specific viral DNA substrate can compete with the binding of a nonspecific ds oligonucleotide (20-mer), a 6-fold increase in substrate concentration had no effect on the inhibition by T30177, DS5000 (Table 1), or ds 35-mer T35/SK70 (data not shown). This may reflect a strong preference of IN to bind to longer DNA molecules (e.g., the ds 35-mer T35/SK70) or polyanions (e.g., DS5000) regardless of DNA structure and sequence.

Can the inhibitory effect of T30177 be attributed to a mere polyanion effect? Although both DS and T30177 seem to inhibit HIV-1 IN activity via a similar mechanism (i.e., prevention of ISC formation), the following differences exist between the compounds. At first, DS inhibits DNA strand-transfer, whereas T30177 does not. Second, although both polyanions are of the same length as T30177, the 17-mer KS and T30677 are much less inhibitory in the IN assay. In fact, a structure-activity relationship for the G-quartets has been reported (16). A direct correlation among thermal stability, the ability to fold into the proposed box-like structure, and activity in the IN assay has been confirmed with biophysical measurements (33a). Intramolecular folding of the G-quartets seems to be mediated via coordination of the K^+ ions. The capacity of T30695 to bind additional K^+ ions seems to be responsible for the increased thermal stability of T30695. Our data on the inhibition of IN activity by G-quartets (Table 1) confirm this structure-activity relationship. The highly folded structure of the G-quartets may thus be responsible for the specific interaction with HIV-1 IN. Undoubtedly, the intrinsic structural properties of T30177 and T30695 are also important for the inhibition of HIV-1 replication in cell culture by G-quartets. However, according to results of our gel filtration experiments (data not shown), T30177 has a tendency to oligomerize; therefore, at this point, we cannot exclude that conformations of T30177 other than the proposed G-quartet contribute to the observed inhibition of the IN activity and antiviral effect in cell culture.

We present a hypothetical scheme for the consecutive steps of the integration process *in vitro* and the inhibition by T30177: (i) Formation of a functional IN oligomer is represented by $IN_x + IN_x \rightarrow IN_x - IN_x$, where x is the number of protomers of IN in solution. (ii) Formation of IN/LTR complex is represented by $IN_x - IN_x + LTR \rightarrow IN_{2x} - LTR$. (iii) Maturation of IN/LTR nucleoprotein complex into the functional ISC is represented by $IN_{2x} - LTR \rightarrow ISC$. (iv) The cleavage reaction occurs in the ISC; the intermediate complex is the PSC: ISC → PSC, and PSC + target DNA → strand-transfer products.

Our experimental results indicate that the first two processes can be aborted by the addition of T30177 through a direct interaction with IN: IN_x + T30177 → inactive complex and/or $IN_x - IN_x$ + T30177 → inactive complex.

It is possible that the G-quartets interact with IN before the enzyme forms oligomers capable of a functional interaction with the LTR DNA. Alternatively, T30177 may block the binding of IN oligomers to the LTR. The oligomerization of IN, interaction with the LTR, and maturation of the complex are likely to be interconnected processes. Because the inhibition by G-quartets cannot be competed out by the addition of an excess LTR, it is unlikely that the compounds bind to the DNA binding domain. Interestingly, a recent report suggests an interaction of T30177 with the zinc finger domain of IN (34). This domain has been invoked in the functional oligomerization of HIV-1 IN (35). Further experiments are in progress in our laboratory to identify the binding site of T30177 on HIV-1 IN. The inclusion of the third step in the reaction scheme is based on the observation that the accumulation of the functionally active ISC is a slow reaction (Fig. 3). We postulate that the reaction rate is limited by the maturation into productive IN-LTR complexes. Cleavage and DNA strand-transfer are not inhibited by T30177.

It remains to be shown whether the potent activity against HIV-1 IN is responsible for the observed antiviral effect in cell culture because, alternatively, these G-quartet-containing oligonucleotides may interfere with entry of the virus in the target cell. In fact, in the original report on the antiviral effect of T30177, arguments for both modes of action were presented (16). Although T30177 was found to be inhibitory in a cell fusion assay, the IC_{50} concentration for inhibition of fusion was 100-fold higher than the IC_{50} value in cell culture, whereas the *in vitro* HIV-1 IN activity was inhibited at submicromolar concentrations. Moreover, in infected cells, circularized unintegrated HIV DNA seemed to accumulate after treatment with T30177 (16). To decipher unequivocally the mode of action of T30177 in cell culture, we selected for an HIV-1 strain that is ~500-fold less sensitive to the compound. When sequencing the IN gene of this resistant strain, we found no alterations in the IN amino acid sequence compared with the wild-type strain. The mutations observed in the viral envelope gp120 glycoprotein probably account for the observed resistance in cell culture.[1] These resistance data indicate that the main antiviral target of G-quartets in cell culture is located on gp120, although at this point, a

secondary antiviral target on HIV-1 IN cannot be ruled out completely.

T30177 may finally prove to have a mechanism of action analogous to that of another oligonucleotide, the phosphorothioate TTGGGGTT (ISIS 5320), which is also a potent inhibitor of HIV replication in cell culture (23). This oligonucleotide adopts a parallel-stranded, intermolecular tetrameric G-quartet structure. The compound was shown to inhibit virus entry by binding to the cationic V3 loop of the envelope glycoprotein (26); we have now shown that ISIS 5320 inhibits the 3′-processing reaction of HIV-1 IN as well (Table 1). Although T30177 and ISIS 5320 may target the same replication step, some distinctive features must be noted. Although ISIS 5320 forms tetrameric G-quartets, T30177 is folded into an intramolecular G-quartet. Experimentally, T30177 retains anti-IN activity after heat-denaturation, whereas ISIS 5320 does not. Undoubtedly, the intrinsic structural biophysical properties of both classes of G-quartets are important for the inhibition of HIV replication in cell culture; therefore, the studied G-quartets form interesting lead compounds, and the study of their interaction with proteins should facilitate their development. The results obtained with T30177, and especially the difficulties encountered in ascribing unequivocally a mode of action to these compounds, emphasize the need for developing an IN assay in an intact cell system in which intracellular IN activity and the inhibition of this activity can be monitored.

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