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Probing HIV-1 Integrase Inhibitor Binding Sites with Position-Specific Integrase-DNA Cross-Linking Assays

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ABSTRACT

HIV-1 integrase binds site-specifically to the ends of the viral cDNA. We used two HIV-1 integrase-DNA cross-linking assays to probe the binding sites of integrase inhibitors from different chemical families and with different strand transfer selectivities. The disulfide assay probes cross-linking between the integrase residue 148 and the 5′-terminal cytosine of the viral cDNA, and the Schiff base assay probes cross-linking between an integrase lysine residue and an abasic site placed at selected positions in the viral cDNA. Cross-linking interference by eight integrase inhibitors shows that the most potent cross-linking inhibitors are 3′-processing inhibitors, indicating that cross-linking assays probe the donor viral cDNA (donor binding site). In contrast, strand transfer-selective inhibitors provide weak cross-linking interference, consistent with their binding to a specific acceptor (cellular DNA) site. Docking and crystal structure studies illustrate specific integrase-inhibitor contacts that prevent cross-linking formation. Four inhibitors that prevented Schiff base cross-linking to the conserved 3′-terminal adenine position were examined for inhibition at various positions within the terminal 21 bases of the viral cDNA. Two of them selectively inhibited upper strand cross-linking, whereas the other two had a more global effect on integrase-DNA binding. These findings have implications for elucidating inhibitor binding sites and mechanisms of action. The cross-linking assays also provide clues to the molecular interactions between integrase and the viral cDNA.

HIV-1 integrase is required for insertion of the viral cDNA into host chromosomes. Integrase catalyzes this insertion in two steps. First, an endonucleolytic reaction cleaves both ends of the viral cDNA immediately 3′ from a conserved CA dinucleotide sequence (underlined in Fig. 1A). This releases a terminal 5′-GT dinucleotide from the end of each viral long terminal repeat (LTR) in a reaction called 3′-processing (3′-P). Subsequently, the viral and host DNAs are joined by insertion of both nucleophilic viral cDNA 3′-hydroxyl ends into a host chromosome (termed strand transfer, ST) (Lewinski and Bushman, 2005; Pommier et al., 2005; Van Maele and Debyser, 2005; Marchand et al., 2006).

Despite many attempts, no crystal structure of integrase bound to its DNA substrates has been resolved. Nevertheless, several specific interactions between the viral cDNA and integrase have been revealed biochemically. The deoxyadenosine of the conserved 5′-CA motif immediately 5′ to the 3′-P site (referred to as 3′-A; see Fig. 1A) contacts integrase near residue Lys-159 as shown by photocross-linking of a substituted 5-iododeoxyuracil at this position (Jenkins et al., 1997). A crystal structure of integrase suggested that Lys-159 also contacts the phosphate 5′ to the conserved 3′-A (Wang et al., 2001). Photocross-linking indicated contacts between integrase residues Tyr-143 and Gln-148 and the adenine at the 5′-end of the uncleaved strand (Esposito and Craigie, 1998). We have reported, after using disulfide cross-linking, the proximity of the integrase amino acid residue 148 (mutated from glutamine to cysteine) to the second base (referred to as the 5′-C) from the 5′ end of the "lower strand" of the duplex (thiol-modified C; see Fig. 1A) (Gao et al., 2001; Johnson et al., 2006a). Given the lack of cocrystal structures, such information is important for understanding interactions between integrase and the viral cDNA.

Many integrase inhibitors have been discovered during the past 10 years, with two of them presently in clinical trials (for

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ABBREVIATIONS: LTR, long terminal repeat; 3′-P, 3′-processing; MOPS, 4-morpholinepropanesulfonic acid; DKA, diketo acid; L-CA, L-chicoric acid repeat; ST, strand transfer.

review, see Pommier et al., 2005; Dayam et al., 2006; DeJesus et al., 2006; Semenova et al., 2006b). The binding sites of only two inhibitors, 5CITEP and Y-3, have been determined by X-ray crystallography (Lubkowski et al., 1998; Goldgur et al., 1999). Integrase inhibitors can be classified as those that inhibit both 3′-P and ST reactions (defined as 3′-P inhibitors) and those that inhibit ST efficiently but have little or no effect on 3′-P (defined as ST-selective inhibitors). ST-selective inhibitors, such as diketo acid and naphthyridine carboxamide derivatives (Hazuda et al., 2000, 2004a), have been proposed to bind at the interface of the integrase-DNA complex (Pommier et al., 2005). As part of our ongoing effort to characterize the molecular interactions between integrase, inhibitors, and the U5 LTR viral cDNA end (Johnson et al., 2004, 2006a,b), here we describe the use of disulfide (Johnson et al., 2006a) and Schiff base cross-linking (Mazumder et al., 1996) to examine interference upon cross-link formation of various integrase inhibitors.

For the disulfide cross-linking assay, we used site-directed mutagenesis to create a Gln-148-Cys integrase mutant that forms a disulfide cross-link with the 5′-C of the U5 LTR (Johnson et al., 2006a) (see Fig. 1). Integrase-mediated 3′-P releases a terminal 5′-GT dinucleotide, resulting in a 5′-AC overhang. This overhang is required for ST (Bushman and Craigie, 1992). We recently proposed that a hydrogen bond between integrase Gln-148 and the 5′-C is required for ST in the presence of magnesium (Johnson et al., 2006a). Because the two interacting groups [residue 148 of integrase and the second base from the 5′ end of the LTR (the 5′-C)] are defined, we reasoned that valuable information could be obtained about drug binding site(s) from interference of protein/DNA interactions in the presence of inhibitors (Johnson et al., 2006a).

To further examine drug-DNA interactions with integrase, we also used a Schiff base cross-linking assay by inserting a reactive abasic site at various positions within the viral cDNA substrate (Mazumder et al., 1996). A covalent linkage between an integrase lysine residue and the amino-reactive abasic site (generated by uracil DNA glycosylase) enables formation of a Schiff base that can be reduced by sodium borohydride (Mazumder et al., 1996) (Fig. 1C). The position of the abasic site can be varied to examine cross-link positional effects. The position of the cross-linking lysines can be estimated from structural and molecular modeling studies.

We demonstrate that potent 3′-P inhibitors are also the most potent cross-linking inhibitors, whereas ST-selective inhibitors such as L-708,906 (Hazuda et al., 2000) and L-870,810 (Hazuda et al., 2004a) are weak inhibitors of cross-linking. Inhibition of the specific contacts required for disulfide and Schiff base cross-linking is supported by inhibitor-integrase contacts identified through crystal structures and docking studies for several inhibitors. Results from the Schiff base cross-link assays using an abasic site at defined positions suggest that 3′-P inhibitors interfere with the binding of integrase at sites distal from the active site. The findings reported here indicate the value of cross-linking assays to gain insight into inhibitor binding sites and interactions between integrase and the viral cDNA.

Materials and Methods

Oligonucleotide Synthesis. Unmodified and uracil-containing (for Schiff base cross-linking studies) oligonucleotides were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). For

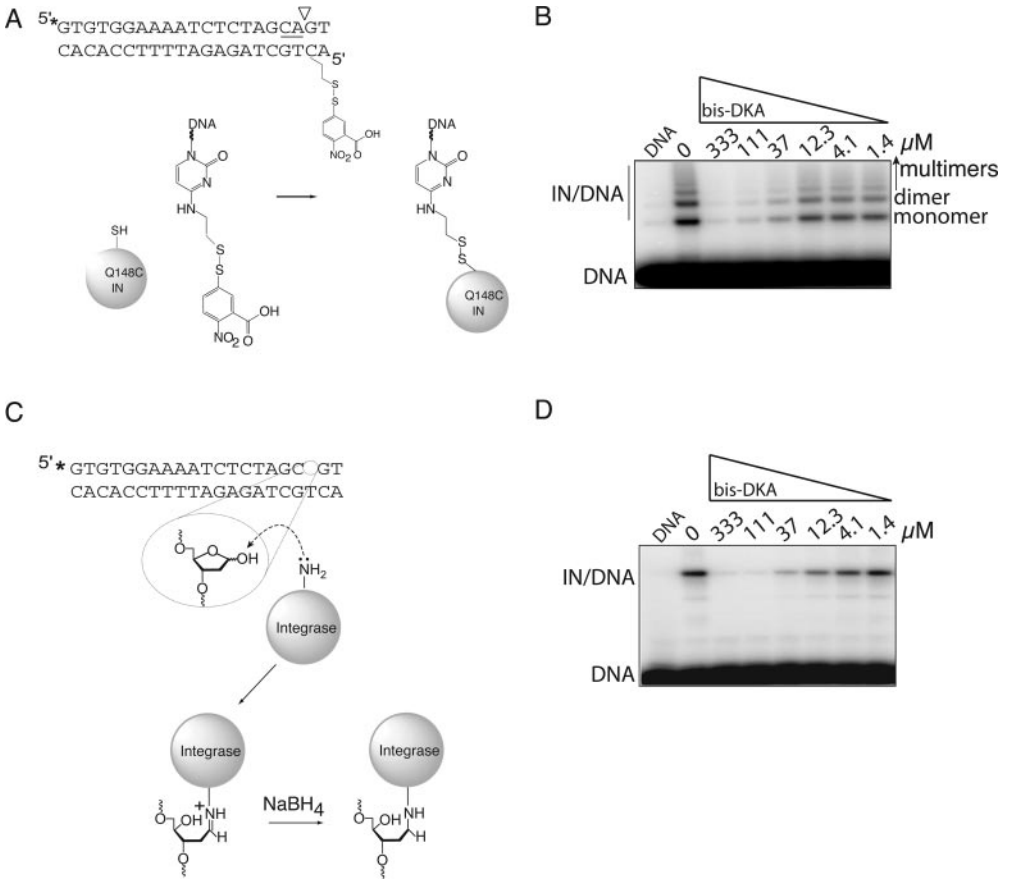


Fig. 1. Principle of the two integrase-viral cDNA cross-linking assays (Mazumder et al., 1996; Johnson et al., 2006a) and inhibition by the integrase inhibitor, bis-DKA (Marchand et al., 2002; Pais et al., 2002). A, schematic diagram illustrating disulfide cross-link formation between a thiol-modified cytosine and an integrase Gln-148-Cys mutant. The star indicates the position of ^{32}P labeling. B, representative gel showing inhibition of disulfide cross-link formation by the bis-DKA inhibitor (see chemical structure in Fig. 4). Drug concentrations are indicated above each lane. The samples are not boiled before nonreducing SDS-PAGE resulting in a ladder of integrase multimers cross-linked to DNA. C, schematic diagram illustrating Schiff base cross-link formation. The DNA substrate contains an abasic site (circle) at the 3′-A position created by uracil DNA glycosylase. An imine linkage forms between an integrase lysine residue and the aldehydic abasic site and is stabilized by sodium borohydride. The star indicates the position of ^{32}P labeling. D, representative gel showing inhibition of Schiff base cross-linking by bis-DKA. Drug concentrations are indicated above each lane.

disulfide cross-linking, an oligonucleotide containing the convertible nucleoside *O*4-triazole-dU was synthesized by Midland Certified Reagent Company, Inc. (Midland, TX). A two-carbon cystamine tether was added postsynthetically using the convertible nucleoside approach (Ferentz and Verdine, 1991), and the resulting cystamine-modified oligonucleotide was gel-purified. The cystamine was reduced with 100-fold molar excess of DTT for 20 min at 37°C and purified using a mini Quickspin Oligo column (Roche, Indianapolis, IN). The thiol modification was activated by the addition of a 10-fold molar excess of 5,5′-dithiobis(2-nitrobenzoic acid) in phosphate buffer, pH 8.5, for 1 h at 37°C. The activated DNA was purified by a mini Quickspin Oligo column (Roche, Indianapolis, IN).

All oligonucleotides were purified on denaturing 20% polyacrylamide gels. Single-stranded oligonucleotides were 5′-labeled using T4 polynucleotide kinase (Invitrogen, Carlsbad, CA) with [γ-^{32}P]ATP (PerkinElmer Life and Analytical Sciences, Boston, MA) according to the manufacturers' instructions. Unincorporated nucleotides were removed by mini Quickspin Oligo columns (Roche). The duplex DNA was annealed by addition of an equal concentration of the complementary strand, heating to 95°C, and slow cooling to room temperature.

Inhibitors. L-708,906, L-870,810, 5CITEP, bis-DKA and L-chicoric acid were synthesized as described previously (Zhao and Burke, 1998; Lin et al., 1999; Anthony et al., 2002; Pais et al., 2002; Hazuda et al., 2004a). Compounds RDS-1997 and RDS-1625 were synthesized by the Di Santo group (Di Santo et al., 2005, 2006). Conocurvone was obtained as a gift from Dr. Jonathan Coates of AMRAD Operations Pty Ltd (Victoria, Australia). All drug solutions were prepared from powder in 100% dimethyl sulfoxide. Drug concentrations producing 50% inhibition (IC_{50} values, Table 1) are from previous reports (Marchand et al., 2002; Di Santo et al., 2006; Johnson et al., 2006b; Stagliano et al., 2006) or were determined as described below. Note that all the compounds shown display anti-HIV activity, except for bis-DKA and 5CITEP (summarized with references in Table 1).

Production of Recombinant Integrase. Recombinant wild-type HIV-1 integrase was purified from *E. coli* as described previously (Leh et al., 2000) with the addition of 10% glycerol to all buffers. Wild-type integrase was used for Schiff base and standard catalytic assays. Site-directed mutagenesis was used to create the integrase mutant Q148C/C56S/C65S/C280S ["QSSS," described previously by Johnson et al. (2006a)] for disulfide cross-linking. The three cysteine-to-serine mutations provide a cross-linking-free background before the addition of the desired cysteine at position 148 (Johnson et al., 2006a). The QSSS mutant was purified using the same method as wild type but with the omission of reducing agents from all buffers after bacterial lysis.

Schiff Base Cross-Linking Assay. Oligonucleotides containing a single uracil at the indicated positions (see Fig. 5) were 5′-[^{32}P]-labeled as described above. After annealing, uracil DNA glycosylase was added to create an abasic site at the uracil position. The abasic site forms a Schiff base cross-link between the deoxyribose aldehyde group and a nearby integrase lysine.

The Schiff base cross-linking experiments were performed as described previously (Mazumder et al., 1996). Inhibitors were preincubated for 20 min at room temperature with 500 nM wild-type integrase, 7.5 mM $MgCl_2$, 14 mM 2-mercaptoethanol, and 20 mM MOPS, pH 7.2. Abasic-site containing duplex DNA (final concentration, 20 nM) was added to each reaction and incubated at room temperature for 5 min. The cross-links were reduced by adding 100 mM sodium borohydride (final concentration) before the addition of tricine-SDS gel loading buffer (1× final concentration). The cross-linked integrase-DNA products were separated from the substrate DNA by SDS-PAGE using 16% tricine gels (Invitrogen, Carlsbad, CA).

Disulfide Cross-Linking. Q148C/SSS integrase (500 nM) was incubated with inhibitors in the presence of 20 mM Tris, pH 7.4, 7.5 mM $MgCl_2$ and 10% glycerol for 20 min at room temperature. DNA duplexes (20 nM) containing a 5′-^{32}P label on the "upper strand" and a thiol-modified cytosine on the other strand (see Fig. 1A) were added to each reaction. The thiol-activated DNA was not labeled itself to avoid reduction of the thiol modification during the labeling reaction. After 2 min, reactions were capped by the addition of 20 mM methylmethanethiosulfonate (MMTS). Nonreducing gel loading buffer (100 mM Tris-Cl, pH 6.8, 4% SDS, 0.2% bromphenol blue, and 20% glycerol) was added and samples were loaded directly onto 16% polyacrylamide gels (Invitrogen). Dried gels were visualized using a 445 SI PhosphorImager (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Quantitation was performed by densitometric analysis using the ImageQuant software from GE Healthcare.

Integrase Reactions. Integrase was incubated with DNA substrates for 20 min on ice. The reaction conditions were 500 nM integrase, 20 nM duplex DNA, 7.5 mM $MgCl_2$, 5 mM NaCl, 14 mM 2-mercaptoethanol, and 20 mM MOPS, pH 7.2. Nine microliters of integrase-DNA mixture was aliquoted into tubes containing 1 μl of inhibitor, and the reactions proceeded at 37°C for 1 h. Reactions were quenched by the addition of an equal volume of gel loading dye (formamide containing 1% SDS, 0.25% bromphenol blue, and xylene cyanol). Products were separated on 20% polyacrylamide denaturing sequencing gels. Dried gels were visualized using a 445 SI PhosphorImager (GE Healthcare). Densitometric analysis was performed using ImageQuant software from GE Healthcare.

Statistical Analysis. Correlations between inhibition of the two cross-linking assays and integrase catalytic assays were examined using one-tailed Pearson's correlation coefficients for each compari-

TABLE 1
Summary of IC_{50} values

Inhibitor	S-S	Schiff Base	Schiff/S-S ratio	3′-P	ST	3′-P/ST Ratio	Antiviral Activity
	μM			*μM*			
L-708,906	300.0	113.0	0.4	2.5[a]	0.06[a]	42	Yes[b]
L-870,810	500.0	500.0	1.0	>12.3	0.06	>205	Yes[c]
5CITEP	13.0	280.0	22.0	400[a]	97[a]	4	No[d]
bis-DKA	22.0	6.2	0.3	110[a]	10[a]	11	No[d]
RDS-1997	1.0	6.3	6.0	0.4[e]	0.02[e]	20	Yes[e]
RDS-1625	>333.0	48.0	<0.2	4.4	0.06	73	Yes[e]
Conocurvone	6.9	129.0	19.0	1.0[f]	1.0[f]	1	Yes[g]
L-Chicoric acid	0.1	4.8	48.0	0.4[i]	0.19[i]	2	Yes[j]

S-S, disulfide cross-linking.
[a] Marchand et al. (2002).
[b] Hazuda et al. (2000).
[c] Hazuda et al. (2004b).
[d] Pais et al. (2002).
[e] Di Santo et al. (2005, 2006).
[f] Stagliano et al. (2006).
[g] Decosterd et al. (1993).
[i] IC_{50} values for 3′-P and ST were obtained in the presence of manganese (Johnson et al., 2006b).
[i] Johnson et al. (2006b).
[j] King et al. (1999); Lin et al. (1999).

son. The Pearson's r values are listed in Fig. 6. The P values are listed in the legend.

Results

Inhibition of Disulfide Cross-Link Formation by Integrase Inhibitors. The assay is based on the formation of a disulfide bond between residue 148 (Gln-148-Cys mutant) within the integrase catalytic core domain and a thiol-modified cytosine located at the tip (5′-C) of the HIV LTR (Johnson et al., 2006a) (Fig. 1A). We recently reported the functional importance of this interaction, and proposed that a hydrogen bond between integrase Gln-148 and the 5′-C is required for ST in the presence of magnesium (Johnson et al., 2006a). The cross-linking complexes between integrase and the ^{32}P-labeled-DNA are observed as a ladder of oligomeric integrase products in nonreducing SDS-PAGE gels (Fig. 1B). Figure 1B demonstrates inhibition of disulfide cross-linking by the bis-DKA derivative (see chemical structure in Fig. 4), which acts as a potent 3′-P inhibitor (inhibitor of both 3′-P and ST; see Introduction) (Marchand et al., 2002).

Inhibition of Schiff Base Formation by Integrase Inhibitors. The Schiff base-mediated cross-linking assay detects covalent bonds between integrase and a DNA abasic site substitution. In the experiment shown in Fig. 1, the abasic site is placed at the position of the conserved adenine of the HIV LTR (Mazumder et al., 1996) (Fig. 1C). The 3′-side of the adenine is the site of 3′-P, providing the 3′-OH nucleophile required for ST. The formation of an integrase-DNA complex is observed as a band with retarded migration in SDS-PAGE gels (Fig. 1D) (Mazumder et al., 1996). Figure 1D shows inhibition of Schiff base cross-linking by the bis-DKA.

Integrase Inhibition Activities of L-870,810. Integrase ST-selective inhibitors are being pursued for clinical development (Hazuda et al., 2004a,b; Pommier et al., 2005; Dayam et al., 2006; DeJesus et al., 2006; Semenova et al., 2006a). The naphthyridine carboximide L-870,810 (Fig. 2A) has been reported as a highly ST-selective inhibitor (Hazuda et al., 2004a). However, actual inhibition experiments have never been reported. Figure 2 (B and C) shows that L-870,810 is indeed a potent ST-selective inhibitor. IC_{50} values for ST and 3′-P are 0.06 and >12.3 μM, respectively, giving a selectivity ratio of at least 200-fold (Fig. 2C and Table 1). Figure 2 also shows that L-870,810 is a weak inhibitor in both disulfide and Schiff base cross-linking assays (D and E, respectively). The contrast between the results obtained with L-870,810 and the bis-DKA suggests that ST-selective inhibitors produce minimal interference with viral cDNA binding as determined by cross-linking inhibition assays.

Integrase Inhibition Activities of RDS-1625. The monofunctional diketo acid RDS-1625 (Fig. 3A) is structurally related to the previously reported bifunctional DKA RDS-1997 (Di Santo et al., 2006) (see Fig. 4). Both compounds are potent ST inhibitors (Di Santo et al., 2006) (Fig. 3, B and C, and Table 1). However, the second DKA group of RDS-1997 also provides potent inhibition of 3′-P (Di Santo et al., 2006) whereas RDS-1625 has a 70-fold selectivity for ST (Fig. 3, B and C, and Table 1). Consistent with the results obtained for L-870,810 (see Fig. 2), we find that RDS-1625 is a weak inhibitor of either disulfide or Schiff base cross-linking assays (Fig. 3, D and E), suggesting the ST-selective inhibitor RDS-1625 may bind in a similar region as L-870,810.

Comparison of Different Inhibitors in the Disulfide and Schiff Base Cross-linking Assays. Eight integrase inhibitors from different families and with differential selectivity for ST and 3′-P were evaluated in the disulfide and

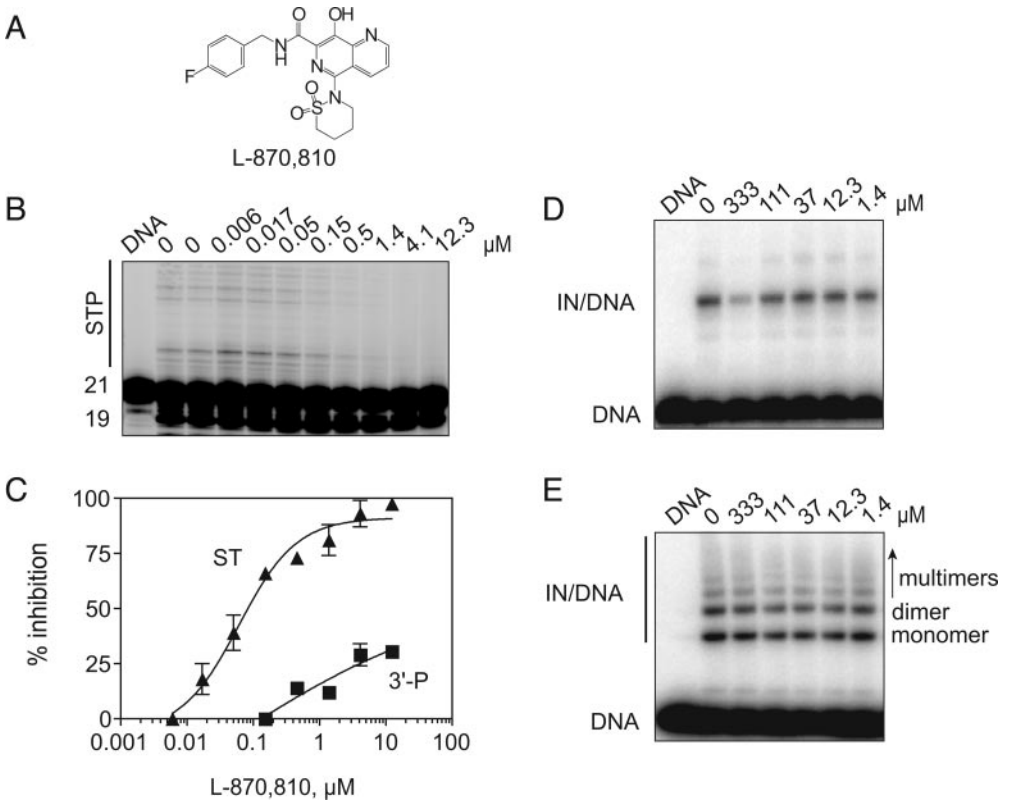


Fig. 2. Inhibition of integrase activities and cross-linking by L-870,810. A, structure of L-870,810. B, representative gel showing the potent and selective inhibition of integrase ST by L-870,810. C, quantification of 3′-P and ST inhibition by L-870,810. D and E, representative gels showing inhibition of Schiff base and disulfide cross-linking by L-870,810, respectively. Error bars correspond to S.D. for at least three independent experiments.

Schiff base cross-linking assays. Figure 4 shows the dose-response curves, and Table 1 summarizes the IC_{50} values for inhibition of 3′-P, ST, disulfide, and Schiff base cross-linking. The most ST-selective inhibitors include the DKA derivative L-708,906 (Hazuda et al., 2000, 2004b; Grobler et al., 2002; Marchand et al., 2002, 2003; Pais et al., 2002), the naphthy-

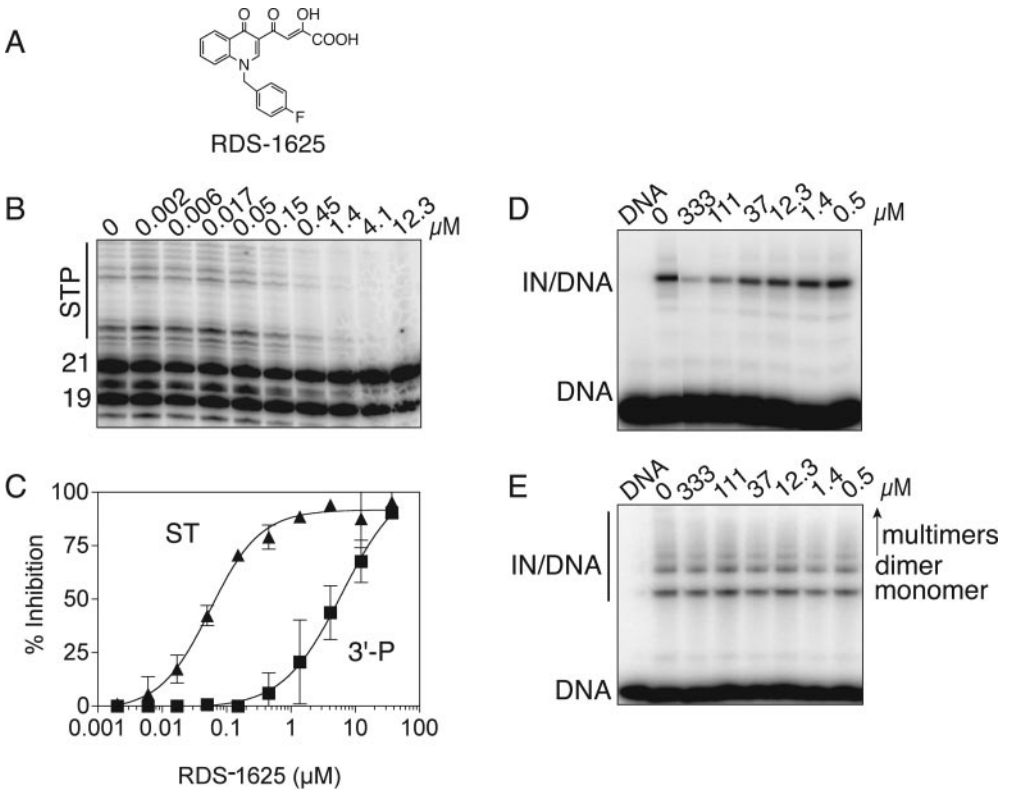


Fig. 3. Inhibition of integrase activities and cross-linking by RDS-1625. A, structure of RDS-1625. B, representative gel showing the selective inhibition of integrase ST by RDS-1625. C, quantification of 3′-P and ST inhibition by RDS-1625. D and E, representative gels showing inhibition of Schiff base and disulfide cross-link formation by RDS-1625, respectively. Error bars correspond to S.D. for at least three independent experiments.

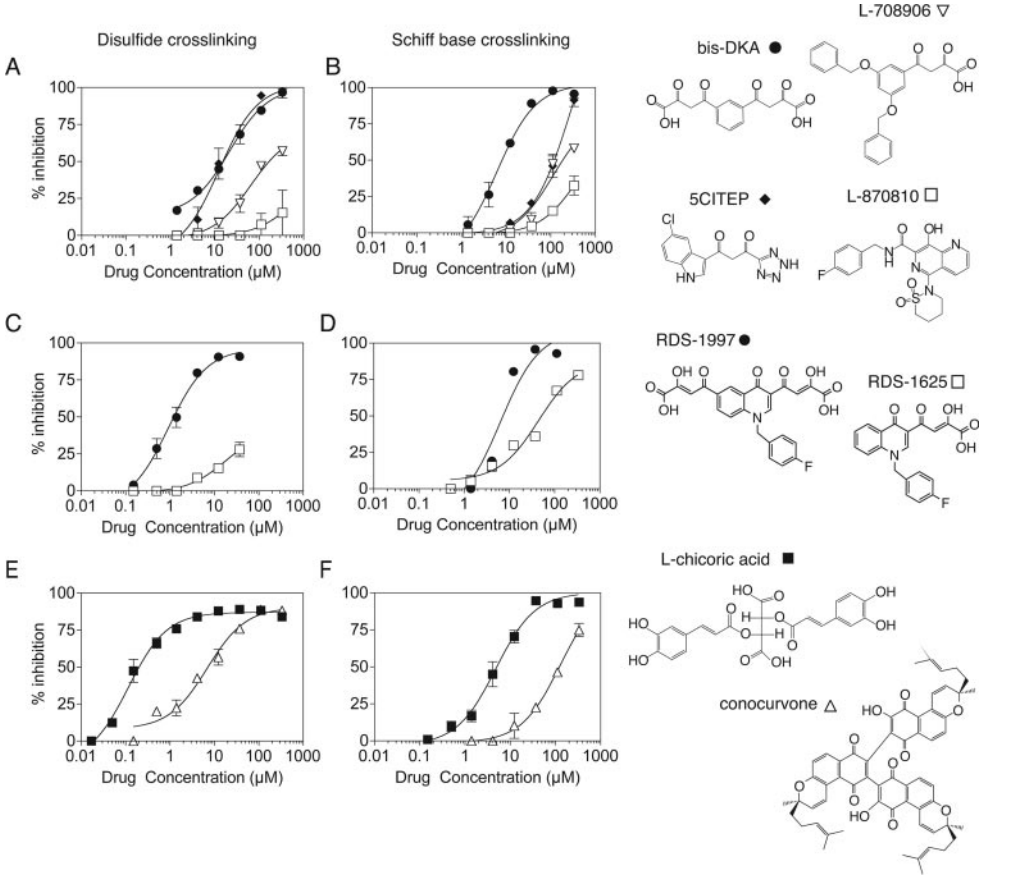


Fig. 4. Comparison of integrase-DNA cross-linking inhibition by various integrase inhibitors: A and B, bis-DKA, 5CITEP, L-708,906, and L-870,810. C and D, DKA derivatives RDS-1625 and RDS-1997. E and F, natural products L-chicoric acid (L-CA) and conocurvone. Symbols for each drug are indicated next to the drug names at right. Error bars correspond to S.D. for at least three independent experiments.

ridine carboxamide L-870,810 (Fig. 2) (Hazuda et al., 2004a), RDS-1625 (Fig. 3), and, to a lesser extent, RDS-1997 (Di Santo et al., 2006). The 3′-P inhibitors (dual inhibitors of ST and 3′-P, Table 1) include conocurvone (Stagliano et al., 2006), L-chicoric acid (King et al., 1999; Pais et al., 2002), bis-DKA (Marchand et al., 2002; Marchand et al., 2003), and 5CITEP (Marchand et al., 2002; Marchand et al., 2003). Indeed the ST-selectivity of 5CITEP is markedly reduced when the divalent metal cofactor is switched from Mn^{2+} to Mg^{2+} (Marchand et al., 2003).

As described previously in Fig. 1, the bis-DKA is a relatively potent inhibitor of both cross-link assays (Fig. 4, A and B, ●). By contrast, 5CITEP is a potent inhibitor of the disulfide cross-link (Fig. 4A, ♦) (Johnson et al., 2006a) and a relatively weak inhibitor of Schiff base cross-linking (Fig. 4B, ♦). In comparison, L-708,906 (Fig. 4, A and B, ▽) and L-870,810 (Fig. 4, A and B, □) are weak inhibitors of both disulfide and Schiff base cross-linking assays. Therefore, the DKA and naphthyridine carboxamide derivatives illustrate differences in cross-linking inhibition among the ST-selective inhibitors.

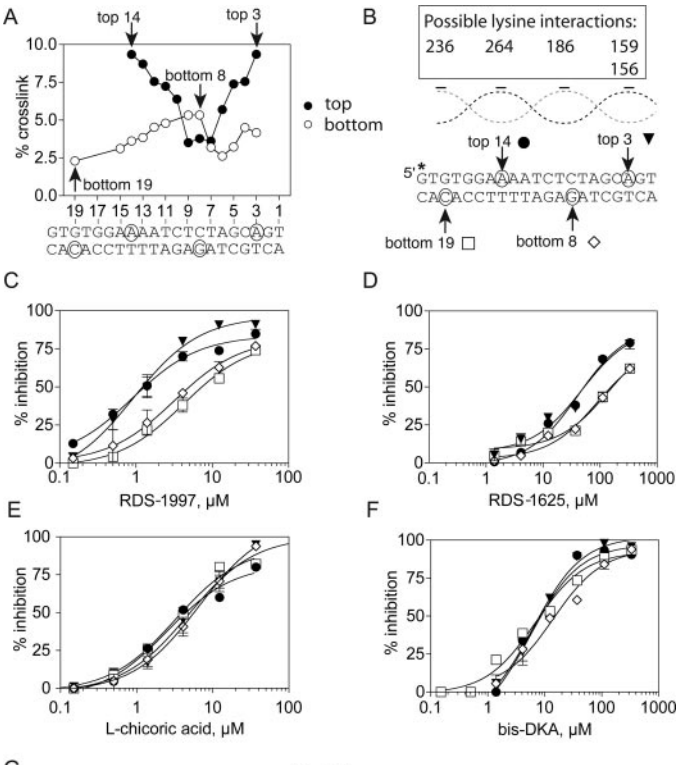
The DKA RDS-1997 (Fig. 4, C and D, ●) containing two diketo acid moieties (similar to the bis-DKA) inhibits both disulfide and Schiff base cross-linking efficiently compared with RDS-1625 (Fig. 4, C and D, □), which contains only one DKA moiety. Thus, addition of a second DKA group (as in bis-DKA and RDS-1997) provides interactions between integrase and the viral cDNA as determined by cross-linking inhibition.

Finally, 3′-P inhibitors (dual inhibitors of ST and 3′-P) showed drug-specific differences. The synthetic dicaffeoyl derivative L-chicoric acid (L-CA) (Zhao and Burke, 1998; King et al., 1999; Lin et al., 1999) (Fig. 4, E and F, ■) is the most potent inhibitor of disulfide cross-linking among the eight compounds examined (compare E, C, and A in Fig. 4; Table 1). L-CA is also among the most potent inhibitors of Schiff base cross-link formation. The natural product conocurvone (Stagliano et al., 2006) (Fig. 4, E and F, △) is a relatively potent inhibitor of disulfide cross-link formation and a weaker inhibitor of Schiff base cross-linking.

Effects of Schiff Base Cross-Linking Position within the Substrate DNA. First, we scanned the integrase-DNA contacts in the absence of drug using different Schiff base substrates containing a single abasic site at various positions. Figure 5A summarizes multiple experiments where cross-linking efficiency was examined over the indicated positions of the terminal 21 base pairs of the U5 LTR DNA. In the absence of drug, cross-linking to the "top" strand of the duplex results in two cross-linking peaks at the 3rd and 14th positions from the end of the viral cDNA (Fig. 5A, ●). Cross-linking to the "bottom" strand (○) is weaker in general, and results in maximum cross-linking at the 8th position. The highest efficiency sites for each strand (top 3 and 14 and bottom 8) lie on the same side of a double helix in standard B-form DNA and the lysines likely to interact with each cross-link position are noted in Fig. 5B (Karki et al., 2004).

The three positions giving maximum cross-linking (top 3, top 14, and bottom 8) plus the 19th position of the bottom strand (Fig. 5, A and B) were then used to examine Schiff base cross-linking inhibition as a function of the cross-linking position. The drugs producing inhibition of Schiff base cross-linking at the 3rd position (RDS-1997, RDS-1625, bis-DKA, and L-chicoric acid; see Figs. 1–4) were tested for inhibition of Schiff base cross-linking at these four positions (Fig. 5). RDS-1997 and RDS-1625 inhibit Schiff base cross-linking more effectively at the top 3rd and 14th positions (Fig. 5, C and D; ▼ and ●, respectively) compared with the bottom 8th and 19th positions (Fig. 5, C and D; ◇ and □, respectively). This differential inhibition was not observed with bis-DKA or L-chicoric acid (Fig. 5, E and F). These results suggest that RDS-1997 and RDS-1625 tend to interfere preferentially with upper strand cross-linking, whereas L-chicoric acid and bis-DKA inhibit overall viral cDNA binding.

Comparison of 3′-P and ST Inhibition and Cross-Linking Inhibition for Integrase Inhibitors. Figure 6 shows graphical comparisons of inhibition parameters to highlight differences in inhibitor potencies. A is a plot of the 3′-P versus ST IC_{50} values for the eight compounds tested. Conocurvone and L-chicoric acid show no selectivity for ST and therefore sit on the X = Y line (3′-P = ST). By contrast, L-870,810 is the most ST-selective inhibitor studied (see Fig. 2 and Table 1), and is the furthest from the X = Y line. The



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IC_{50}, μM

Position	RDS-1997	RDS-1625	bis-DKA	L-chicoric acid
▼ top 3	0.93	48.3	6.3	4.8
◇ bottom 8	3.2	90.9	13.9	6.6
● top 14	0.91	41.1	5.6	2.5
□ bottom 19	4.2	139.5	6.9	3.2

Fig. 5. Effect of position of the abasic site for Schiff base cross-linking and inhibition by integrase inhibitors. A, summary of cross-linking efficiency as a function of the position for each of the abasic sites. B, DNA sequence of the viral cDNA substrate highlighting the four positions examined for cross-linking inhibition. The lysines likely interacting with each region are noted at the top. C to F, graphs showing inhibition of Schiff base cross-linking at each of the four positions by RDS-1997, RDS-1625, bis-DKA, and L-chicoric acid, respectively. G, summary of IC_{50} values derived from C to F. Error bars correspond to S.D. for at least three independent experiments.

novel DKA derivative RDS-1997 is the most potent inhibitor of ST (IC_{50} of 0.02 μM) but is also one of the most potent inhibitors of 3′-P (IC_{50} of 0.4 μM; see Fig. 2 and Table 1). The ratio for 3′-P/ST selectivity is listed in Table 1, where a high value indicates true ST-selectivity.

The plot of Schiff base versus disulfide cross-linking inhibition (Fig. 6B) illustrates an overall positive correlation between inhibition of Schiff base and disulfide cross-linking for the eight compounds studied ($r = 0.73$, $p < 0.05$). L-chicoric acid is the best inhibitor in both assays, whereas L-708,906 and L-870,810 fail to block either cross-linking reaction effectively. L-chicoric acid, RDS-1997, conocurvone, and 5CITEP inhibit disulfide cross-linking at least 10 times more efficiently than Schiff base cross-linking (Fig. 6B).

Pair-wise comparisons for inhibition of cross-linking (in each of the two assays) versus inhibition of integrase reactions (3′-P and ST) are shown in Fig. 6, C to F. Potent 3′-P inhibitors (L-chicoric acid, RDS-1997, and conocurvone) effectively blocked viral cDNA binding (cross-linking), whereas ST-selective inhibitors with weak inhibition of 3′-P (L-708,906 and L-870,810) had minimal effect on viral cDNA binding.

Discussion

Inhibition of integrase-DNA cross-linking demonstrates interference with viral cDNA binding. In particular, inhibition of disulfide cross-linking indicates that an inhibitor prevents the normal interaction between the integrase 148 residue and the conserved 5′-cytosine (5′-C) two bases from the end of the viral cDNA (Johnson et al., 2006a). Inhibition of cross-linking via Schiff base formation at an abasic site placed at the A position of the conserved CA (3′-A, position top 3 in Fig. 5B) indicates that an inhibitor blocks bond formation between integrase residues Lys-156 and Lys-159 (presumably) and the DNA at the 3rd position from the end of the viral cDNA. Because the 3′-A and 5′-C belong to adjacent base pairs, cross-linking assays can be used together to probe drug binding sites with precision. Moreover, because both contacts are important for specific recognition of the viral LTR cDNA end and integrase activity (Mazumder and Pommier, 1995; Johnson et al., 2006a), it is plausible that alteration of these interactions is the primary mechanism of action for 3′-P inhibitors such as L-chicoric acid, bis-DKA, conocurvone, and RDS-1997 (see Fig. 6B).

L-Chicoric acid is the most potent inhibitor of disulfide cross-linking, with approximately 50-fold selectivity for disulfide over Schiff base cross-linking (Fig. 6B, Table 1). Consistent with this result, docking experiments suggest that L-chicoric acid forms a hydrogen bond with Gln-148 (Sotriffer et al., 2000). Moreover, HIV in cell culture gains a Gly-140-Ser resistance mutation when cultured with L-chicoric acid for extended periods (King and Robinson, 1998). The finding that L-chicoric acid also inhibits Schiff base cross-linking with the conserved 3′-A is also consistent with docking experiments showing interactions of chicoric acid with Lys-156 and Lys-159 (Sotriffer et al., 2000). Because L-chicoric acid inhibits Schiff base cross-linking irrespective of the position of the abasic site from the end of the viral cDNA (Fig. 5E), it is likely that L-chicoric acid binds near residues ranging from

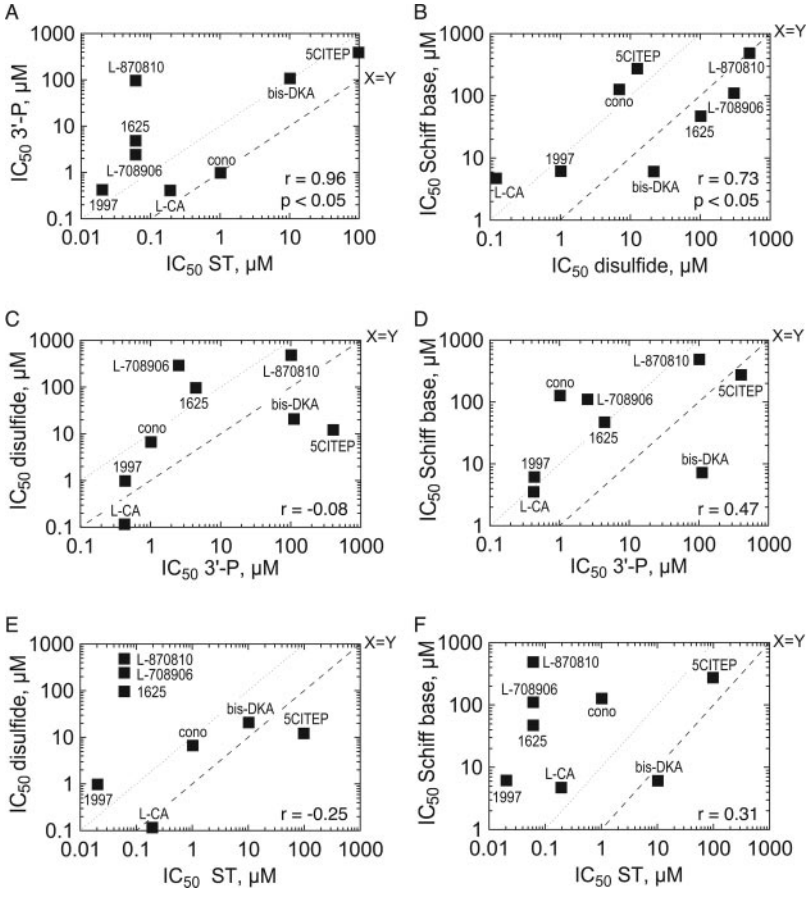

Fig. 6. Correlations between inhibition for each of the two cross-linking assays and the two integrase reactions (IC_{50} values are represented as paired-scattered plots). A, 3′-P versus ST inhibition. B, Schiff base versus disulfide cross-linking inhibition. C, disulfide cross-linking versus 3′-P inhibition. D, Schiff base cross-linking versus 3′-P inhibition. E, disulfide cross-linking versus ST inhibition. F, Schiff base cross-linking versus ST inhibition. The dashed lines indicate equivalent inhibition (X = Y). Pearson's one-tailed correlation coefficients are indicated for each comparison at the bottom right of each. The *P* values are 0.0002 and 0.0389 for A and B, respectively.

140 to 160 and produces global alterations of the integrase-viral cDNA interactions.

Bis-DKA is a 3′-P inhibitor that inhibits both the integrase 3′-P and ST reactions (Fig. 6A and Table 1) (Marchand et al., 2002), as well as both disulfide and Schiff base cross-linking within the same concentration range (Fig. 6B). We also find that bis-DKA inhibits Schiff base cross-linking over several positions of the viral cDNA (Fig. 5F). In agreement with these results, bis-DKA has been proposed to interfere with both the acceptor and donor DNA binding sites of integrase (Marchand et al., 2002). Docking of bis-DKA to an integrase-DNA-Mg^{2+} complex suggests that bis-DKA extends from the metal chelation site toward the viral LTR conserved 3′-A (Marchand et al., 2003). Together, these results suggest that bis-DKA, like L-chicoric acid, has a global effect on integrase-DNA binding.

5CITEP is one of two integrase inhibitors cocrystallized with integrase (Lubkowski et al., 1998; Goldgur et al., 1999). 5CITEP is a ST-selective inhibitor in the presence of Mn^{2+}. However, this selectivity is markedly reduced in the presence of Mg^{2+} (Table 1) (Marchand et al., 2003). 5CITEP was cocrystallized in the integrase catalytic site in the absence of donor DNA (Goldgur et al., 1999). In this cocrystal, 5CITEP contacts Gln-148 of the integrase flexible loop (Greenwald et al., 1999). As 5CITEP inhibits cross-linking of this same amino acid residue 148 in our disulfide cross-linking assay (Fig. 4A), contacts between 5CITEP and the flexible loop probably play an important role for the integrase inhibition. 5CITEP also exhibits approximately 22-fold selectivity for disulfide cross-linking inhibition over Schiff base cross-linking inhibition with the abasic site placed at the conserved 3′-A position (Fig. 6B). Because the 3′-A position corresponds to the base pair adjacent to the 5′-C, these results suggest that 5CITEP binds near the 5′-C and Gln-148 of integrase but has only minor interaction with the 3′-A and Lys-159/156. Such a selective inhibition of disulfide cross-linking versus Schiff base cross-linking is consistent with the cocrystal structure (Goldgur et al., 1999).

Conocurvone is a dual inhibitor of 3′-P and ST with antiviral activity (Stagliano et al., 2006). We find that conocurvone inhibits disulfide cross-linking in the same concentration range as for 3′-P and ST inhibition (Fig. 6, A and C, and Table 1). Because conocurvone exhibits an approximately 20-fold selectivity for disulfide versus Schiff base cross-linking, it is likely that conocurvone preferentially interferes with and inhibits the contacts between integrase Gln-148 and the terminal 5′-C of the viral cDNA. As conocurvone inhibits 3′-P, it is a candidate for cocrystallization with the core domain of integrase.

Among the diketo acid derivatives, RDS-1997 is the only compound with significant cross-linking inhibition (Fig. 6B). RDS-1997 is also the most potent inhibitor of both 3′-P and ST (Fig. 6A) (Di Santo et al., 2006). Docking with integrase suggests that RDS-1997 interacts with Lys-156 and Lys-159 (Di Santo et al., 2006), the lysines that are thought to form Schiff base cross-linking with the abasic site substitution at the conserved 3′-A position. Scanning of Schiff base cross-linking inhibition with RDS-1997 showed strongest inhibition when the abasic site is placed at the 3′-A position (Fig. 5C). Hence, we propose that RDS-1997 binds near and interacts with Lys-156/159. Because RDS-1997 is an effective 3′-P inhibitor with remarkable inhibition of Schiff base cross-linking at the 3′-A position, this compound might be a good candidate for cocrystallization with the integrase core domain.

The ST-specific inhibitors L-870,810 and L-708,906 dock within the integrase active site by extending away from the region containing Lys-156 and Lys-159 (Marchand et al., 2002; Marchand et al., 2003; Hazuda et al., 2004a). This is probably the case for RDS-1625, which is also a ST-specific inhibitor (Figs. 3 and 6A). All three compounds are weak inhibitors of cross-linking both in the disulfide and Schiff base assays (Fig. 6B). They may bind the integrase-DNA-metal complex in an orientation that does not interfere with viral DNA binding and 3′-P. Selective inhibitors of ST are therefore inefficient cross-link inhibitors because they may have a separate set of binding interactions, such as with the acceptor DNA site and integrase, enabling specificity for ST inhibition. Thus, it is possible that L-708,906, L-870,810, and RDS-1625 prevent ST by blocking acceptor DNA binding.

The experiments presented here provide novel information for drug binding sites in the integrase-viral cDNA complex. In the absence of integrase-DNA and integrase-inhibitor cocrystal structures, the cross-linking inhibition analyses provide unique tools to probe drug binding interactions and mechanistic understanding of integrase inhibition by various classes of drugs.

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