HIV-1 Integrase Complexes with DNA Dissociate in the Presence of Short Oligonucleotides Conjugated to Acridine†

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ABSTRACT: The human immunodeficiency virus type 1 (HIV-1) integrase is an essential enzyme in the life cycle of the virus and is therefore an attractive target for the development of new antiviral drugs. Among them, inhibitors which are capable of targeting the preassembled integrase/DNA complex are of particular interest, because they could suppress integrase activity in the context of the HIV-1 preintegration complex. Here, we study the mechanism of action of 11-mer oligonucleotides, which are efficient inhibitors of the catalytic activity of integrase, provided that they are conjugated to a hydrophobic compound, acridine. To understand the mechanism of the conjugate inhibitory action, we used a steady-state fluorescence anisotropy assay, which allowed us to study the stability of the integrase/DNA complex in various conditions. We found that oligonucleotide−acridine conjugates induced the efficient dissociation of preassembled integrase/DNA complexes. The simultaneous presence of both acridine and an oligonucleotidic moiety is required for the inhibitory activity of conjugates. However, the dissociation effect is not dependent on the oligonucleotide sequence. Finally, our results suggest that the conjugates bind directly to integrase within its complex with DNA at a site different from the viral DNA binding site.

Human immunodeficiency virus replication requires the activity of three viral enzymes, reverse transcriptase, protease, and integrase (IN)[1], which are privileged targets for anti-HIV drugs. The discovery of reverse transcriptase and protease inhibitors led to the development of combined therapy directed against these two enzymes. Although this treatment suppresses HIV replication for long periods of time, it cannot eradicate it completely (*1*−*3*). Therefore, the identification of inhibitors of the third enzyme, integrase, which is responsible for the integration of viral DNA into the genome of infected cells, is one of the major goals of HIV pharmacology.

Integrase binds to conserved sequences located on U3 and U5 long terminal repeats (LTR) at the extremities of the reverse-transcribed viral DNA, thereby forming a nucleoprotein complex, which constitutes the main component of the preintegration complex (PIC) (*4*). The PIC undergoes two spatially and temporally independent reactions, relying on the catalytic activity of IN. The first reaction, named 3′-processing, takes place in the cytoplasm of infected cells and consists of elimination of 3′-terminal GT dinucleotides from both LTR extremities of the viral DNA. The PIC subsequently migrates into the nucleus, wherein IN catalyses a strand transfer reaction that eventually leads to the covalent insertion of the processed viral DNA (substrate DNA) into the host-cell DNA (target DNA). At this point of the retroviral DNA integration, IN within the PIC must simultaneously accommodate both viral DNA ends, U3 and U5, and the host-cell DNA (*5*).

Although the organization of the integrase active unit is not yet known, the molecular docking of a dinucleotide onto the IN core domain led to the identification of two possible nucleic acid-binding sites on a single monomer (*6*, *7*). One of these sites spans a previously described nucleotide-binding site and participates in the binding of the viral DNA (*7*−*9*). The second site is a putative binding site for the cellular DNA. This is consistent with IN−DNA interaction models, according to which both the viral and target DNAs bind to a single IN monomer (*10*). Nevertheless, a multimeric form of IN, most likely tetrameric, is required for the concomitant insertion of both U3 and U5 extremities into the target DNA.

The capability of IN to accommodate nucleic acids suggests that nucleotidic compounds may possess pharmacological potential. Indeed, nucleotides, dinucleotides and their analogues can bind to the substrate-binding site within IN, consequently acting as competitive inhibitors of the enzyme. However, although some progress has recently been made in the design of more active dinucleotides, their anti-

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[1] ABBREVIATIONS: Acr, acridine; DTT, dithiothreitol; FITC, fluorescein isothiocyanate; Fl, fluorescein; HIV-1, Human Immunodeficiency Virus type 1; IN, integrase; LTR, Long Terminal Repeat; PIC, preintegration complex.

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integrase activity remains moderate (*7*, *11*, *12*). Natural oligonucleotides derived from the U5 LTR also inhibit IN, probably through a similar, competitive mechanism (*13*). Single-stranded oligonucleotides containing less than 20 bases are moderately active. Modification of the sugar−phosphate backbone does not significantly improve their potency, as shown with phosphorothioate oligonucleotides *(14)*. The inhibitory activity may be enhanced by the formation of secondary structures such as G-quadruplex, for which IN displays high affinity (*15*−*17)*. Triple-helix formed by triplex-forming oligonucleotides bound to the IN binding site on viral DNA is another kind of secondary structure which blocks IN activity (*18*−*20*). However, despite their anti-IN activity in vitro, none of these oligonucleotides are capable of blocking IN activity in cells. In particular, the antiviral effect of G-quartet oligonucleotides was eventually demonstrated to originate in an interference with the viral entry (*21*).

Finally, polynucleotides can inhibit the catalytic activity of IN in vitro by acting either in competitive or noncompetitive mode. The noncompetitive mechanism results from their ability to bind to and to disrupt the IN−viral DNA complex (*22*). This mode of action is of particular interest because the most efficient IN inhibitors in cells identified to date are the recently discovered IN−DNA complex ligands (*23*−*25*). Thus, shorter oligonucleotides retaining the potential of polynucleotides to disrupt the IN−DNA complex would provide a new way to block IN activity in vivo. Some progress has been made in the field of IN inhibition by short oligonucleotides. For example, 11-mer oligonucleotides containing a modified heterocyclic base, 6-oxocytosine, inhibit the catalytic activity of integrase by directly interacting with and disrupting the integrase/viral DNA complex (*26*, *27*). The presence of 6-oxocytosine within the inhibitor is thought to play a key role in its interactions with integrase, by forming supplementary contacts between the enzyme and the oligonucleotide (*26*).

Because integrase interacts with hydrophobic aromatic molecules (*28*, *29*), we hypothesized that the conjugation of short single-stranded oligonucleotides with such molecules might confer the ability to target and to disrupt the IN−DNA complex in the same way as 6-oxocytosine-containing oligonucleotides (*26*). Here, we studied the effect of oligonucleotide-acridine conjugates on the stability of the IN−DNA complex. Using a steady-state fluorescence anisotropy assay, we demonstrated that these conjugates may actually induce the dissociation of IN−DNA complexes. In agreement, the conjugates inhibited the catalytic activity of integrase within preassembled enzyme−substrate complexes in the same concentration range. The efficiency of the IN activity inhibition and the IN−DNA complex dissociation depended on the presence of both oligonucleotidic and aromatic moieties. It is noteworthy that the conjugates neither interact with the viral DNA nor form any self-structures under conditions when they induce the IN−DNA complex dissociation. Thus, the potency of these compounds as HIV-1 integrase inhibitors is mediated by the direct interaction of single-stranded oligonucleotide-acridine conjugates with the IN−viral DNA complex and disturbance of its integrity and activity.

MATERIALS AND METHODS

Unmodified Oligonucleotides. Oligonucleotides were synthesized using the phosphoramidite technique on an automatic DNA synthesizer (ABI 380B, Applied Biosystems). The resulting oligonucleotides were then purified by electrophoresis on an 18% polyacrylamide/7 M urea gel. The following oligonucleotides were prepared: U5B (5′-GTGTGGAAAATCTCTAGCAGT-3′), U5B-2 (5′-GTGTGGAAAATCTCTAGCA-3′), U5A (5′-ACTGCTAGAGATTTTCCACAC-3′), U3B (5′-GAGTGAATTAGCCCTTCCAGT-3′), and U3A (5′-ACTGGAAGGGCTAATTCACTC-3′) mimicking the extremities of HIV-1 U5 and U3 LTRs; nonspecific oligonucleotide nsA (5′-GGAATCTAGCGGCGCATAGGT-3′) and unmodified analogues of inhibitors, GTGT (5′-GGTTTTTGTGT-3′), CTGA (5′-CTGACTGCATC-3′), and TCTC (5′-TCTCTAGCAGT-3′).

Modified Oligonucleotides. Oligonucleotides bearing fluorescein on their 5′-end: Fl-U5B (Fl-5′-GTGTGGAAAATCTCTAGCAGT-3′) and Fl-nsB (Fl-5′-ACCTATGCGCCGCTAGATTCC-3′) and the 3′-biotinylated oligonucleotide U5A-Biot (5′-ACTGCTAGAGATTTTCACAC-3′-Biot) were purchased from Eurogentec and further purified by electrophoresis on an 18% polyacrylamide/7M urea gel.

Synthesis of 3′ and/or 5′-Acridine-Oligonucleotide Conjugates. An acridine (Acr) residue was incorporated into the 3′- and/or 5′-ends of oligonucleotides by use of a solid phase support "Acridine-CPG" and a 5′-Acridine-modified agent (Glen Research), respectively. After the synthesis of conjugates, the support was treated with 1 mL of 0.4 M sodium hydroxide solution in methanol for 17 h at room temperature. The supernatant was neutralized with 1.5 mL of 2 M triethylammonium acetate, and conjugates were precipitated with ethanol. The reaction mixtures were analyzed by reverse phase HPLC (ion pair mode) and purified by electrophoresis in a 20% polyacrylamide/7M urea gel. The presence of the Acr-group in conjugates was confirmed by spectrophotometry. The ratio between the absorption of the Acr-group at 344 nm and the total absorption of the acridine−oligonucleotide conjugate at 260 nm was used to determine whether each oligonucleotide contained one or two acridine residues.

Assays of 3′-Processing and Strand Transfer Integrase Activities. The recombinant full-length IN was expressed in an eukaryotic system that was infected with a recombinant baculovirus and purified near to homogeneity as described in (*30*). Oligonucleotides U5B or U3B and U5B-2 were 5′-labeled by T4 polynucleotide kinase (New England Biolabs) using [γ^{32}P]ATP (specific activity 3000 Ci/mol), and the enzyme was inactivated by adding EDTA and heating at 65°C for 5 min. Each of the oligonucleotides were then annealed with an equimolar quantity of the complementary U5A or U3A oligonucleotide in a buffer containing 20 mM Tris-HCl pH 7.2, 100 mM NaCl. The resulting duplexes, U5B/A (U3B/A) and U5B-2/A, were purified from unincorporated [γ^{32}P]ATP on Micro Bio-Spin6 columns (Amersham Biosciences). The same reaction conditions were used for 3′-processing and strand transfer reactions. The ^{32}P-labeled substrates, U5B/A or U3B/A (2 nM) for 3′-processing and U5B-2/A (10 nM) for strand transfer, were incubated in the presence of IN (100 nM) and increasing concentrations of an oligonucleotide inhibitor (0.1−30 μM) in 20 μL of a buffer containing 20 mM Hepes pH 7.2, 20 mM NaCl, 1

mM DTT, and 10 mM $MgCl_2$ at 37 °C for 1 h. The reaction was stopped by the addition of 80 μL of a solution containing 10 mM Tris-HCl pH 7.5, 0.3 M sodium acetate and 1 mM EDTA. IN was phenol-extracted and the reaction products were precipitated with ethanol and resuspended in a formamide/water (4:1 v/v) solution. Products were separated on an 18% polyacrylamide/7M urea gel. Images were recorded on a Storm 840 Phosphoimager (Molecular dynamics) and quantified using the ImageQuant software. The averages of three independent experiments were determined, and isotherm inhibition curves were fitted with the Origin 6.0 software.

Assay of 3′-Processing Activity within the Preassembled Integrase−Substrate Complex. The oligonucleotide U5B was 5′-end radiolabeled with ^{32}P, and its duplex with an equimolar quantity of the complementary oligonucleotide U5A bearing biotin on its 3′-end was prepared as described above. The ^{32}P-labeled biotinylated duplex ^{32}P-U5B/A-Biot (2 nM) was incubated with IN (100 nM) in the presence of Streptavidin Dynabeads M-280 (DYNAL) at 25 °C for 15 min in a buffer containing 20 mM Hepes pH 7.2, 20 mM NaCl, 1 mM DTT and 10 mM $MgCl_2$. The IN−substrate complex fixed on the beads was separated from the excess of free IN, washed with the same buffer and mixed with an oligonucleotide inhibitor (0.1 μM to 30 μM in the buffer used for the complex formation). The reaction mixture was incubated at 37 °C for 1 h. The next steps were carried out as described above for the standard IN activity test.

Steady-State Fluorescence Anisotropy Assay. DNA duplexes containing fluorescein Fl-U5B/A or Fl-nsB/A (2 nM) were mixed with integrase (100 nM) in a buffer containing 20 mM Hepes pH 7.2, 10 mM $MgCl_2$ and 1 mM DTT. The mixture was incubated at 25 °C for 20 min, then the temperature was increased to 37 °C and an oligonucleotide inhibitor was added to a final concentration of between 0.1 μM and 10 μM. The fluorescence anisotropy values corresponding to the dissociation of the IN−Fl-duplex complex were measured by the Beacon 2000 Fluorescence Polarization System (Pan Vera part #*P*2300), and dissociation curves were constructed.

RESULTS

Integration Inhibition by Short Oligonucleotides and Their Conjugates with Acridine. It was previously shown that short oligonucleotides of certain sequences conjugated to hydrophobic aromatic molecules possessing intercalating properties are able to inhibit 3′-processing and strand transfer reactions in vitro (*18*−*20*, *31*). Under peculiar conditions, their inhibitory effect was due to the formation of a triple-helix between the conjugate and the viral DNA (*18*−*20*).

Here, we examined an inhibitory activity of oligonucleotides conjugated with a such hydrophobic molecule as acridine in conditions not compatible with triplex formation (*26*). Acridine was coupled to three 11-mer oligonucleotides of different sequences: 5′-GGTTTTTGTGT-3′ (GTGT), an anti-integration activity of which was earlier reported (*31*), 5′-TCTCTAGCAGT-3′ (TCTC), which displays a substrate-like sequence mimicking the 11-mer terminus of the U5 LTR, and 5′-CTGACTGCATC-3′ (CTGA) with a random sequence. Using physicochemical methods such as UV-melting, CD-spectroscopy and nondenaturing gel electrophoresis, we demonstrated that the conjugates did not interact with viral DNA as well as they did not form any intra- and intermolecular secondary structures in our conditions (i.e., pH 7.2, 20 mM NaCl, 10 mM $MgCl_2$, 37 °C; data not shown).

Table 1. Inhibition of HIV-1 IN Activity by Oligonucleotide−Acridine Conjugates

		IC_{50} values (μM)[a]		
		3′-processing		strand transfer
name	sequence 5′ → 3′	U5	U3	U5
GTGT	GGTTTTTGTGT	25		
GTGT−Acr	GGTTTTTGTGT−Acr	2.5	2.5	2
CTGA	CTGACTGCATC	45		
CTGA−Acr	CTGACTGCATC−Acr	5	7.5	5
TCTC	CTGACTGCATC	25		
TCTC−Acr	CTGACTGCATC−Acr	5	8	5
Acr	Acr−NH_2	90		

[a] IC_{50} values are average of three independent experiments. Standard deviation between experiments was 5−10%.

The inhibitory effect of the conjugates was determined in both the 3′-processing and strand transfer reactions (Table 1). 3′-Processing was carried out on both U5B/A and U3B/A duplexes to rule out any effect related to the substrate DNA sequence. All acridine−oligonucleotide conjugates inhibited IN activity with similar efficiency for 3′-processing of both U5 and U3 substrates and for the strand transfer reaction (IC_{50} comprised between 2 and 8 μM), thereby confirming that the inhibitory activity of the conjugates was not due to a sequence specific interaction with DNA. As can be observed in Figure 1, which shows a typical result obtained with modified oligonucleotides, the presence of acridine reinforced the inhibitory activity, because a 5−10-fold higher concentration of unmodified oligonucleotides was required to inhibit IN activity. However, it is important to note that acridine alone was characterized by a poor anti-IN activity ($IC_{50} = 90$ μM). Recently, we introduced the hypothesis that short oligonucleotides may target and dissociate IN−DNA complexes, provided that they contain modifications stabilizing their interactions with IN (*26*). In this report, we investigated whether the oligonucleotide coupling to acridine plays a similar role, allowing the short oligonucleotide conjugates to target and subsequently disrupt the IN−DNA complexes.

Effect of Oligonucleotide−Intercalator Conjugates on the Stability of IN−DNA Complexes. To determine whether oligonucleotides conjugated to acridine affect the dissociation rate of the IN−viral DNA complex, it was first necessary to evaluate the stability of the complex. The formation of IN−DNA complexes has been previously studied by several methods, including DNA filtration and UV-*cross*-linking (*22*, *32*). Here, we used an assay based on steady-state fluorescence anisotropy measurements. This technique is based on the measurement of the light depolarization and allowed us to detect DNA−protein interactions directly in solutions in conditions corresponding to that of enzyme activity assay (*33*−*35*). To form the IN−viral DNA complex, the fluorescein-labeled U5 substrate, Fl-U5B/A, mimicking the blunt-ended U5 LTR terminus, was incubated with IN at 25 °C in an Mg^{2+}-containing buffer (*35*). Complex formation was accompanied by an increase of fluorescence anisotropy from 0.05, corresponding to the free DNA, to a higher value, close to 0.21, which characterizes the protein−DNA complex. This

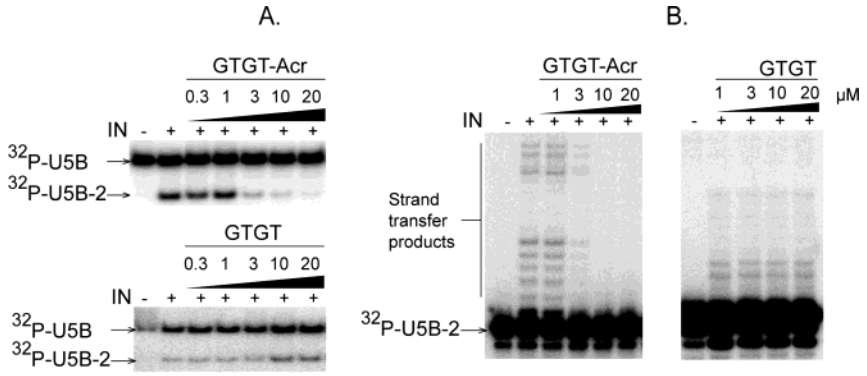

FIGURE 1: Inhibition by the 11-mer oligonucleotide GTGT and its acridine conjugate GTGT−Acr of 3′-processing (A) and strand transfer (B) carried out by HIV-1 IN. 2 nM of ^{32}P-labeled U5 (3′-processing) or U5−2 (strand transfer) double-stranded substrates were incubated with 100 nM integrase in a buffer containing 20 mM Hepes pH 7.2, 20 mM NaCl, 1 mM DTT, and 10 mM $MgCl_2$ at 37 °C for 1 h in the presence of increasing concentrations of either GTGT or GTGT−Acr.

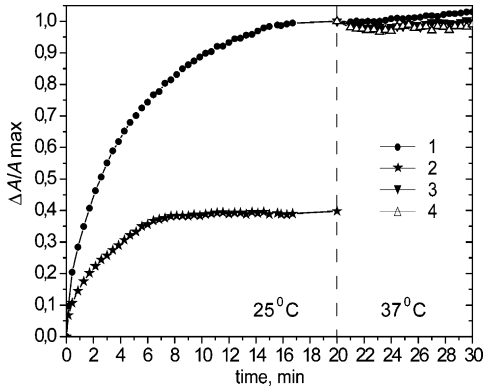

FIGURE 2: Influence of heparin on the stability of the integrase−U5 substrate complex. Fluorescein-labeled U5 substrate Fl-U5B/A (2 nM) was incubated with integrase (100 nM) at 25 °C for 20 min alone (curve 1) or in the presence of heparin (250 μg/L) (curve 2). The preformed complex of integrase and Fl-U5B/A substrate was incubated at 37 °C in the presence of heparin at following concentrations: 50 μg/L (curve 3) and 250 μg/L (curve 4). $\Delta A/\Delta A_{max}$ represents the fractional saturation where ΔA is the difference between the anisotropy value of the complex minus the anisotropy value of the free DNA.

higher value of the steady-state anisotropy can be explained by a combination of two phenomena, the slower global tumbling of the IN−DNA complex and the more restricted local motion of fluorescein. It is important to note that free and bound DNA display similar fluorescence lifetimes (*36*). Moreover, at the concentration of integrase used in this assay (i.e., 100 nM), no protein aggregates were observed, thus ruling out the possibility of unspecific depolarization due to scattering. For these reasons, the change in static anisotropy is directly correlated to degree of DNA saturation with the protein, allowing fractional saturation curves to be derived. Such curves are reported in Figure 2. Equilibrium was reached after 20 min (Figure 2, curve 1), indicating very slow kinetics of IN binding to DNA, which is in agreement with previous results (*26*). Complex dissociation was subsequently monitored by measuring the reequilibration rate. Reequilibration occurs either after complex dilution or after the addition of a ligand that shifts the equilibrium by trapping a free component. Our experimental conditions preclude the use of the dilution method. Therefore, the second approach with heparin as a ligand of IN was used to monitor the IN−DNA complex dissociation.

Heparin is a polyanionic heteropolysaccharide that binds to positively charged tracts in DNA-binding proteins, thereby impairing DNA-protein interplay (*37*). Both the catalytic and the C-terminal domains of integrase are rich in basic amino acids, which can interact with negatively charged molecules such as suramin and heparin (*22*, *38*). In agreement, a significant decrease of the steady-state anisotropy was observed when heparin (250 μg/L) was preincubated with IN before addition of the fluorescein-labeled DNA, Fl-U5B/A, (Figure 2, curve 2). This result confirms that heparin interacts with IN, impairing its binding to the U5 substrate. Therefore, in the case of the reversible binding of IN to DNA, heparin could interact with free IN molecules and consequently shift the equilibrium toward the IN−DNA complex dissociation. However, the addition of heparin up to 250 μg/L concentration did not induce IN−U5 complex disruption, showing that the preassembled complex was highly stable in our experimental conditions (Figure 2, curves 3−4).

Then, we investigated whether short oligonucleotides conjugated to acridine could induce the dissociation of the IN−DNA complex in conditions close to physiological ones. This study was started by using an acridine conjugate of the GGTTTTTGTGT oligonucleotide (GTGT−Acr), the anti-integration activity of which had been already demonstrated (Table 1). The fluorescein-labeled U5 substrate, Fl-U5B/A, was incubated with IN at 25 °C until the binding reached equilibrium, then the temperature was increased to 37 °C, and the conjugate GTGT−Acr or its unmodified counterpart GTGT was added to the complex at concentrations ranging from 0.1 to 5 μM. Changes in steady-state fluorescence anisotropy values were measured and dissociation curves were constructed. The addition of the conjugate resulted in the rapid dissociation of the preassembled IN−DNA complex (Figure 3A). The rate of complex dissociation was dose-dependent. In the presence of 5 μM concentration of GTGT−Acr, the complex was completely disrupted after one minute (Figure 3A, curve 5). Because no dissociation was detected in the absence of the conjugate (Figure 3A, curve 1), it indicates that the conjugate was responsible for the complex dissociation. It is important to note that the unmodified oligonucleotide had only a limited effect on the complex stability even at the highest concentration tested (Figure 3B), thereby demonstrating that the presence of acridine is essential for the complex dissociation.

We verified that no significant change of the overall fluorescence intensity is observed during the dissociation process (data not shown). Therefore, the variation of ani-

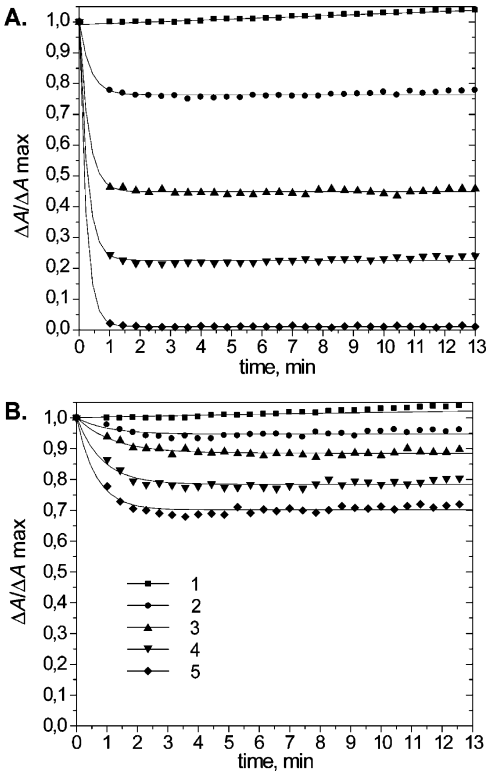

FIGURE 3: Disruption of the integrase−U5 substrate complex by the oligonucleotide−intercalator conjugate GTGT−Acr (A) and the nonmodified oligonucleotide GTGT (B). Fluorescein-labeled U5 substrate Fl-U5B/A (2 nM) was incubated with integrase (100 nM) at 25 °C for 20 min and then at 37 °C in the presence of either GTGT−Acr (A) or GTGT (B) at following concentrations: curve 1, no oligo; curve 2, 0.1 μM; curve 3, 0.5 μM; curve 4, 1 μM; curve 5, 5 μM.

sotropy, ΔA, was directly correlated to the fractional saturation (i.e., the fraction of IN−DNA complexes). Thus, affinity constants for the oligonucleotides tested can be calculated by plotting $\Delta A/\Delta A_{max}$ versus oligonucleotide concentration. An apparent Kd of 500 nM was obtained for the acridine-conjugated oligonucleotide, whereas the nonmodified oligonucleotide was characterized by an apparent Kd value higher than 5 μM. Finally, acridine alone did not affect the IN−DNA complex stability at concentrations up to 500 μM (data not shown) which is in agreement with the its poor inhibitory activity in IN activity assays (Table 1). These results demonstrate that the simultaneous presence of both the oligonucleotide and the acridine moieties is required in a synergistic way to induce the efficient IN−DNA complex dissociation.

To determine whether the IN−viral DNA complex disruption results from an active interplay of the GTGT−Acr conjugate with IN, we studied the IN−DNA complex dissociation in the presence of both heparin and the conjugate. As demonstrated above, heparin interacts with free IN (Figure 2, curve 2). Thus, when added to the complex simultaneously with the conjugate, it may trap free IN molecules resulting from the conjugate-induced complex dissociation, thereby preventing the complex re-formation. The fluorescein-labeled U5 substrate, Fl-U5B/A, was incubated with IN until the binding reached equilibrium, and then both the conjugate GTGT−Acr (5 or 10 μM) and heparin (250 μg/L) were simultaneously added to the complex. As shown in Figure 4, in the presence of a concentration of

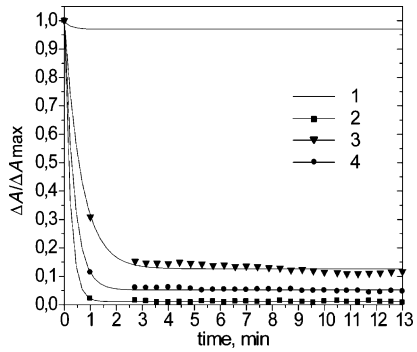

FIGURE 4: Disruption of the integrase−U5 substrate complex by the oligonucleotide−acridine conjugate GTGT−Acr alone and in the presence of heparin. Fluorescein-labeled U5 substrate Fl-U5B/A (2 nM) was incubated with integrase (100 nM) at 25 °C for 20 min and then at 37 °C. Curve 1, heparin (250 μg/L); curve 2, GTGT−Acr (5 μM); curve 3, heparin (250 μg/L) and GTGT−Acr (5μM); curve 4, heparin (250 μg/L) and GTGT−Acr (10μM).

heparin that did not alter the complex stability (250 μg/L), the addition of GTGT−Acr resulted in IN−DNA complex disruption, with the almost complete dissociation reached in the presence of 10 μM of the conjugate (curve 4). Interestingly, the presence of heparin decreased the efficiency of the complex disruption under the influence of the conjugate (Figure 4, compare curves 2 and 3). Indeed, a higher concentration of the conjugate (10 μM) was necessary to disrupt the complex as compared to the same experiment performed in absence of heparin (curve 4). This result suggests that heparin also interacted with IN within its complex with DNA and competed with the conjugate for the binding to the complex. Nevertheless, in contrast to the conjugate, heparin even being bound to the complex did not trigger its dissociation.

As the oligonucleotide−acridine conjugate, GTGT−Acr, was able to disrupt the IN−viral DNA complex, we investigated whether this mode of the conjugate action is dependent on the oligonucleotide sequence. Other conjugates capable of inhibiting IN activity, TCTC−Acr and CTGA−Acr, were tested (see Table 1). All of these oligonucleotide−acridine conjugates disrupted the IN−substrate complex in the presence of heparin with comparable efficiencies (Figure 5, curves 2−4). These results suggest that any short oligonucleotide−acridine conjugate can provoke the IN−viral DNA complex dissociation, and as a consequence, inhibit the IN catalytic activity in the context of the preassembled complex.

To determine whether the conjugates can target and disturb IN complexes with any DNA, we studied the effect of the GTGT−Acr conjugate on the stability of the IN complex with a random duplex Fl-nsB/A lacking the specific integrase-binding site. It was found that this complex dissociated in the presence of the conjugate GTGT−Acr with the same efficiency and velocity as the IN complex with its cognate substrate U5 (data not shown). This result indicates that oligonucleotide−acridine conjugates dissociate the complexes of IN with DNA independently of the nature of DNA (substrate or target) involved in this complex.

Inhibition of the Catalytic Activity of HIV-1 Integrase within its Complex With DNA by Oligonucleotides Conjugated to Acridine. As conjugates disrupted the IN−DNA complex regardless of their nucleotidic sequence, we inves-

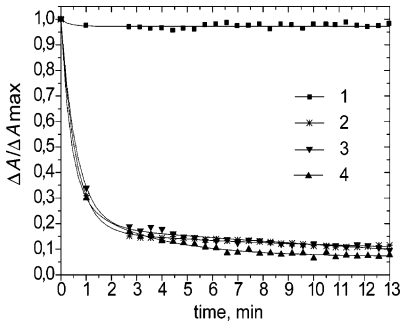

FIGURE 5: Disruption of the integrase−U5 substrate complex by oligonucleotide−acridine conjugates of different sequence: GTGT−Acr, TCTC−Acr, or CTGA−Acr in the presence of heparin. Fluorescein-labeled U5 substrate Fl-U5B/A (2 nM) was incubated with integrase (100 nM) at 25 °C for 20 min and then at 37 °C in the presence of heparin and oligonucleotides. Curve 1, heparin (250 μg/L); curve 2, heparin (250 μg/L) and GTGT−Acr (5 μM); curve 3, heparin (250 μg/L) and TCTC−Acr (5 μM); curve 4, heparin (250 μg/L) and CTGA−Acr (5 μM).

Table 2. Inhibition of the U5 Substrate 3′-Processing in the Preassembled and Nonpreassembled Integrase−U5 Substrate Complex by Oligonucleotide−Intercalator Conjugates

	IC_{50} values (μM): 3′-processing U5[a]	
name	preassembled complex	nonpreassembled complex
GTGT	>40	25
GTGT−Acr	4	2.5
CTGA−Acr	15	5
TCTC−Acr	15	5

[a] The IC_{50} value shown is an average of three independent experiments, the standard deviation between experiments was 5−10%.

tigated their effect on the IN catalytic activity in the context of the preassembled IN−substrate complex. For this purpose, we used ^{32}p−U5B/A-Biot, a U5 substrate bearing biotin at the 3′-end of the nonprocessed strand. This substrate was preincubated with IN in the presence of streptavidin magnetic beads for 15 min, under conditions where the enzyme activity cannot take place, then the complex was separated from the free enzyme and placed in the conditions used for the 3′-processing reaction (Mg^{2+}-containing buffer, 37 °C (*26*)). This isolated IN−substrate complex was stable and active in providing the 3′-processing reaction for at least 3 h (data not shown). The addition of increasing concentrations of oligonucleotide-acridine conjugates (GTGT−Acr, TCTC−Acr, or CTGA−Acr) to the preassembled and isolated IN−U5 complex resulted in the inhibition of the IN catalytic activity (4 μM < IC50 < 15 μM, see Table 2). The inhibitory effect of the nonmodified oligonucleotide GTGT was significantly weaker. The inhibitory activity of the oligonucleotide−acridine conjugates in the context of the preassembled IN−viral DNA complex corresponds to their effect on the complex stability, thus confirming that they may act as IN inhibitors by targeting and disrupting the IN−DNA complex.

Finally, we also studied the effect of heparin on the IN activity either when heparin and the U5 substrate were added to IN simultaneously or when heparin was added to the preassembled IN−U5 complex. It was found that in the case of the non-preassembled complex, heparin inhibited 3′-processing with an IC_{50} value of 2.5 μg/L (Figure 6A). When heparin was added to the preassembled IN−U5 complex,

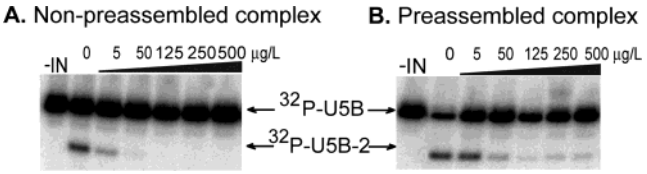

FIGURE 6: Heparin influence on the U5 substrate 3′-processing in the non-preassembled (A) and preassembled integrase−U5 substrate complex (B). U5 substrate (2 nM) containing the ^{32}P-labeled U5B strand was incubated with integrase (100 nM) in a buffer containing 20 mM Hepes pH 7.2, 20 mM NaCl, 1 mM DTT and 10 mM $MgCl_2$ at 37 °C for 1 h in the presence of increasing concentrations of heparin.

the reaction was inhibited at higher heparin concentration (IC_{50} = 10 μg/L) and not completely, because a small amount of the processed U5 substrate was detected even in the presence of up to 500 μg/L heparin (Figure 6B). This result confirms the ability of heparin to interact with and to affect IN within its complex with viral DNA. However, in contrast to the acridine−oligonucleotide conjugates, heparin did not disrupt the IN−viral DNA complex (Figure 2, curves 3 and 4) at both concentrations, 50 μg/L and 250 μg/L, for which the IN activity was suppressed, thus suggesting a different mechanism of inhibition. This mechanism is further discussed in the next section.

DISCUSSION

In this study, we investigated the HIV-1 IN inhibition by several 11-mer oligonucleotides conjugated to acridine. We found that such conjugates can block IN activity in vitro with a GTGT−Acr conjugate, with guanosine and thymidine nucleosides being the most potent (IC_{50} value of 2.5 μM) (Tables 1 and 2).

During the early steps of HIV-1 replication, IN forms a highly stable complex with viral DNA in such a way that once properly assembled, they remain bound throughout the integration (*39*). The IN−viral DNA complex is a relevant target for anti-integrase inhibitors in vivo. There are only a few examples of inhibitors discovered to date that are able to bind to IN within its complex with viral DNA. Among them, the most potent are diketo acid derivatives (*25*). Here, we present evidence suggesting that the inhibitory effect of short oligonucleotides conjugated to acridine originates in their capability to target and subsequently to disrupt IN−DNA complexes. In contrast to known G-quartet-forming oligonucleotides, these conjugates do not appear to form any intra- and/or intermolecular secondary structures. Thus, the overall inhibitory effect can be actually attributed to linear single-stranded modified oligonucleotides.

Previous studies using either gel filtration or UV-cross-linking have shown that the stability of IN−DNA complexes may depend on the cationic cofactor present during the interaction of both partners. Mn^{2+} enhance the stability of the complex by stimulating a cooperative binding, which leads to the formation of oligomers as large as octamers on short double-stranded oligonucleotides mimicking the viral substrate (*22*, *32*). In contrast, Mg^{2+}promotes the formation of smaller complexes, which can be reduced to a monomer at 25 °C and to a monomer/dimer equilibrium at 37 °C (*36*). Although the multimeric state of IN in active complexes remains undetermined, Mg^{2+} is believed to the relevant physiological cofactor, and the dimeric form of IN on the

21-bp substrate is likely to represent the active structure of the enzyme. For these reasons, we investigated the effect of the conjugates against the stability of IN−DNA complexes formed in Mg^{2+}-containing buffers. These conditions were similar to the ones used to determine the action of the conjugates on the complex. A steady-state anisotropy assay was used for this purpose. The stability of the IN−DNA complex formed with Mg^{2+} was investigated in the presence of heparin, an anionic polysaccharide that might cause protein−DNA complex dissociation by shifting the equilibrium in the case of the reversible binding of IN to the substrate. The absence of the dissociation following the addition of heparin showed that the IN−viral DNA complex was very stable. Nevertheless, the oligonucleotide−acridine conjugates alters the preassembled complex stability resulting in its rapid dissociation in the presence or absence of heparin, thus indicating that the complex was actively disrupted by oligonucleotide conjugates. The effect of conjugates on the IN−substrate complex stability and activity depends on the simultaneous presence of both the oligonucleotidic and acridine moieties because each isolated moiety displays only a weak dissociating effect. The capacity to disrupt the IN−DNA complex was observed with different oligonucleotide sequences, suggesting that this is a common property of conjugates of any short single-stranded oligonucleotide.

It must be noted the apparent K_d corresponding to the affinity of the conjugate for the complex (500 nM), estimated from the disruption experiment, did not match the IC_{50} evaluated for IN inhibition (see Table 1). Given the fact that IN shows practically no turn over, one would expect both parameters to be comparable if all complexes, formed in vitro and subsequently disrupted by the conjugate, were competent for IN activity. However, it has been previously demonstrated that IN forms at least two different types of complex with DNA in vitro (*40*). Furthermore, it was suggested that both types of complexes are not equally competent for IN activity. Thus, the difference between the apparent K_d and IC_{50} can be explained if the active complexes are more resistant to the conjugate-mediated disruption than the inactive ones. Indeed, in this case, inactive complexes would be disrupted at concentrations for which IN activity is not yet affected. This hypothesis is in agreement with previous observations which suggested that the IN−DNA complexes can be differentiated by their different stability toward single-stranded DNA-mediated disruption (*40*). In any case, because static anisotropy assay does not differentiate between the different complexes, it is difficult to correlate the apparent K_d, obtained with this method, with the IC_{50} measured against IN activity.

The dissociation of the IN−DNA complex can occur in two ways; that is, the conjugate may displace the double-stranded DNA from its binding site in the integrase structure or it may interact with other site(s), altering the integrase conformation and thus resulting in the IN−DNA complex disruption. The comparison of the effects of heparin and the conjugates on the complex stability revealed several interesting features. Heparin inhibited the activity of integrase within the preassembled complex without affecting the integrity of the complex. Thus, heparin does not displace the substrate but probably bind to a different site on the enzyme. In contrast, the oligonucleotide-acridine conjugates being added to the IN−DNA complex induced its rapid and efficient dissociation. In the presence of heparin, these conjugates still disrupted the complex, although the dissociation efficiency was lower than in absence of heparin. It points to the possibility that integrase sites involved in the conjugate and heparin binding may coincide, although these compounds do not display a similar mechanism of inhibition of the IN−DNA complex activity. Indeed, heparin is unable to disrupt the IN−DNA complex at concentrations for which inhibition is observed. It is thus likely that heparin binding to the complex blocks this one in an inactive state, whereas the conjugate binding triggers the complex dissociation.

Electrostatic interactions are sufficient to bind strongly a polyanion to the complex surface, as attested by the inhibitory effect of heparin on the preassembled IN−DNA complex. However, this is not sufficient to trigger dissociation as heparin does not disrupt the complex, even though it interacts with it. Thus, since oligonucleotides are also polyanionic compounds, their inhibitory effect must originate in additional structural features that could allow them to form stronger contacts with IN provoking the complex dissociation. This hypothesis is confirmed by the key role of the oligonucleotide modification: only oligonucleotides conjugated to aromatic hydrophobic molecules (acridine) or containing a modified base (6-oxocytosine (*26*)) are able to provoke IN−DNA complex dissociation. Furthermore, although the activity of the integrase−viral DNA complex was inhibited by conjugates with different oligonucleotide sequences, the elimination of either the hydrophobic (acridine) or the oligonucleotide part resulted in the complete loss of the inhibitory activity. Thus, both the oligonucleotide and hydrophobic domains have a synergetic effect on the ability of the conjugate to interact with IN and to dissociate its complex with DNA. The negatively charged oligonucleotide part probably binds to basic amino acids within IN via predominantly electrostatic contacts, whereas the hydrophobic moiety takes root in the hydrophobic domain of the enzyme anchoring the oligonucleotide. Such interactions between the conjugate and the IN−DNA complex may plausibly lead to conformational changes within the IN structure resulting in the complex dissociation. The same mechanism can be also postulated in the case of 6-oxocytosine-containing oligonucleotides, for which electrostatic contacts might be supplemented by additional hydrogen bonds (*26*).

Our results do not rule out the possibility that modified single-stranded oligonucleotides may also act as competitive inhibitors as it was previously suggested for unmodified oligonucleotides (*13*). Indeed, as the overall inhibitory activity of the oligonucleotide−acridine conjugates was higher when they were tested in competitive conditions than with the preassembled IN−substrate complex (see Table 2), it indicates that their inhibition mechanism may involve in part competitive inhibition.

In conclusion, we believe that the capacity of short modified oligonucleotides to alter the IN−DNA complex stability may have implications for the design of anti-HIV integrase drugs as this complex is likely to be the relevant target in vivo. IN−DNA complexes formed in vitro does not strictly match the physiological preintegration complex, as this one is more stable and is likely to involve viral and/or cellular cofactors which remain to be identified. Therefore, more experiments such as the effect of acridine−oligonucle-

otides against purified PIC activity must be performed to determine whether such compounds may have antiviral potential. Nevertheless, the ability of IN to interact with DNA as well as with hydrophobic molecules opens new possibilities for designing combined compounds possessing properties of both. Modifications of the oligonucleotidic part as well as replacement of acridine by other hydrophobic group should be performed, to overcome such drawbacks of oligonucleotide inhibitors as cytotoxicity, low cellular delivery and stability. The choice of the modifications and hydrophobic groups should be directed toward those that could increase the inhibitory activity of short oligonucleotides. Taking into account some progress reached in the field of inhibition of gene expression by modified antisense oligonucleotides and siRNAs (*41*−*43*), we believe that the further development of oligonucleotide inhibitors as anti-HIV theurapeutic agents should not be excluded.

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