Molecular Pharmacology Fast Forward. Published on December 15, 2006 as DOI: 10.1124/mol.106.030817
Molecular Pharmacology Fast Forward. Published on December 15, 2006 as doi:10.1124/mol.106.030817
This article has not been copyedited and formatted. The final version may differ from this version.

Title Page

PROBING HIV-1 INTEGRASE INHIBITOR BINDING SITES WITH POSITION-SPECIFIC INTEGRASE-DNA CROSSLINKING ASSAYS

Allison A. Johnson*, Christophe Marchand*, Sachindra S. Patil, Roberta Costi, Roberto Di Santo, Terrence R. Burke, Jr., Yves Pommier

Laboratory of Molecular Pharmacology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD (A.A.J., C.M., Y.P.)

Laboratory of Medicinal Chemistry, Center for Cancer Research, National Cancer Institute-Frederick, National Institutes of Health, Bethesda, MD (S.S.P., T.R.B.)

Instituto Pasteur - Fondazione Cenci Bolognetti, Dipartimento di Studi Farmaceutici, Universita di Roma “La Sapienza”, Roma, Italy (R.C., R.D.S.)

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 23, 2024

Copyright 2006 by the American Society for Pharmacology and Experimental Therapeutics.

Molecular Pharmacology Fast Forward. Published on December 15, 2006 as DOI: 10.1124/mol.106.030817
This article has not been copyedited and formatted. The final version may differ from this version.

Running Title Page

Running Title : Inhibition of HIV-1 integrase/DNA crosslinking

Corresponding Author: Dr. Yves Pommier, Laboratory of Molecular Pharmacology, Building 37, Room 5068, National Institutes of Health, Bethesda, MD 20892. Tel: 301-496-5944; Fax: 301-402-0752; E-mail: pommier@nih.gov

Number of pages: 30

Number of tables: 1

Number of figures: 6

Number of references: 41

Number of words:

in the abstract: 220

in the introduction: 837

in the discussion: 1076

Abbreviations: Abbreviations: 3'-P, 3'-processing; cono, conocurvone; DKA, diketo acid; L-CA, L-chicoric acid; LTR, long terminal repeat; ST, strand transfer

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 23, 2024

Molecular Pharmacology Fast Forward. Published on December 15, 2006 as DOI: 10.1124/mol.106.030817
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

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

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 23, 2024

Molecular Pharmacology Fast Forward. Published on December 15, 2006 as DOI: 10.1124/mol.106.030817
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction

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; Marchand et al., 2006; Pommier et al., 2005; Van Maele and Debyser, 2005).

In spite of many attempts, there is no crystal structure of integrase bound to its DNA substrates. 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 photocrosslinking of a substituted 5-iododeoxyuracil at this position (Jenkins et al., 1997). Crystal structure of integrase suggested that Lys-159 also contacts the phosphate 5' to the conserved 3'-A (Wang et al., 2001). Photocrosslinking 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). Recently, using disulfide crosslinking, we reported 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 co-crystal structures, such information is important for understanding interactions between integrase and the viral cDNA.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 23, 2024

Molecular Pharmacology Fast Forward. Published on December 15, 2006 as DOI: 10.1124/mol.106.030817
This article has not been copyedited and formatted. The final version may differ from this version.

Many integrase inhibitors have been discovered during the past 10 years, with two of them presently in clinical trials [see review (Dayam et al., 2006; DeJesus et al., 2006; Pommier et al., 2005; Semenova et al., 2006b)]. The binding sites of only two of these inhibitors, 5CITEP and Y-3 have been determined by x-ray crystallography (Goldgur et al., 1999; Lubkowski et al., 1998). Integrase inhibitors can be discriminated between the ones that inhibit both 3'-P and ST reactions (defined as 3'-P inhibitors) and the ones that inhibit ST efficiently but have a low or no effect on 3'-P (defined as ST-selective inhibitors). ST-selective inhibitors such as diketo acid and naphtyridine carboxamide derivatives (Hazuda et al., 2004a; Hazuda et al., 2000) 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., 2006a; Johnson et al., 2004; Johnson et al., 2006b), here we describe the use of disulfide and Schiff base crosslinking (Johnson et al., 2006a; Mazumder et al., 1996) to examine interference upon crosslink formation of various integrase inhibitors.

For the disulfide crosslinking assay, we used site-directed mutagenesis to create a Gln-148-Cys integrase mutant that forms a disulfide crosslink 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).

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 23, 2024

Molecular Pharmacology Fast Forward. Published on December 15, 2006 as DOI: 10.1124/mol.106.030817
This article has not been copyedited and formatted. The final version may differ from this version.

To further examine drug-DNA interactions with integrase, we also used a Schiff base crosslinking 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 crosslink positional effects. The position of the crosslinking lysines can be estimated from structural and molecular modeling studies.

We demonstrate that potent 3'-P inhibitors are also the most potent crosslinking inhibitors, while ST-selective inhibitors like L-708,906 (Hazuda et al., 2000) and L-870,810 (Hazuda et al., 2004a) are weak inhibitors of crosslinking. Inhibition of the specific contacts required for disulfide and Schiff base crosslinking is supported by inhibitor-integrase contacts identified through crystal structures and docking studies for several inhibitors. Results from the Schiff base crosslink assays using 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 crosslinking assays to gain insight into inhibitor binding sites and interactions between integrase and the viral cDNA.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 23, 2024

Molecular Pharmacology Fast Forward. Published on December 15, 2006 as DOI: 10.1124/mol.106.030817
This article has not been copyedited and formatted. The final version may differ from this version.

Materials and Methods

Oligonucleotide Synthesis: Unmodified and uracil-containing (for Schiff base crosslinking studies) oligonucleotides were synthesized by IDT (Coralville, IA). For disulfide crosslinking, an oligonucleotide containing the convertible nucleoside O4-triazole-dU was synthesized by Midland Certified Reagent Company, Inc. (Midland, TX). A two carbon cystamine tether was added post-synthetically using the convertible nucleoside approach (Ferentz, 1991) and the resulting cystamine-modified oligonucleotide was gel purified. The cystamine was reduced with 100-fold molar excess of DTT for 20 minutes at 37 ^{o}C, and purified using a mini Quickspin oligo column (Roche, Indianapolis, IN). The thiol-modification was activated by the addition of 10-fold molar excess of 5,5'-dithiobis(2-nitrobenzoic acid) in pH 8.5 phosphate buffer for 1 hour at 37 ^{o}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 (Gibco BRL, Rockville, MD) with [γ-^{32}P]ATP (PerkinElmer, Wellesley, MA) according to the manufacturers' instructions. Unincorporated nucleotides were removed by mini Quickspin oligo columns (Roche, Indianapolis, IN). The duplex DNA was annealed by addition of an equal concentration of the complementary strand, heating to 95 $^{\circ}$C, and slow cooling to room temperature.

Inhibitors. L-708,906, L-870,810, 5CITEP, bis-DKA and L-chicoric acid were synthesized according to literature methods (Anthony et al., 2002; Hazuda et al., 2004a; Lin et al., 1999; Pais et al., 2002; Zhao and Burke, 1998). Compounds RDS-1997 and RDS-1625 were synthesized by the Di Santo group (Di Santo et al., 2006; Di Santo et

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 23, 2024

Molecular Pharmacology Fast Forward. Published on December 15, 2006 as DOI: 10.1124/mol.106.030817
This article has not been copyedited and formatted. The final version may differ from this version.

al., 2005). Conocurvone was obtained as a gift from Dr. Jonathan Coates of AMRAD Operations Pty Ltd (Victoria, Australia, 26). All drug solutions were prepared from powder in 100% DMSO. Drug concentrations producing 50% inhibition (IC_{50} values, Table 1) are from previous reports (Di Santo et al., 2006; Johnson et al., 2006b; Marchand et al., 2002; Stagliano et al., 2006) or were determined as described below. Note that all the compounds shown display anti-HIV activity, with the exception of 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 (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 in (Johnson et al., 2006a)] for disulfide crosslinking. The three serine to cysteine mutations provide a crosslinking-free background prior to 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 following bacterial lysis.

Schiff Base Crosslinking 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 glycolylase (UDG) was added to create an abasic site at the uracil position. The abasic site forms a Schiff base crosslink between the deoxyribose aldehyde group and a nearby integrase lysine.

The Schiff base crosslinking experiments were performed as described (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

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 23, 2024

Molecular Pharmacology Fast Forward. Published on December 15, 2006 as DOI: 10.1124/mol.106.030817
This article has not been copyedited and formatted. The final version may differ from this version.

MOPS, pH 7.2. Abasic-site containing duplex DNA (20 nM final concentration) was added to each reaction and incubated at room temperature for 5 minutes. The crosslinks were reduced by adding 100 mM sodium borohydride (final concentration) prior to the addition of tricine-SDS gel loading buffer (1X final concentration). The crosslinked integrase-DNA products were separated from the substrate DNA by SDS-PAGE using 16% tricine gels (Invitrogen, Carlsbad, CA).

Disulfide Crosslinking. 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 twenty minutes 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 medification during the labeling reaction. After two minutes, reactions were capped by the addition of 20 mM methyl-methanethiosulfonate (MMTS). Non-reducing gel loading buffer (100 mM Tris-Cl, pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol) was added and samples were loaded directly onto 16% polyacrylamide gels (Invitrogen). Dried gels were visualized using a Molecular Dynamics 445 SI phosphorimager (Sunnyvale, CA). Quantitation was performed by densitometric analysis using the ImageQuant software from Molecular Dynamics.

Integrase Reactions: Integrase was incubated with DNA substrates for twenty minutes 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 one microliter of inhibitor and the reactions proceeded at 37 $^{\circ}$C for one hour. Reactions were quenched by the addition of an equal volume of gel loading dye (formamide containing 1% SDS,

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 23, 2024

Molecular Pharmacology Fast Forward. Published on December 15, 2006 as DOI: 10.1124/mol.106.030817
This article has not been copyedited and formatted. The final version may differ from this version.

0.25% bromophenol blue and xylene cyanol). Products were separated on 20% polyacrylamide denaturing sequencing gels. Dried gels were visualized using a Molecular Dynamics 445 SI phosphorimager. Densitometric analysis was performed using ImageQuant software from Molecular Dynamics.

Statistical Analysis: Correlations between inhibition of the two crosslinking assays and integrase catalytic assays were examined using one-tailed Pearson's correlation coefficients for each comparison. The Pearson's r value are listed on each panel of Figure 6. The p values are listed in the legend.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 23, 2024

Molecular Pharmacology Fast Forward. Published on December 15, 2006 as DOI: 10.1124/mol.106.030817
This article has not been copyedited and formatted. The final version may differ from this version.

Results

Inhibition of Disulfide Crosslink 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 crosslinking complexes between integrase and the [^{32}P]-labeled-DNA are observed as a ladder of oligomeric integrase products in non-reducing SDS-PAGE gels (Fig. 1B). Figure 1B demonstrates inhibition of disulfide crosslinking 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 crosslinking assay detects covalent bonds between integrase and a DNA abasic site substitution. In the experiment shown in Figure 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 crosslinking by the bis-DKA.

Integrase Inhibition Activities of L-870,810. Integrase ST-selective inhibitors are being pursued for clinical development (Dayam et al., 2006; DeJesus et al., 2006; Hazuda et al., 2004a; Hazuda et al., 2004b; Pommier et al., 2005; Semenova et al., 2006a). The

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 23, 2024

Molecular Pharmacology Fast Forward. Published on December 15, 2006 as DOI: 10.1124/mol.106.030817
This article has not been copyedited and formatted. The final version may differ from this version.

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 (panels B & 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 crosslinking assays (panels 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 crosslinking 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. 3B 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. 3B & C, 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 crosslinking assays (Fig. 3D & 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 Crosslinking Assays. Eight integrase inhibitors from different families and with differential selectivity for ST and 3'-P were evaluated in the disulfide and Schiff base crosslinking 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 crosslinking. The most ST-selective inhibitors include the DKA derivative (L-708,906) (Grobler et al., 2002; Hazuda et al., 2000; Hazuda et al.,

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 23, 2024

Molecular Pharmacology Fast Forward. Published on December 15, 2006 as DOI: 10.1124/mol.106.030817
This article has not been copyedited and formatted. The final version may differ from this version.

2004b; Marchand et al., 2003; Marchand et al., 2002; Pais et al., 2002), the naphthyridine carboxamide (L-870,810; Fig. 2) (Hazuda et al., 2004a), RDS-1625 (Fig. 3) and to a lesser extend 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., 2003; Marchand et al., 2002) and 5CITEP (Marchand et al., 2003; Marchand et al., 2002). 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 Figure 1, the bis-DKA is a relatively potent inhibitor of both crosslink assays (Fig. 4A & B, circle symbols). By contrast, 5CITEP is a potent inhibitor of the disulfide crosslink (Fig. 4A, diamond symbols) (Johnson et al., 2006a), while being a relatively weak inhibitor of Schiff base crosslinking (Fig. 4B, diamond symbols). In comparison, L-708,906 (Fig. 4A & B, triangle symbols) and L-870,810 (Fig. 4A & B, square symbols) are weak inhibitors of both disulfide and Schiff base crosslinking assays. Therefore, the DKA and naphthyridine carboxamide derivatives illustrate differences in crosslinking inhibition among the ST-selective inhibitors.

The DKA RDS-1997 (Fig. 4C & D, circle symbols) containing two diketo acid moieties (similar to the bis-DKA) inhibits both disulfide and Schiff base crosslinking efficiently as compared to RDS-1625 (Fig. 4C & D, square symbols), 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 crosslinking 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) (King et al., 1999; Lin et al., 1999; Zhao and Burke, 1998) (Fig. 4E & F, square symbols) is the most potent inhibitor of disulfide crosslinking among the eight compounds examined (compare

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 23, 2024

Molecular Pharmacology Fast Forward. Published on December 15, 2006 as DOI: 10.1124/mol.106.030817
This article has not been copyedited and formatted. The final version may differ from this version.

panels E, C and A in Fig. 4; Table 1). L-CA is also among the most potent inhibitor of Schiff base crosslink formation. The natural product conocurvone (Stagliano et al., 2006) (Fig. 4E & F, triangle symbols) is a relatively potent inhibitor of disulfide crosslink formation and a weaker inhibitor of Schiff base crosslinking.

Effects of Schiff Base Crosslinking 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 crosslinking efficiency was examined over the indicated positions of the terminal 21 base pairs of the U5 LTR DNA. In the absence of drug, crosslinking to the 'top' strand of the duplex results in two crosslinking peaks at the 3rd and 14th positions from the end of the viral cDNA (Fig. 5A, closed circles). Crosslinking to the 'bottom' strand (open symbols) is weaker in general, and results in maximum crosslinking 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 interacting with each crosslink position are noted in Figure 5B (Karki et al., 2004).

The three positions giving maximum crosslinking (top3, top14 and bottom 8) plus the 19th position of the bottom strand (Fig. 5A & B) were then used to examine Schiff base crosslinking inhibition as a function of the crosslinking position. The drugs producing inhibition of Schiff base crosslinking 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 crosslinking at these four positions (Fig. 5). RDS-1997 and RDS-1625 inhibit Schiff base crosslinking more effectively at the top 3rd and 14th positions (Fig. 5C & D; filled triangles and filled circles, respectively) compared to the bottom 8th and 19th positions (Fig. 5C & D; open diamond and open square symbols, respectively). This differential inhibition was

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 23, 2024

Molecular Pharmacology Fast Forward. Published on December 15, 2006 as DOI: 10.1124/mol.106.030817
This article has not been copyedited and formatted. The final version may differ from this version.

not observed with bis-DKA or L-chicoric acid (Fig. 5E & F). These results suggest that RDS-1997 and RDS-1625 tend to interfere preferentially with upper strand crosslinking, whereas L-chicoric acid and bis-DKA inhibit overall viral cDNA binding.

Comparison of 3'-P and ST Inhibition and Crosslinking Inhibition for Integrase Inhibitors. Figure 6 shows graphical comparisons of inhibition parameters to highlight differences in inhibitor potencies. Panel 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 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 crosslinking inhibition (Fig. 6B) illustrates an overall positive correlation between inhibition of Schiff base and disulfide crosslinking for the eight compounds studied ($r=0.73$, $p<0.05$). L-chicoric acid is the best inhibitor in both assays, while L-708,906 and L-870,810 fail to block either crosslinking reaction effectively. L-chicoric acid, RDS-1997, conocurvone and 5CITEP inhibit disulfide crosslinking at least 10 times more efficiently than Schiff base crosslinking (Fig. 6B).

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

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 23, 2024

Molecular Pharmacology Fast Forward. Published on December 15, 2006 as DOI: 10.1124/mol.106.030817
This article has not been copyedited and formatted. The final version may differ from this version.

Discussion

Inhibition of integrase-DNA crosslinking demonstrates interference with viral cDNA binding. Specifically, inhibition of disulfide crosslinking 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, 2006 #13>. Inhibition of crosslinking via Schiff base formation at an abasic site placed at the A position of the conserved CA (3'-A, position top3 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. Since the 3'-A and 5'-C belong to adjacent base pairs, crosslinking 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 (Johnson et al., 2006a; Mazumder and Pommier, 1995), 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 crosslinking with approximately 50-fold selectivity for disulfide over Schiff base crosslinking (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 crosslinking 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 crosslinking irrespective of the position of the abasic site from the end of the viral cDNA (Fig. 5E), it is likely that L-chicoric acid

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 23, 2024

Molecular Pharmacology Fast Forward. Published on December 15, 2006 as DOI: 10.1124/mol.106.030817
This article has not been copyedited and formatted. The final version may differ from this version.

binds near residues ranging from 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; Table 1; (Marchand et al., 2002)], as well as both disulfide and Schiff base crosslinking within the same concentration range (Fig. 6B). We also find that bis-DKA inhibits Schiff base crosslinking 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 towards 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 co-crystallized with integrase (Goldgur et al., 1999; Lubkowski et al., 1998). 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 co-crystallized in the integrase catalytic site in the absence of donor DNA (Goldgur et al., 1999). In this co-crystal 5CITEP contacts Gln-148 of the integrase flexible loop (Greenwald et al., 1999). As 5CITEP inhibits crosslinking of this same amino acid residue 148 in our disulfide crosslinking 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 crosslinking inhibition over Schiff base crosslinking inhibition with the abasic site placed at the conserved 3'-A position (Fig. 6B). Since 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 while having only minor interaction with the 3'-A and Lys-159/156. Such a

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 23, 2024

Molecular Pharmacology Fast Forward. Published on December 15, 2006 as DOI: 10.1124/mol.106.030817
This article has not been copyedited and formatted. The final version may differ from this version.

selective inhibition of disulfide crosslinking versus Schiff base crosslinking is consistent with the co-crystal 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 crosslinking in the same concentration range as for 3'-P and ST inhibition (Fig. 6A & C, Table1). Because conocurvone exhibits an approximately 20-fold selectivity for disulfide versus Schiff base crosslinking, 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 co-crystallization with the core domain of integrase.

Among the diketo acid derivatives, RDS-1997 is the only compound with significant crosslinking 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 crosslinking with the abasic site substitution at the conserved 3'-A position. Consistently, scanning of Schiff base crosslinking 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. Since RDS-1997 is an effective 3'-P inhibitor with remarkable inhibition of Schiff base crosslinking at 3'-A position, this compound might be a good candidate for co-crystallization 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 (Hazuda et al., 2004a; Marchand et al., 2003; Marchand et al., 2002). 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 crosslinking both in the disulfide and Schiff base assays (Fig. 6B).

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 23, 2024

Molecular Pharmacology Fast Forward. Published on December 15, 2006 as DOI: 10.1124/mol.106.030817
This article has not been copyedited and formatted. The final version may differ from this version.

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 crosslink inhibitors as 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 co-crystal structures, the crosslinking inhibition analyses provide unique tools to probe drug binding interactions and mechanistic understanding of integrase inhibition by various classes of drugs.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 23, 2024

Molecular Pharmacology Fast Forward. Published on December 15, 2006 as DOI: 10.1124/mol.106.030817
This article has not been copyedited and formatted. The final version may differ from this version.

References

Anthony NJ, Gomez RP, Young S, Egbertson M, Wai JS, Zhuang L, Embrey M, Tran L, Melamed JY, Langford HM, Guare JP, Fisher TE, Jolly SM, Kuo MS, Perlow DS, Bennett JJ and Funk TW (2002) Aza- and polyaza-naphtalenyl carboxamides useful as HIV integrase inhibitors, Merck & CO., INC.

Bushman FD and Craigie R (1992) Integration of human immunodeficiency virus DNA: adduct interference analysis of required DNA sites. *Proc Natl Acad Sci U S A* **89**(8):3458-3462.

Dayam R, Deng J and Neamati N (2006) HIV-1 integrase inhibitors: 2003-2004 update. *Med Res Rev* **26**(3):271-309.

Decosterd LA, Parsons IC, Gustafson KR, Cardellina JH, II, McMahon JB, Cragg GM, Murata Y, Pannell LK, Steiner JR, Clardy J and Boyd MR (1993) Structure, absolute stereochemistry, and synthesis of conocurvone, a potent, novel HIV-inhibitory naphthoquinone trimer from a Conospermum sp. *J Am Chem Soc* **115**:6673-6679.

DeJesus E, Berger D, Markowitz M, Cohen C, Hawkins T, Ruane P, Elion R, Farthing C, Zhong L, Cheng AK, McColl D and Kearney BP (2006) Antiviral activity, pharmacokinetics, and dose response of the HIV-1 integrase inhibitor GS-9137 (JTK-303) in treatment-naive and treatment-experienced patients. *Journal of acquired immune deficiency syndromes (1999)* **43**(1):1-5.

Di Santo R, Costi R, Roux A, Artico M, Lavecchia A, Marinelli L, Novellino E, Palmisano L, Andreotti M, Amici R, Galluzzo CM, Nencioni L, Palamara AT, Pommier Y and Marchand C (2006) Novel bifunctional quinolonyl diketo acid derivatives as HIV-1 integrase inhibitors: design, synthesis, biological activities, and mechanism of action. *J Med Chem* **49**(6):1939-1945.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 23, 2024

Molecular Pharmacology Fast Forward. Published on December 15, 2006 as DOI: 10.1124/mol.106.030817
This article has not been copyedited and formatted. The final version may differ from this version.

Di Santo R, Pommier Y, Marchand C, Artico M and Costi R (2005) Quinolin-4-ones as inhibitors of retroviral integrase for the treatment of HIV, AIDS and AIDS related complex (ARC), The Government of the United States of America.

Esposito D and Craigie R (1998) Sequence selectivity of viral end DNA binding by HIV-1 integrase reveals critical regions for protein-DNA interactions. *EMBO J* **17**:5832-5843.

Ferentz AEV, G.L. (1991) Disulfide-linked oligonucleotides. *J Amer Chem Soc* **113**(10):4000-4002.

Gao K, Butler SL and Bushman F (2001) Human immunodeficiency virus type 1 integrase: arrangement of protein domains in active cDNA complexes. *EMBO J* **20**(13):3565-3576.

Goldgur Y, Craigie R, Cohen GH, Fujiwara T, Yoshinaga T, Fujishita T, Sugimoto H, Endo T, Murai H and Davies DR (1999) Structure of the HIV-1 integrase catalytic domain complexed with an inhibitor: a platform for antiviral drug design. *Proc Natl Acad Sci U S A* **96**(23):13040-13043.

Greenwald J, Le V, Butler SL, Bushman FD and Choe S (1999) The mobility of an HIV-1 integrase active site loop is correlated with catalytic activity. *Biochemistry* **38**(28):8892-8898.

Grobler JA, Stillmock K, Hu B, Witmer M, Felock P, Espeseth AS, Wolfe A, Egbertson M, Bourgeois M, Melamed J, Wai JS, Young S, Vacca J and Hazuda DJ (2002) Diketo acid inhibitor mechanism and HIV-1 integrase: implications for metal binding in the active site of phosphotransferase enzymes. *Proc Natl Acad Sci U S A* **99**(10):6661-6666.

Hazuda DJ, Anthony NJ, Gomez RP, Jolly SM, Wai JS, Zhuang L, Fisher TE, Embrey M, Guare JP, Jr., Egbertson MS, Vacca JP, Huff JR, Felock PJ, Witmer MV, Stillmock KA, Danovich R, Grobler J, Miller MD, Espeseth AS, Jin L, Chen IW,

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 23, 2024

Molecular Pharmacology Fast Forward. Published on December 15, 2006 as DOI: 10.1124/mol.106.030817
This article has not been copyedited and formatted. The final version may differ from this version.

Lin JH, Kassahun K, Ellis JD, Wong BK, Xu W, Pearson PG, Schleif WA, Cortese R, Emini E, Summa V, Holloway MK and Young SD (2004a) A naphthyridine carboxamide provides evidence for discordant resistance between mechanistically identical inhibitors of HIV-1 integrase. *Proc Natl Acad Sci U S A* **101**(31):11233-11238.

Hazuda DJ, Felock P, Witmer M, Wolfe A, Stillmock K, Grobler JA, Espesath A, Gabryelski L, Schlelf W, Blau C and Miller MD (2000) Inhibitors of strand transfer that prevent integration and inhibit HIV-1 replication in cells. *Science* **287**:646-650.

Hazuda DJ, Young SD, Guare JP, Anthony NJ, Gomez RP, Wai JS, Vacca JP, Handt L, Motzel SL, Klein HJ, Dornadula G, Danovich RM, Witmer MV, Wilson KA, Tussey L, Schleif WA, Gabryelski LS, Jin L, Miller MD, Casimiro DR, Emini EA and Shiver JW (2004b) Integrase inhibitors and cellular immunity suppress retroviral replication in rhesus macaques. *Science* **305**(5683):528-532.

Jenkins TM, Esposito D, Engelman A and Craigie R (1997) Critical contacts between HIV-1 integrase and viral DNA identified by structure-based analysis and photo-crosslinking. *EMBO J* **16**(22):6849-6859.

Johnson AA, Santos W, Pais GC, Marchand C, Amin R, Burke TR, Jr., Verdine G and Pommier Y (2006a) Integration requires a specific interaction of the donor DNA terminal 5'-cytosine with glutamine 148 of the HIV-1 integrase flexible loop. *J Biol Chem* **281**(1):461-467.

Johnson AA, Sayer JM, Yagi H, Kalena GP, Amin R, Jerina DM and Pommier Y (2004) Position-specific suppression and enhancement of HIV-1 integrase reactions by minor groove benzo[a]pyrene diol epoxide deoxyguanine adducts: implications for molecular interactions between integrase and substrates. *J Biol Chem* **279**(9):7947-7955.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 23, 2024

Molecular Pharmacology Fast Forward. Published on December 15, 2006 as DOI: 10.1124/mol.106.030817
This article has not been copyedited and formatted. The final version may differ from this version.

Johnson AA, Sayer JM, Yagi H, Patil SS, Debart F, Maier MA, Corey DR, Vasseur JJ, Burke TR, Jr., Marquez VE, Jerina DM and Pommier Y (2006b) Effect of DNA modifications on DNA processing by HIV-1 integrase and inhibitor binding: role of DNA backbone flexibility and an open catalytic site. *J Biol Chem* **281**(43):32428-32438.

Karki RG, Tang Y, Burke TR, Jr. and Nicklaus MC (2004) Model of full-length HIV-1 integrase complexed with viral DNA as template for anti-HIV drug design. *J Comput Aided Mol Des* **18**(12):739-760.

King PJ, Ma G, Miao W, Jia Q, McDougall BR, Reinecke MG, Cornell C, Kuan J, Kim TR and Robinson WE, Jr. (1999) Structure-activity relationships: analogues of the dicaffeoylquinic and dicaffeoyltartaric acids as potent inhibitors of human immunodeficiency virus type 1 integrase and replication. *J Med Chem* **42**(3):497-509.

King PJ and Robinson WE, Jr. (1998) Resistance to the anti-human immunodeficiency virus type 1 compound L-chicoric acid results from a single mutation at amino acid 140 of integrase. *J Virol* **72**(10):8420-8424.

Leh H, Brodin P, Bischerour J, Deprez E, Tauc P, Brochon JC, LeCam E, Coulaud D, Auclair C and Mouscadet JF (2000) Determinants of Mg2+-dependent activities of recombinant human immunodeficiency virus type 1 integrase. *Biochemistry* **39**(31):9285-9294.

Lewinski MK and Bushman FD (2005) Retroviral DNA integration--mechanism and consequences. *Adv Genet* **55**:147-181.

Lin Z, Neamati N, Zhao H, Kiryu Y, Turpin JA, Aberham C, Strebel K, Kohn K, Witvrouw M, Pannecouque C, Debyser Z, De Clercq E, Rice WG, Pommier Y and Burke TR, Jr. (1999) Chicoric acid analogues as HIV-1 integrase inhibitors. *J Med Chem* **42**(8):1401-1414.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 23, 2024

Molecular Pharmacology Fast Forward. Published on December 15, 2006 as DOI: 10.1124/mol.106.030817
This article has not been copyedited and formatted. The final version may differ from this version.

Lubkowski J, Yang F, Alexandratos J, Wlodawer A, Zhao H, Burke TR, Jr., Neamati N, Pommier Y, Merkel G and Skalka AM (1998) Structure of the catalytic domain of avian sarcoma virus integrase with a bound HIV-1 integrase-targeted inhibitor. *Proc Natl Acad Sci U S A* **95**(9):4831-4836.

Marchand C, Johnson AA, Karki RG, Pais GCG, Zhang X, Cowansage K, Patel TA, Nicklaus MC, Burke TR, Jr. and Pommier Y (2003) Metal-dependent inhibition of HIV-1 integrase by beta-diketo acids and resistance of the soluble double-mutant (F185K/C280S). *Mol Pharmacol* **64**(3):600-609.

Marchand C, Johnson AA, Semenova EA and Pommier Y (2006) Mechanisms and inhibition of HIV integration. *Drug Discov Today: Disease Mecha* **3**(2):253-260.

Marchand C, Zhang X, Pais GC, Cowansage K, Neamati N, Burke TR, Jr. and Pommier Y (2002) Structural determinants for HIV-1 integrase inhibition by beta-diketo acids. *J Biol Chem* **277**(15):12596-12603.

Mazumder A, Neamati N, Pilon A, Sunder S and Pommier Y (1996) Chemical trapping of ternary complexes of human immunodeficiency virus type 1 integrase, divalent metal, and DNA substrates containing an abasic site. *J Biol Chem* **271**:27330-27338.

Mazumder A and Pommier Y (1995) Processing of deoxyuridine mismatches and abasic sites by human immunodeficiency virus type-1 integrase. *Nucleic Acids Res* **23**(15):2865-2871.

Pais GC, Zhang X, Marchand C, Neamati N, Cowansage K, Svarovskaia ES, Pathak VK, Tang Y, Nicklaus M, Pommier Y and Burke TR, Jr. (2002) Structure activity of 3-aryl-1,3-diketo-containing compounds as HIV-1 integrase inhibitors. *J Med Chem* **45**(15):3184-3194.

Pommier Y, Johnson AA and Marchand C (2005) Integrase inhibitors to treat HIV/AIDS. *Nat Rev Drug Discov* **4**(3):236-248.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 23, 2024

Molecular Pharmacology Fast Forward. Published on December 15, 2006 as DOI: 10.1124/mol.106.030817
This article has not been copyedited and formatted. The final version may differ from this version.

Semenova EA, Johnson AA, Marchand C, Davis DA, Yarchoan R and Pommier Y (2006a) Preferential inhibition of the magnesium-dependent strand transfer reaction of HIV-1 integrase by alpha-hydroxytropolones. *Mol Pharmacol* **69**(4):1454-1460.

Semenova EA, Johnson AA, Marchand C and Pommier Y (2006b) Integration of human immunodeficiency virus as a target for antiretroviral therapy. *Curr Opin HIV AIDS* **1**:380-387.

Sotriffer CA, Ni H and McCammon JA (2000) Active site binding modes of HIV-1 integrase inhibitors. *J Med Chem* **43**(22):4109-4117.

Stagliano KW, Emadi A, Lu Z, Malinakova HC, Twenter B, Yu M, Holland LE, Rom AM, Harwood JS, Amin R, Johnson AA and Pommier Y (2006) Regiocontrolled synthesis and HIV inhibitory activity of unsymmetrical binaphthoquinone and trimeric naphthoquinone derivatives of conocurvone. *Bioorg Med Chem* **14**(16):5651-5665.

Van Maele B and Debyser Z (2005) HIV-1 integration: an interplay between HIV-1 integrase, cellular and viral proteins. *AIDS Rev* **7**(1):26-43.

Wang JY, Ling H, Yang W and Craigie R (2001) Structure of a two-domain fragment of HIV-1 integrase: implications for domain organization in the intact protein. *EMBO J* **20**(24):7333-7343.

Zhao H and Burke TR (1998) Facile syntheses of (2R,3R)-(-)-and (2S,3S)-(+)-chicoric acids. *Synth Commun* **28**:737-740.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 23, 2024

Molecular Pharmacology Fast Forward. Published on December 15, 2006 as DOI: 10.1124/mol.106.030817
This article has not been copyedited and formatted. The final version may differ from this version.

Footnotes

* These authors contributed equally to this work

This research was supported by the NIH Intramural Program, Center for Cancer Research, National Cancer Institute.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 23, 2024

Molecular Pharmacology Fast Forward. Published on December 15, 2006 as DOI: 10.1124/mol.106.030817
This article has not been copyedited and formatted. The final version may differ from this version.

Legends for figures

Figure 1. Principle of the two integrase-viral cDNA crosslinking assays (Johnson et al., 2006a; Mazumder et al., 1996) and inhibition by the integrase inhibitor, bis-DKA (Marchand et al., 2002; Pais et al., 2002). **A.** Schematic diagram illustrating disulfide crosslink 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 crosslink formation by the bis-DKA inhibitor (see chemical structure in Fig. 4). Drug concentrations are indicated above each lane. The samples are not boiled prior to non-reducing SDS-PAGE resulting in the ladder of integrase multimers crosslinked to DNA. **C.** Schematic diagram illustrating Schiff base crosslink 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 crosslinking by bis-DKA. Drug concentrations are indicated above each lane.

Figure 2. Inhibition of integrase activities and crosslinking by L-870,810. **A.** Representative gel showing the potent and selective inhibition of integrase ST by L-870,810. **B.** Quantification of 3'-P and ST inhibition by L-870,810. **C & D.** Representative gels showing inhibition of Schiff base and disulfide crosslinking by L-870,810, respectively. Error bars correspond to standard deviation for at least 3 independent experiments.

Figure 3. Inhibition of integrase activities and crosslinking by RDS-1625. **A.** Representative gel showing the selective inhibition of integrase ST by RDS-1625. **B.** Quantification of 3'-P and ST inhibition by RDS-1625. **C.** Representative gels showing

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 23, 2024

Molecular Pharmacology Fast Forward. Published on December 15, 2006 as DOI: 10.1124/mol.106.030817
This article has not been copyedited and formatted. The final version may differ from this version.

inhibition of Schiff base and disulfide crosslink formation by RDS-1625, respectively. Error bars correspond to standard deviation for at least 3 independent experiments.

Figure 4. Comparison of integrase-DNA crosslinking inhibition by various integrase inhibitors: **A.** bis-DKA, 5CITEP, L-708,906 and L-870,810. **B.** DKA derivatives, RDS-1625 and RDS-1997. **C.** 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 standard deviation for at least 3 independent experiments.

Figure 5. Effect of position of the abasic site for Schiff base crosslinking and inhibition by integrase inhibitors. **A.** Summary of crosslinking 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 crosslinking inhibition. The lysines likely interacting with each region are noted at the top. **C, D, E, & F.** Graphs showing inhibition of Schiff base crosslinking 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 panels C-F. Error bars correspond to standard deviation for at least 3 independent experiments.

Figure 6. Correlations between inhibition for each of the two crosslinking assays and the two integrase reactions (IC_{50} values are represented as paired-scattered plots). **A.** 3'-P vs. ST inhibition. **B.** Schiff base vs. disulfide crosslinking inhibition. **C.** Disulfide crosslinking vs. 3'-P inhibition. **D.** Schiff base crosslinking vs. 3'-P inhibition. **E.** Disulfide crosslinking vs. ST inhibition. **F.** Schiff base crosslinking vs. 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 panel. The P values are 0.0002 and 0.0389 for **A** & **B** respectively.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 23, 2024

Molecular Pharmacology Fast Forward. Published on December 15, 2006 as DOI: 10.1124/mol.106.030817
This article has not been copyedited and formatted. The final version may differ from this version.

Tables

Table 1. Summary of IC50 values (μM)

Inhibitor	S-S	Schiff base	Schiff /S-S ratio	3'-P	ST	3'-P/ST ratio	Antiviral activity
L-708,906	300	113	0.4	2.5[1]	0.06[1]	42	Yes[5]
L-870,810	500	500	1	>12.3	0.06	>205	Yes[6]
5CITEP	13	280	22	400[1]	97[1]	4	No[7]
bis-DKA	22	6.2	0.3	110[1]	10[1]	11	No[7]
RDS-1997	1.0	6.3	6	0.4[2]	0.02[2]	20	Yes[2]
RDS-1625	>333	48	<0.2	4.4	0.06	73	Yes[2]
conocurvone	6.9	129	19	1.0[3]	1.0[3]	1	Yes[8]
L-chicoric acid*	0.1	4.8	48	0.4[4]	0.19[4]	2	Yes[9]

IC_{50} values for 3'-P and ST are from (Marchand et al., 2002)[1], (Di Santo et al., 2006; Di Santo et al., 2005)[2], (Stagliano et al., 2006)[3], (Johnson et al., 2006b)[4]
Antiviral activity reported in (Di Santo et al., 2006; Di Santo et al., 2005)[2], (Hazuda et al., 2000)[5], (Hazuda et al., 2004b)[6], (Pais et al., 2002)[7], (Decosterd et al., 1993)[8], (King et al., 1999; Lin et al., 1999)[9].
** L-chicoric acid IC_{50} values for 3'-P and ST were obtained in the presence of Manganese (Johnson et al., 2006b)[4]*

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 23, 2024

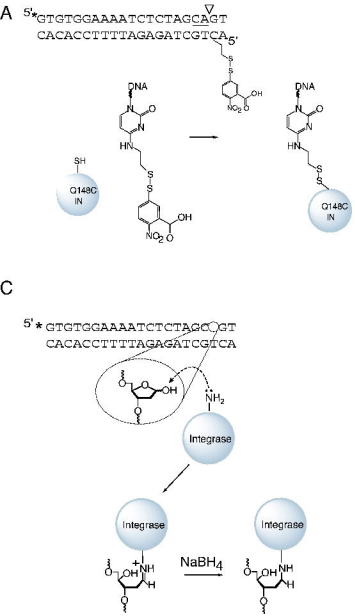

A
5'*GTGTGGAAAATCTCTAGCAGT
CACACCTTTTAGAGATCGTCA5'
DNA
SH
Q148C
IN
OH
NO2
O
S
HN
N
O
B
bis-DKA
DNA
0
333
111
37
12.3
4.1
1.4
μM
multimers
dimer
monomer
IN/DNA
DNA
C
5'* GTGTGGAAAATCTCTAGCAGT
CACACCTTTTAGAGATCGTCA
NH2
Integrase
NaBH4
NH
H
D
bis-DKA
DNA
0
333
111
37
12.3
4.1
1.4
μM
IN/DNA
DNA

Figure 1

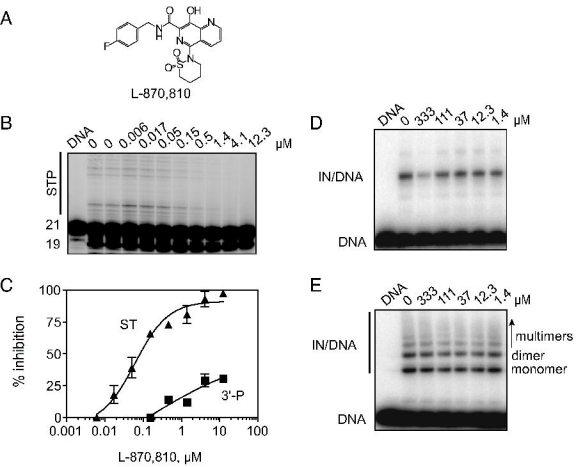

A
L-870,810
B
DNA
0
0
0.006
0.017
0.05
0.15
0.5
1.4
4.1
12.3
μM
STP
21
19
C
% inhibition
100
75
50
25
0
ST
3'-P
0.001
0.01
0.1
1
10
100
L-870,810, μM
D
DNA
0
333
111
37
12.3
1.4
μM
IN/DNA
DNA
E
DNA
0
333
111
37
12.3
1.4
μM
IN/DNA
multimers
dimer
monomer
DNA

Figure 2

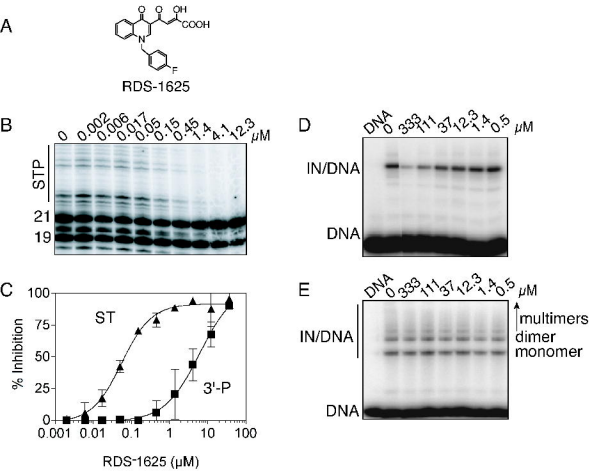

A
O
O
OH
COOH
N
F
RDS-1625
B
0 0.002 0.006 0.017 0.05 0.15 0.45 1.4 4.1 12.3 μM
STP
21
19
C
100
75
50
25
0
% Inhibition
ST
3'-P
0.001 0.01 0.1 1 10 100
RDS-1625 (μM)
D
DNA 0 333 111 37 12.3 1.4 0.5 μM
IN/DNA
DNA
E
DNA 0 333 111 37 12.3 1.4 0.5 μM
multimers
dimer
monomer
IN/DNA
DNA

Figure 3

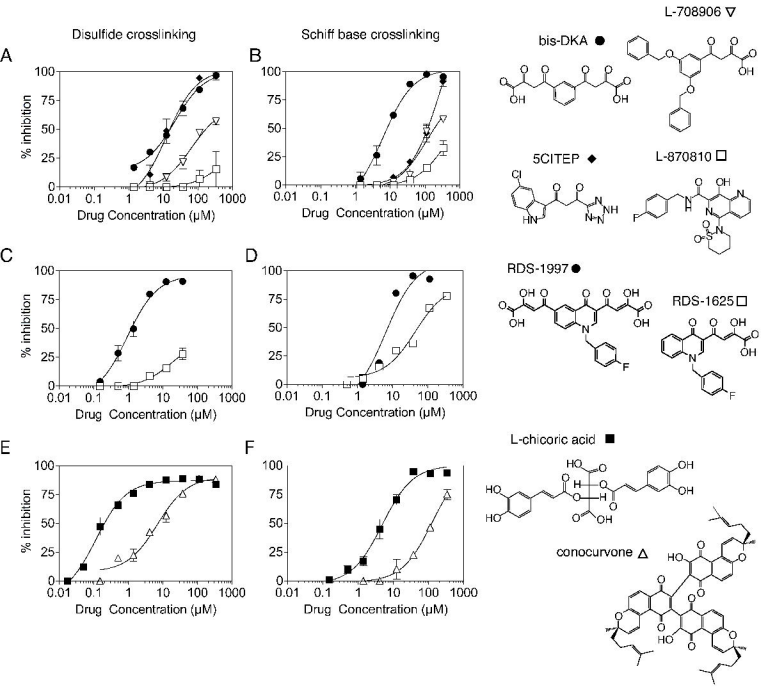
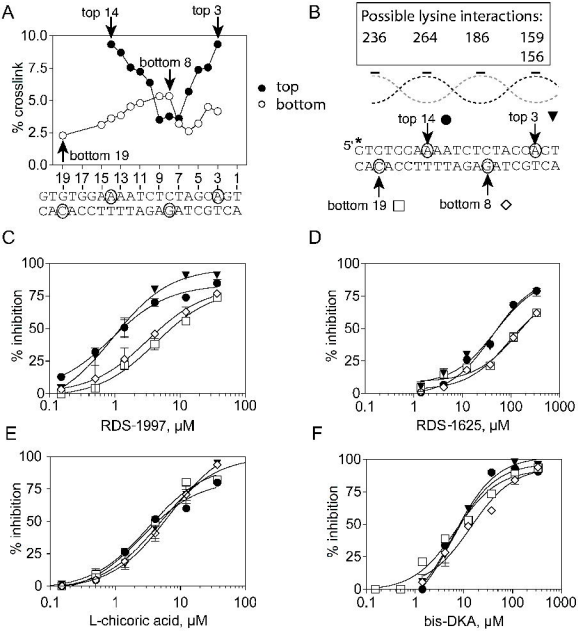

Disulfide crosslinking
Schiff base crosslinking
A
B
C
D
E
F
% inhibition
Drug Concentration (μM)
L-708906 ▽
bis-DKA ●
5CITEP ◆
L-870810 □
RDS-1997 ●
RDS-1625 □
L-chicoric acid ■
conocurvone △

Figure 4



G

Position	IC_{60}, μM 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

Figure 5

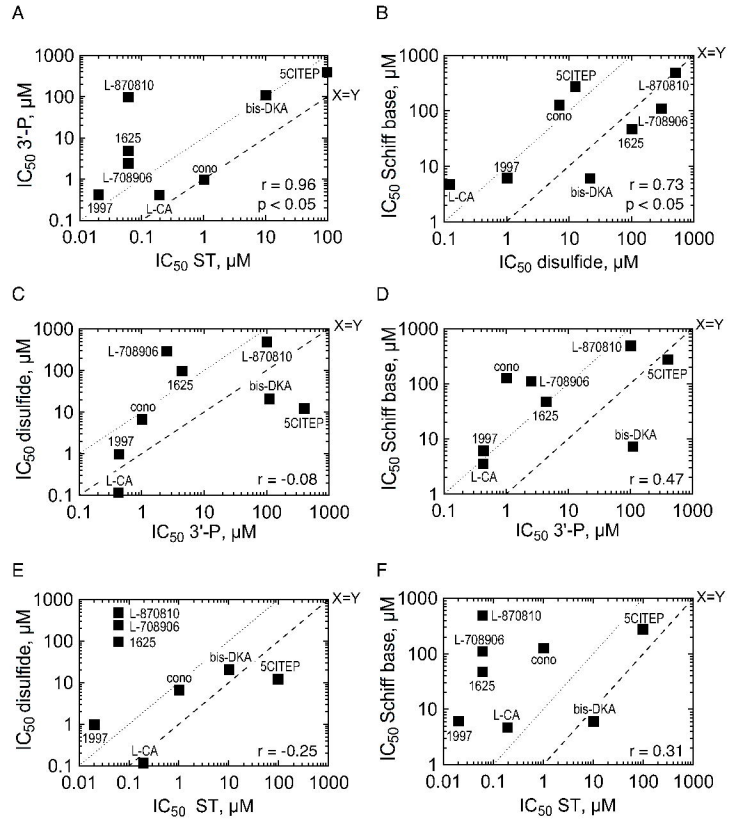

A
B
C
D
E
F
IC50 3'-P, μM
IC50 ST, μM
IC50 Schiff base, μM
IC50 disulfide, μM
IC50 disulfide, μM
IC50 Schiff base, μM
IC50 3'-P, μM
IC50 disulfide, μM
IC50 Schiff base, μM
IC50 ST, μM
X=Y
L-870810
5CITEP
bis-DKA
1625
L-708906
cono
1997
L-CA
r = 0.96
p < 0.05
r = 0.73
p < 0.05
r = -0.08
r = 0.47
r = -0.25
r = 0.31

Figure 6