

Dissecting Tn5 Transposition Using HIV-1 Integrase Diketoacid Inhibitors<sup>†</sup>Agata Czyz,<sup>‡,§</sup> Kara A. Stillmock,<sup>||</sup> Daria J. Hazuda,<sup>||</sup> and William S. Reznikoff<sup>\*:‡</sup>

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**ABSTRACT:** Diketoacid (DKA) compounds have been shown to inhibit HIV-1 integrase by a mechanism that involves sequestration of the active site metals. Because HIV-1 integrase and Tn5 transposase have similar active site architectures and catalytic mechanisms, we investigated whether DKA analogues would inhibit Tn5 transposase activity and provide a model system to explore the mechanisms of action of these inhibitors. A screen of several hundred DKA analogues identified several with activity against Tn5 Tnp. Six DKA inhibitors used in this study manifested a variety of effects on different transposition steps suggesting that different analogues may have different binding contacts with transposase. All DKA compounds inhibited paired end complex (PEC) formation in which the nucleoprotein complex required for catalysis is assembled. Dissociation of PECs by some DKA compounds indicates that these inhibitors can decrease PEC stability. Four DKA compounds inhibited the two cleavage steps releasing transposon DNA from flanking DNA, and one of these four compounds preferentially inhibited the second cleavage step. The differential effect of this inhibitor on the second cleavage event indicates that cleavage of the two transposon–donor DNA boundaries is a sequential process requiring a conformational change. The requirement for a conformational change between cleavage events was also demonstrated by the inability of transposase to perform second cleavage at 25 °C. Finally, all six compounds inhibit strand transfer, the final step of Tn5 transposition. Two of the compounds that inhibited strand transfer have no effect on DNA cleavage. The strand transfer inhibition properties of various DKA compounds was sensitive to the structure of the 5′-non-transferred strand, suggesting that these compounds bind in or near the transposase active site. Other results that probe compound binding sites include the effects of active site mutations and donor DNA on DKA compound inhibition activities. Thus, DKA inhibitors will provide an important set of tools to investigate the mechanism of action of transposases and integrases.

DNA transposition involves the movement of a defined DNA sequence (called a transposon) from one site in the genome to a second site. Transposition is an important process because it has played a critical role in genome evolution (1, 2) and because the transposition process is mechanistically similar to other important DNA transactions such as the integration of HIV-1 DNA into the cellular genome (3). DNA transposition that does not involve an RNA intermediate is typically catalyzed by a protein called transposase (Tnp<sup>1</sup>). Each given type of transposon encodes its own specific Tnp, and the key to this specificity lies in the recognition reaction between the Tnp and the terminal DNA sequences of the transposon (1). Tn5 is an excellent model system for studying transposition because of the extensive genetic, biochemical, and structural work that has been performed on this system (4–7). In fact, Tn5 is the only transposition related system for which there is structural information regarding the full-length protein bound to the

transposon terminal DNA sequences (5, 8, 9). As shown in Figure 1, Tn5 transposes through a multistep process that includes the formation of a dimeric Tnp–transposon recognition end sequence, DNA synaptic complex (a paired end complex, PEC), cleavage of the transposon DNA free of adjacent donor DNA as a result of three phosphoryl transfer catalytic events, target DNA capture, and strand transfer that integrates the transposon into the new DNA site.

In this article, we report the first use of small molecule inhibitors to study the Tn5 transposition process. Our goal is twofold. First, we hope that the inhibitors will enhance

<sup>1</sup> Abbreviations: Tnp, transposase; IN, retroviral integrase; DKA, diketoacid compound; PEC, paired end complex; a complex containing two molecules of transposase bound to two transposon end sequences (ESs); E54K, designating a point mutant version of Tnp that contains an E to K change at residue 54 (other point mutant versions of Tnp include L372P, D97A, D188A, and E326A); ES, a 19 bp Tn5 end sequence that is specifically recognized by Tnp during transposition; DBB, donor backbone (the DNA from which the transposon is cleaved during transposition); SEB, single-end break (cleavage of a transposon from the DBB occurs first at one end (generating a SEB) and then at the second end (generating a double-end break)); DEB, double-end break; STP, strand transfer product (the final product of transposition); ST, strand transfer; SEST, single-end strand transfer (the transfer of one transposon end into the target DNA); DEST, double-end strand transfer (the transfer of both transposon ends into the target DNA).

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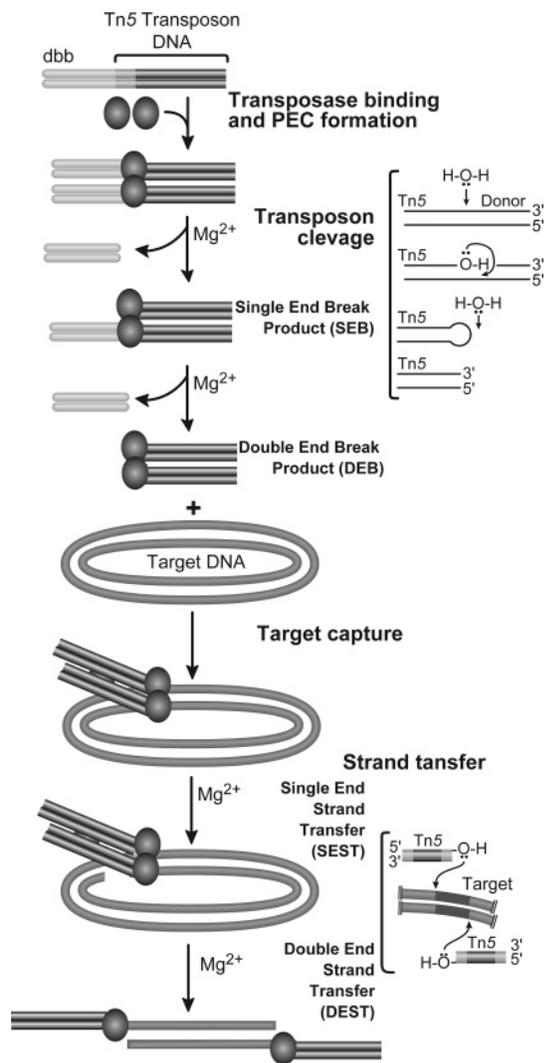


FIGURE 1: Tn5 Tnp catalyzed DNA transposition. Tnp catalyzed transposition occurs through a multistep process. For the experiments in which the transposon ESs are located on short double stranded oligonucleotides, these steps include the following: (a) Formation of a dimeric Tnp–ES PEC (comparable to synaptic complex formation); (b) in the presence of  $Mg^{2+}$ , a three step catalysis of cleavage at the ES–DBB boundary (transferred strand nicking, hairpin formation, and hairpin cleavage) for one oligonucleotide in the PEC yields a SEB product; (c) cleavage of the second ES–DBB boundary to yield a DEB product; (d) target capture; (e) Tnp catalyzed strand transfer to yield one transferred ES (SEST) and both ESs transferred (DEST).

our understanding of the Tn5 transposition mechanism. Second, it is possible that the inhibitor studies with Tn5 Tnp will help in the development of pharmacologically effective compounds for inhibiting HIV-1 integrase (HIV-1 IN).

The first HIV-1 IN inhibitors with reported activity against HIV integration were DKA analogues. The two inhibitors, MK-0518 from Merck and GS9137 from Gilead, that are currently in clinical trials are diketoacid (DKA) isosteres (10, 11). The DKA isostere is critical for the activity of these compounds and is believed to sequester the active site metals. It has been reported that DKA compounds preferentially inhibit IN catalyzed strand transfer of the viral DNA into host DNA *in vitro* (12, 13). A cocrystal structure of a DKA related compound (5CITEP) with the HIV-1 catalytic core (14) and a sequence analysis of DKA resistant mutants of HIV-1 IN (15, 16) suggest that these DKA compounds bind

within the IN active site, although the structure of critical inhibitor-IN residue contacts are not yet resolved.

In order to gain more insight into the mode of action of DKA compounds, we have chosen to use the Tn5 Tnp system as a model. Our study is based on the observation that 15–30% of IN and Tnp inhibitors, including some DKA analogues, inhibit both enzymes (Stillmock and Hazuda, unpublished data, (17)) and that the active site architectures are very similar between HIV-1 IN and Tn5 Tnp (18, 19). Therefore, these inhibitors may possess a similar mechanism of action and interact with similar residues in the active sites of IN and Tnp.

In this article, we show that Tn5 Tnp may be a useful model for studying IN DKA inhibitors. Our data suggest that at least some of the tested DKA compounds interact close to or with the Tnp active site residues that correspond to the IN residues responsible for DKA binding. In addition, the experiments with the HIV-1 IN DKA inhibitors with the Tn5 transposition system extend our understanding of the Tn5 transposition process. Results of these *in vitro* assays demonstrate that the tested compounds can affect various steps of the Tn5 transposition process including PEC formation, the stability of the PEC, and cleavage and/or strand transfer reactions. The efficacy of inhibition of the various transposition steps varied among the DKA compounds, suggesting that these compounds manifest different Tnp binding specificities.

## EXPERIMENTAL PROCEDURES

**DKA Compounds.** All six compounds used in these experiments were identified in a screen of the Merck DKA-collection for inhibitors of Tn5 Tnp-catalyzed strand transfer *in vitro* (Stillmock, K., unpublished data). These compounds were provided by John S. Wai and are described in Figure 2. Before use, all DKA compounds were resuspended in dimethyl sulfoxide (DMSO) to 10 mM and stored at  $-20^{\circ}\text{C}$ .

**DNA Substrates.** Short oligonucleotide substrates used in these experiments were purchased from either Integrated DNA Technology or Operon Biotechnologies Inc. Two different short DNA substrates were used in the experiments. The full-length substrate (60-mer, 5'-GGC CAC GAC ACG CTC CCG CGC **TGT CTC TTA TAC ACA TCT** TGA GTG AGT GAG CAT GCA TGT-3' and its complement) contains 20 bp of donor backbone, the 19 bp transposon mosaic recognition end sequence (ES), and 21 bp of transposon DNA. The ES is presented in bold. The pre-cleaved substrate (40-mer, 5'-C **TGT CTC TTA TAC ACA TCT** TGA GTG AGT GAG CAT GCA TGT-3' and its complement) is identical to the 60-mer, except that it lacks the 20 bp donor backbone (DBB) DNA. Three types of pre-cleaved DNA substrate were used: with a phosphate group on the 5'-end of the non-transferred strand, with an OH group on the 5'-end of the non-transferred strand, or with a 6-FAM fluorescein on the 5'-end of the non-transferred strand. All oligonucleotides were labeled with 6-FAM fluorescein at the 5'-end of the transferred strand. The double-stranded DNA substrates were annealed at a final concentration of  $1\ \mu\text{M}$  in 10 mM NaCl and 10 mM Tris-HCl at pH 7.5 by heating at  $95^{\circ}\text{C}$  for 1 min and then cooling  $0.1^{\circ}\text{C/s}$  to  $4^{\circ}\text{C}$ . For the strand transfer experiments, pUC19 plasmid DNA prepared with a Qiagen maxi prep kit was used.

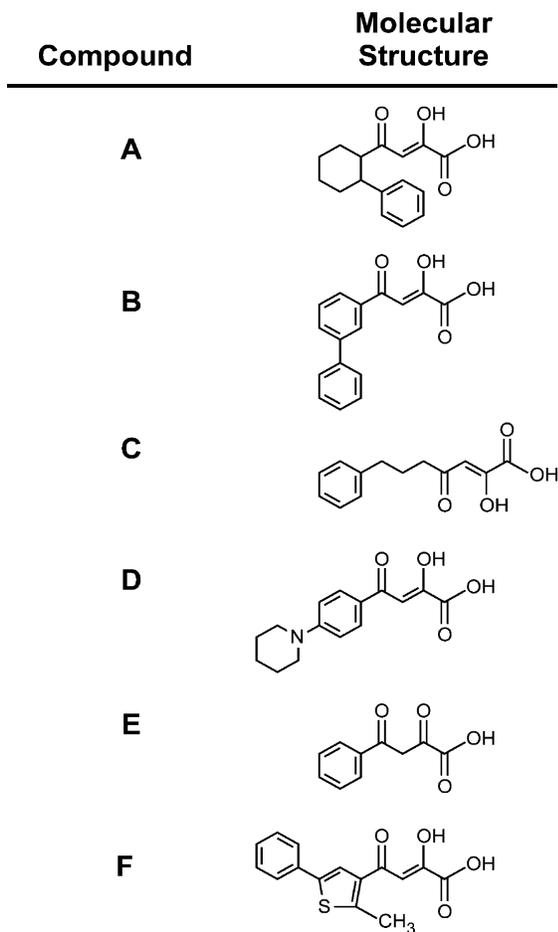


FIGURE 2: DKA compounds used in this study.

**Tnp Purification.** The experiments described in this study were performed with the hyperactive Tn5 E54K/L372P transposase (Tnp) unless otherwise indicated. We also purified the E54K/L372P Tnp derivatives containing the D97A (TnpD97A) or D188A (TnpD188A) or E326A (TnpE326A) mutations. Overexpression and Tnp protein purification were carried out as described previously (20). Protein was quantitated by means of the standard Bradford method (21) using bovine serum albumin (Pierce) as a standard, and the purity was assessed using SDS-PAGE with Coomassie staining.

**Paired End Complex (PEC) Formation Assays.** The ability of the DKA compounds to inhibit PEC formation with both full-length and pre-cleaved DNA substrates was assessed. Full-length or pre-cleaved DNA substrate (30 nM) and 0.01 mM, 0.1 mM, 0.25 mM, 0.5 mM, or 1 mM (final concentrations) DKA compound were mixed in transposition binding buffer lacking  $Mg^{++}$  (100 mM potassium glutamate and 20 mM HEPES at pH 7.5). Each compound (or DMSO for the control) was added such that the final concentration of DMSO was 10%. Then, 225 nM E54K/L372P transposase was added, and the reactions were incubated at 37 °C for 1.5 h. In control experiments, it was demonstrated that the DKA compounds were soluble at 1 mM concentration under these conditions (data not shown). Following incubation, the glycerol loading dye (0.25% Brilliant Blue R-250 in 50% glycerol) was added, and the samples were immediately placed at -20 °C. The reaction products were separated by electrophoresis for 2 h at 300 V on a 7% native polyacry-

lamide gel at 4 °C. The PECs and unbound DNA were visualized using a Typhoon 9410 Variable Mode Imager. All gels were quantitated using Image Quant Total Lab software. To determine the percentage of PECs formed from the DNA substrates, the density of upper (PECs) and lower (free DNA substrate) bands were compared.

**Paired End Complex (PEC) Dissociation Assays.** The ability of the DKA compounds to cause the dissociation of pre-formed PECs was investigated. First, PECs were formed with pre-cleaved or full-length DNA substrates as described above, except that no DKA compounds were included. Twenty microliters of each reaction was removed and added to 5  $\mu$ L of glycerol loading dye. To the remaining complexes, the DMSO (control) or DKA compounds were added to 10% or 1 mM, respectively. Dissociation was then monitored by removing samples at 30 s, 1 min, 5 min, 10 min, and 20 min, mixing with ice-cold glycerol loading dye and electrophoresing on a 7% native polyacrylamide gel at 4 °C. The PECs and unbound DNA were visualized as described above. To establish the concentration of DKA compounds that causes the most effective PEC dissociation, preliminary experiments with lower concentrations of DKA compounds were performed (data not shown). The dissociation assays were performed at 37 °C.

**Cleavage Assays.** To assess the effect of the DKA compounds on DNA cleavage, the following assay was performed. PECs were formed with the full-length substrate, and a 20  $\mu$ L sample was removed as described previously. DMSO (control) or DKA compounds were added to 10% or 0.125 mM, respectively. The 0.125 mM concentration of DKA compounds does not cause the dissociation of PEC complexes pre-formed with the full-length DNA substrate (data not shown). Following a 10 min equilibration period, a 20  $\mu$ L sample was taken and mixed with glycerol loading dye.  $Mg^{++}$  acetate was added to 10 mM, and cleavage was monitored by taking samples at 30 s, 1 min, 10 min, 30 min, 1 h, 2 h, 3 h, 4 h, 6 h, and 24 h and mixing them with ice-cold glycerol loading dye to stop the reactions. All samples were analyzed as described above. Cleavage assays were performed at 37 °C unless otherwise specified.

**Strand Transfer Assays.** PECs were formed with pre-cleaved DNA substrates, and a 20  $\mu$ L sample was removed as described in the previous section. DMSO (control) or DKA compounds (final concentration, 0.125 mM for compounds A, B, D, and E, and 0.05 mM for compounds C and F) were then added and allowed to equilibrate at 37 °C with the PECs as described in the previous section. After equilibration, the second 20  $\mu$ L sample was removed and mixed with glycerol loading dye. Higher concentrations of DKA compounds were found to cause the dissociation of PEC complexes formed with pre-cleaved DNA substrate (data not shown). To the remaining reaction,  $Mg^{++}$  acetate (10 mM, final concentration) and pUC19 plasmid DNA (30 nM, final concentration) were added and incubation was performed at 20 °C. To examine strand transfer over time, 20  $\mu$ L samples were then taken at 30 s, 1, 2, 3, 4, 5, 10, 15, and 20 min and mixed with 5  $\mu$ L glycerol loading dye and 5  $\mu$ L 1% SDS. In the case of the DNA substrate that contained OH or 6-FAM fluorescein on the 5'-end of the non-transferred strand, the reaction was performed at 37 °C. Samples were taken at 10 min, 30 min, 1 h, 2 h, 4 h, 6 h, and, in some experiments, 24 h. To ensure that complex formation had been ap-

proximately equal in all cases for a given experiment and that addition of the DKA compounds had not caused dissociation of PECs, the first two samples were electrophoresed on a 7% native polyacrylamide gel at 4 °C. The remaining samples were separated on a 2% agarose gel at room temperature. The PECs present on the native PAGE gel and the strand transfer products on the agarose gel were visualized using the 526 nm setting on the Typhoon Variable Mode Imager. The agarose gel was also stained with Ethidium Bromide and visualized using the 610 nm setting of the Typhoon Variable Mode Imager. All gels were quantitated using Image Quant Total Lab software.

**PEC Formation Assays with Tnp DDE Motif Mutants.** To evaluate the hypothesis that the inhibition of PEC formation by DKA compounds could be a result of their direct binding to the active site of the transposase, PEC formation assays were performed with Tnp derivatives containing one of the following active site mutations: D97A, D188A, or E326A. The experimental protocol was the same as that for the PEC formation assay described previously for both full-length and pre-cleaved oligonucleotide substrates (either with or without the 5'-phosphate), except that the DKA compound concentrations were changed. The following concentrations of DKA inhibitors were used: 0.01 mM to 1 mM for A, D, and E compounds, 0.01 mM to 0.1 mM for compounds C and F, and 0.01 to 0.2 mM for compound B. Concentrations above 0.1 mM for compounds C and F and above 0.2 mM for compound B cause PEC dissociation (data not shown). Control experiments were performed with E54K/L372P protein.

**PEC Formation Assays in the Presence of Magnesium Ions.** The influence of binding Mg<sup>++</sup> in the active site of E54K/L372P Tnp on DKA compound inhibition of PECs formation was investigated. To avoid cleavage reactions promoted by divalent ions, the experiments were performed with pre-cleaved oligonucleotide substrates only. The protocols were identical to those described previously for the PEC formation assay except that 10 mM Mg<sup>++</sup> acetate was added to the reaction prior to the addition of the DKA compounds. These reactions were compared with the no Mg<sup>++</sup> controls. The experiments were performed at the same concentrations of DKA compounds as those of the experiments with DDE Tnp mutants. For the pre-cleaved DNA substrate with the 5'-phosphate, assays were performed at 20 °C for 5 min. The reduced temperature in these assays reduces the rate of the reaction so that meaningful kinetics can be obtained.

## RESULTS

Previous structural and biochemical studies have led to the conclusion that Tn5 Tnp and HIV-1 IN are members of the same super family of proteins with similar active site catalytic residue architectures and catalytic mechanisms (18, 19). These observations suggest that analogues of inhibitors that are targeted to the active site of either Tnp or IN may have activity on the other protein. Indeed, an initial analysis of 20 Tn5 Tnp inhibitors led to the discovery of 6 compounds that inhibited HIV-1 IN (17), and a screen of the DKA class of HIV-1 IN inhibitors also identified inhibitors of Tn5 Tnp (Stillmock et al., unpublished results). Thus, the biochemically more tractable Tn5 Tnp system offers a potentially

powerful tool for studying the mechanism of inhibition of cross-reacting HIV-1 IN inhibitors. Six DKA IN inhibitors (see Figure 2), which were discovered by a screen to block transposase catalyzed strand transfer, were chosen for further study. These compounds were all potent inhibitors of strand transfer with IC<sub>50</sub> values of 1 μM or less.

**Inhibition of Paired End Complex (PEC) Formation by DKA Compounds.** One of the first steps in Tn5 transposition is the formation of the synaptic complex that contains two molecules of Tnp bound to two 19 bp ESs (see Figure 1). The formation of the synaptic complex is typically studied through PEC assays in which the two 19 bp ESs are located between the transposon and donor DNA on separate oligonucleotides. The formation of the PECs involves multiple transposase–DNA contacts including contacts that are located near the Tnp active site. Therefore, inhibitors that block catalysis through interactions with the Tnp active site will likely block PEC formation. The efficiency of DKA mediated inhibition of PEC formation was also analyzed using pre-cleaved DNA substrates, DNA molecules in which the donor DNA is absent, as would be the case after transposase catalyzed DNA cleavage. If the binding of a given inhibitor is sensitive to the presence of donor DNA, this would be evident in this latter assay.

When small oligonucleotides with the transposon ES are incubated with Tnp in buffer lacking Mg<sup>++</sup>, PECs are formed. These complexes are retarded relative to the unbound DNA following electrophoresis on a native polyacrylamide gel (22). To determine the effect of the DKA compounds on this step of transposition, varying concentrations of the DKA compounds were included in PEC formation reactions with both full-length and pre-cleaved DNA substrates. The reaction products were then analyzed by native polyacrylamide gel electrophoresis. Examples of PEC formation with full-length and pre-cleaved DNA substrates in the presence of 0.25 mM DKA compounds can be seen in Figure 3A and B, respectively. At this concentration, compounds C and F inhibit PEC formation almost completely. Compound B causes a 4-fold reduction for full-length and a 20-fold reduction for pre-cleaved substrates in PEC formation. Compound D causes a 1.5-fold reduction in PEC formation with the full-length DNA substrate and a 3-fold reduction with pre-cleaved substrate. Compound A reduces PEC formation with the full-length DNA substrate 1.5-fold while reducing PEC formation with the pre-cleaved substrate 5.2-fold. Finally, compound E inhibits PEC formation the least, only inhibiting PEC formation with full-length substrate 10% and with pre-cleaved substrate 2.5-fold.

To determine the apparent K<sub>i</sub> parameters for each compound, PEC formation with both full-length and pre-cleaved DNA substrates was quantitated in the presence of several different concentrations (0.01 mM–1 mM) of DKA compounds. See Figure 3C and D for a graphical representation of these data. From the data set for each compound, we calculated the DKA compound concentration that caused 50% inhibition (K<sub>i</sub>). These values can be seen for each DKA compound with full-length and pre-cleaved substrate DNAs in Figure 3. For inhibitors A and D, the presence of donor DNA has a dramatic negative impact on inhibitor efficacy.

Pre-cleaved DNA substrates used in the above experiments did not contain the phosphate group on the 5'-end of non-transferred strand. However, the 5'-phosphate at this position

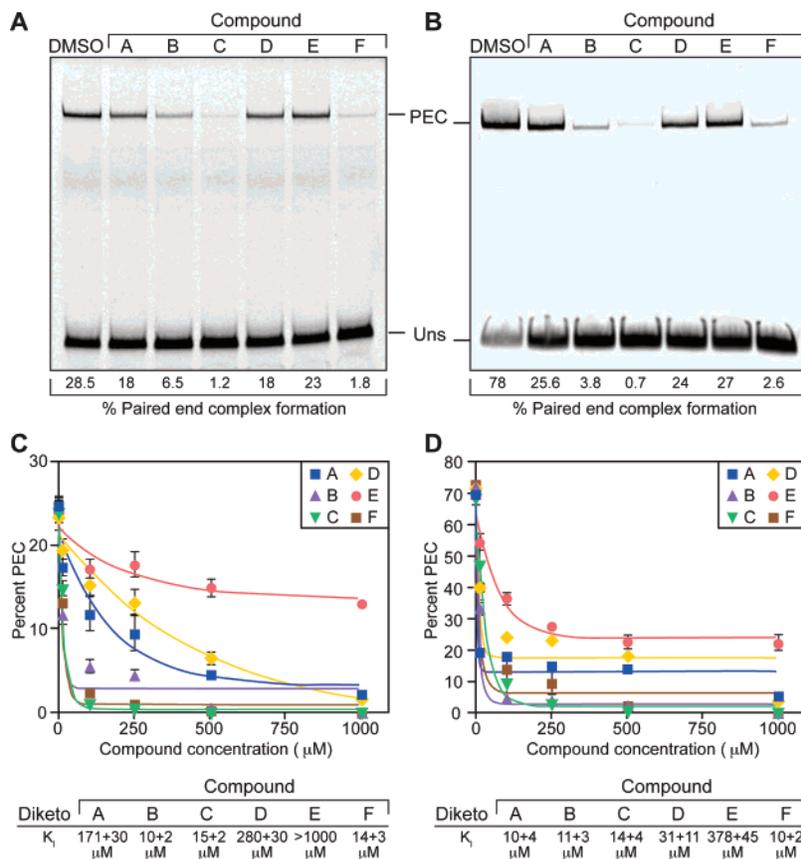


FIGURE 3: Inhibition of PEC formation. PEC formation with 60 bp DNA in which the recognition signal sequence is connected to 20 bp of donor DNA (panel A) and 40 bp pre-cleaved DNA (no donor DNA, panel B) was studied in the presence of the six inhibitors using a gel retardation assay. The percent of the retarded PECs is indicated below the gel lanes. All compounds were dissolved in DMSO. The DMSO lane indicates the no compound control. The presence of 0.25 mM of the various diketo compounds caused inhibition of PEC formation. The inhibition efficiency varies among the chemicals. Tables below the gel figures show the  $K_1$  values calculated for each compound. All compounds demonstrated enhanced inhibition when pre-cleaved DNA was used as a substrate. Abbreviations: DMSO = dimethyl sulfoxide; A = compound A; B = compound B; C = compound C; D = compound D; E = compound E; F = compound F; Uns = unshifted DNA substrate (free oligonucleotides).

is found on the transposon end formed by Tn5 excision from the donor DNA. Therefore, the PEC formation assays with the 5'-phosphate pre-cleaved substrate were performed to determine the importance of the 5'-phosphate. The experimental data shows no significant differences in PEC formation for substrates with and without the 5'-phosphate and no differences in inhibitor sensitivity at this step (data not shown).

**Dissociation of Pre-formed PEC Complexes by DKA Compounds.** To investigate the influence of DKA compounds on pre-formed PEC stability, we performed PEC dissociation assays. PECs were first formed by incubation of DNA substrates with Tnp and then exposed for differing lengths of time to compounds at 1 mM final concentration. (At concentrations  $\leq 500 \mu\text{M}$ , no dissociation is observed; data not shown.) In these experiments, we used either full-length or pre-cleaved DNA substrates as indicated.

For the full-length 60-mer DNA substrate, the only DKA compound that caused significant dissociation of pre-formed PEC complexes was compound F (Figure 4A). At 1 mM concentration, F dissociated 60% of pre-formed PEC complexes in the first 30 s, and then the reaction came to a plateau. PECs formed from pre-cleaved DNA substrates were generally more sensitive to the presence of DKA compounds (Figure 4B). The most effective PEC dissociation agent was compound C, causing 99% dissociation at 1 mM concentra-

tion. The same concentrations of F, B, and D DKA compounds caused approximately 67%, 35%, and 25% PEC dissociation, respectively. It is worth noting that the dissociation properties for compound F were similar for both full-length and pre-cleaved DNA substrates. No effects of compounds A and E on complex dissociation were observed. The presence of the phosphate on the 5'-end of the non-transferable strand for pre-cleaved DNA substrates does not change the results for the PEC dissociation experiments (data not shown).

**DKA Compounds Affect Transposon–Donor DNA Cleavage.** The first catalytic steps in the Tn5 transposition process involve the cleavage of the phosphodiester bonds linking the transposon to the donor backbone (DBB) DNA. The PEC complex may then capture the target DNA, allowing strand transfer to occur (see Figure 1).

Cleavage assays were performed in order to examine whether DKA compounds affect steps after PEC complex formation. In these experiments, the full-length 60-mer DNA substrate and Tnp were incubated together to form PEC complexes (see Experimental Procedures for details). Approximately 30% of the DNA substrate was involved in PEC formation in each experiment (Figure 5A, B, and C, line 1). The amount of DMSO (10%) or the 0.125 mM concentration of DKA compounds used in the assays did not cause PEC dissociation (Figure 5A, B, and C, line 2).

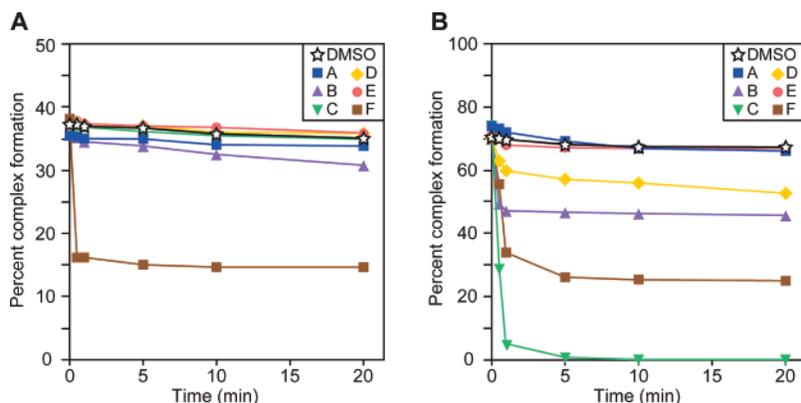


FIGURE 4: DKA inhibitors destabilize PECs. In order to test the effect of DKA compounds on PEC stability, PECs were formed with either 60 bp full-length DNAs (donor DNA present, panel A) or 40 bp pre-cleaved DNA (no donor DNA, panel B). The DKA compounds (1 mM final concentration) were added following PEC formation at time 0, and then samples were removed at 30 s, 1 min, 5, 10, and 20 min, and analyzed through the gel retardation assay. Using the 60 bp DNA that contained the donor DNA, only compound F caused dissociation. Using pre-cleaved DNA, compounds D, B, F, and C caused dissociation in that order.

The addition of  $Mg^{++}$  acetate initiated the cleavage reaction. Products of this reaction run faster on the native polyacrylamide gel than PECs. The first cleavage product is a single-end break (SEB) product with only one of the two DBB DNA–transposon boundaries cleaved (Figure 1). The SEB product is visible on the gel as a band located immediately below the PEC band (Figure 5A, B, and C, line 3). The different compounds had different effects on the amounts and kinetics of SEB product formation. For the control reaction (DMSO), the SEB product band was most visible 1 min after  $Mg^{++}$  acetate was added (Figure 5A, line 4). The amount of the SEB product decreases as time progresses because of the formation of double-end break (DEB) products. Results similar to that seen for the control were obtained for compounds A and C. For compounds D and E, the maximum amount of the SEB product was observed at 10 min, whereas for compounds B and F (Figure 5B and C, line 7), the peak was at 1 h. Increases in the amount of SEB product corresponded with decreases in PEC abundance. Representative graphical representations of the cleavage assay results are presented in Figure 6.

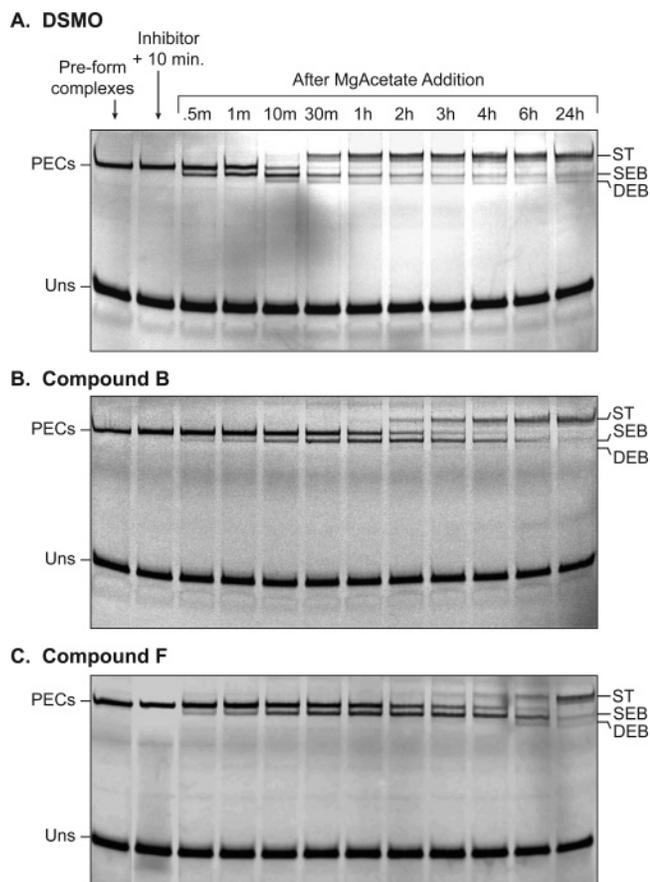
The DEB product, visible on the gel as a band under the SEB product band, was detectable for the DMSO control (Figure 5A, line 5) as well as for most of the compounds 10 min after  $Mg^{++}$  acetate was added. A significant delay in the formation of the DEB product was observed only for compounds B (Figure 5B) and F (Figure 5C) (see also Figure 6C). DEB products were detectable 3 h (compound B) and 6 h (compound F) after the  $Mg^{++}$  ions were added.

The strand transfer products (STPs) are formed after the excision of the transposon from the DBB and the capture of the new target DNA (believed to include both excess free linear 60-mer substrate DNA and perhaps DNA bound in other PECs). Although the DKA compound-dependent effect on the concentration of DEB substrates makes the strand transfer results in the assays difficult to quantify, qualitative information is available. STPs are visible on the native polyacrylamide gel as bands running more slowly than the PEC band. The first strand transfer products in the control experiment were formed during the first 10 min after  $Mg^{++}$  acetate addition. The maximum amount of STPs was reached at 4 h, and then the reaction came to a plateau (Figure 5A, lines 6–12, upper band; in addition, see Figure 6D). A

decrease in the level of PECs, SEB, and DEB products with time was noticed along with increasing amounts of STPs. For compounds B and F, STP bands became visible in the second (compound B) or the third (compound F) hour after  $Mg^{++}$  acetate had been added and reached the maximum density at the end of the experiment (Figure 5B, lines 9–12 for compound B, and Figure 5C, lines 9–12 for compound F). Compounds A and C do not appear to affect STP formation, while compounds D and E modestly inhibit STP formation (Figure 6D).

*Temperature Effect on Transposon-Donor DNA Cleavage.* The delay in the SEB to DEB cleavage reaction imposed by inhibitor F is compatible with F inhibiting a conformational change in the PEC required for Tnp to catalyze the second DNA cleavage (see Discussion). In order to test whether a conformational change might be required prior to the DEB cleavage event, the cleavage process was examined for temperature sensitivity. Cleavage experiments were performed at 37 °C (as was performed in the previous experiment) and at 25 °C in the absence of any DKA compounds. As shown in Figure 7, the generation of SEB products were moderately impaired at 25 °C; however, the generation of DEB and ST products were almost completely blocked. The extreme temperature sensitivity of DEB generation would explain the failure to produce ST products since the DEB product is a required substrate for strand transfer. The easiest explanation for the inability to perform SEB to DEB cleavage at 25 °C is that this step in transposition requires a prior conformational change in the PEC, and the conformational change process is impaired at 25 °C.

*DKA Compounds Affect Strand Transfer.* The final step in transposition is strand transfer in which Tnp in the presence of  $Mg^{++}$  activates the 3'-OH groups of the transposon DNA to act as nucleophiles in the phosphotransfer reaction that integrates the transposon into target DNA (see Figure 1). The cleavage assays reported above provide a glimpse of the inhibitor compound effects on strand transfer, but the analysis is complicated by the compound effects on cleavage resulting in the generation of varying levels of cleavage substrate. In addition, supercoiled DNA (and not a linear oligonucleotide) is the preferred target for strand transfer and is more biologically relevant. Thus, we used another assay, which specifically examines the effects of



**FIGURE 5:** DKA inhibition of DBB DNA–transposon cleavage; compounds B and F. DKA compounds are capable of inhibiting catalysis by pre-formed PECs at concentrations lower than that needed to cause complex dissociation. DMSO or 0.125 mM inhibitors (final concentration) in DMSO were added to pre-formed PECs with donor DNA attached.  $Mg^{++}$  acetate was added to allow catalysis, and then samples were removed (and the reaction was stopped) at various times. The samples were analyzed by native PAGE electrophoresis. Panel A represents the reaction in the presence of DMSO. Panel B represents the reaction in the presence of 0.125 mM compound B. Panel C represents the reaction in the presence of 0.125 mM compound F. Lane 1 = pre-formed PEC. Lane 2 = PEC plus inhibitor or DMSO. Lanes 3–12 = various time points after the addition of  $Mg^{++}$  acetate. The expected sequence of events: one DNA in the PEC is cleaved, then both DNAs are cleaved, and then strand transfer occurs. The different reaction products are detectable by alterations in the complex gel mobility. Abbreviations: PECs = paired end complexes; Uns = free DNA; ST = strand transfer products; SEB = single-end cleavage product; DEB = double-end cleavage product. Compound B impairs catalysis in general. Compound F is particularly interesting in that it has a modest inhibitory effect on the first cleavage step but a strong inhibitory effect on the second cleavage step.

potential inhibitor compounds on the combined steps of target capture and strand transfer using supercoiled DNA as a target.

First, PECs were formed using pre-cleaved 40-mer DNAs containing a phosphate at the 5'-end of the non-transferred strand. The various DKA compounds (or DMSO as a control) were added to a final concentration of 0.125 mM (compounds A, B, C, and E) or 0.05 mM (compounds C and F) inhibitor or 10% DMSO. These concentrations of inhibitors were chosen because they are known to not cause the dissociation of PEC complexes pre-formed with pre-cleaved DNA substrate for the respective compounds (data not shown). The

target capture and strand transfer were initiated by the addition of the target DNA (supercoiled pUC19 DNA) in the presence of  $Mg^{++}$  acetate. Because of the rapid kinetics of the reaction, the assays were performed at 20 °C.

The strand transfer of the two activated 3'-OH ends in a PEC does not occur in a concerted fashion since one typically observes both single-end strand transfer (SEST), generating nicked target DNA in which one labeled 40-mer DNA is covalently linked to one strand of pUC19 DNA (the product runs as labeled relaxed circular DNA structure), and double-end strand transfer (DEST), generating a labeled linear form of the target (Figure 1). The kinetics of the appearance of the two products is consistent with the SEST products being precursors of the DEST products (see Figures 8 and 9). For the DMSO control (and incubation in the presence of compounds B, C, D, and F), SEST and DEST are evident as early as 30 s. Compounds A and E severely inhibit strand transfer. Compounds B, D, F, and perhaps C show modest inhibitory effects on both SEST and DEST.

The same assays were performed with pre-cleaved DNA substrates containing either 5'-OH or 5'-FAM on the non-transferred strand. For both the 5'-OH and 5'-FAM substrates, the kinetics of strand transfer was dramatically slowed for both the control and for reactions containing the inhibitors. The general sensitivity of the strand transfer process to the 5'-end structure of the non-transferred strand will be addressed in more detail in a subsequent communication. In addition, the relative sensitivity to the compounds was altered. In particular, compounds B and F blocked all strand transfer for up to 24 h, and compound D manifested severe inhibition. In the presence of a 5'-phosphate, compounds B, D, and F have modest inhibitory properties. These results indicate that the presence of a 5'-phosphate impairs the inhibitory effects of compounds B, F, and D (see Discussion). It is also noteworthy that compound A is a potent inhibitor for the 5'-FAM and 5'-phosphate substrates but demonstrates little or no inhibition for the 5'-OH substrate (Figure 9).

*DKA Inhibition Is Sensitive to the Active Site Structure.* Data collected during previous experiments indicated that the DKA compound inhibition of PEC formation and stimulation of disassembly of pre-formed PECs are for some compounds more efficient for PECs constructed with pre-cleaved DNA substrates. Thus, the presence of donor DNA impairs the activity of some compounds. In addition, the presence of a 5'-phosphate on the non-transferred strand impairs the strand transfer inhibitory activity of compounds B, F, and D. These observations suggest that the inhibitors bind within or near the Tnp active site. We have investigated this possibility in more detail by examining whether active site mutations or the presence of  $Mg^{++}$  alters the PEC formation sensitivity to various DKA compound inhibitors.

In order to investigate the impact of active site mutations on DKA compound activity, we have performed PEC formation assays using D97A or D188A or E326A active site Tnp mutants (23). For these experiments, DKA compound concentrations were chosen that caused significant reduction but not complete inhibition of PEC formation with E54K/L372P Tnp.

Table 1, section A presents the results for experiments performed with the full-length DNA substrate. This data is normalized to the DMSO control for each mutant (and in brackets, to the E54K/L372P Tnp DMSO value) for com-

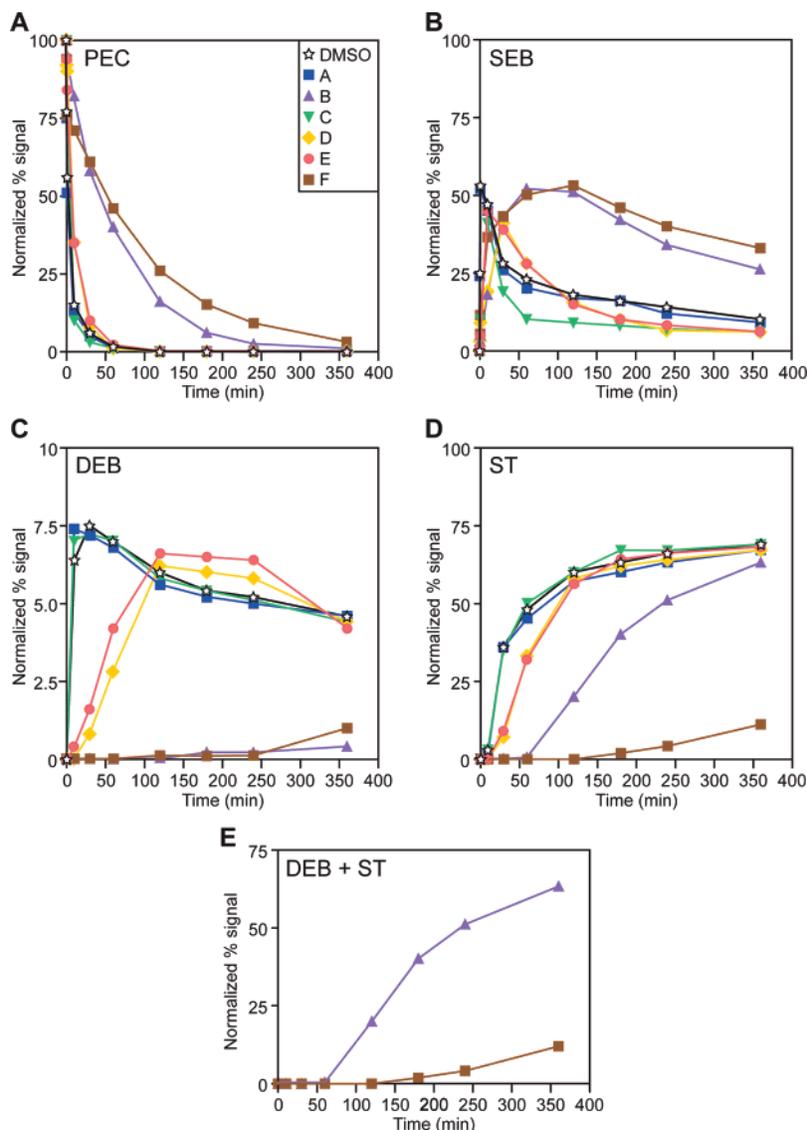


FIGURE 6: DKA inhibitors and DBB—transposon DNA cleavage. A plot of the DNA cleavage inhibition results for all six DKA compounds determined as described in Figure 4. Panel A presents the rate of loss PECs. Panel B presents the production and subsequent loss of SEB products (since one fluorescent out of 4 is removed during SEB generation, the results were normalized to PECs by multiplying the fluorescence units by 1.33). Panel C presents the production and subsequent loss of DEB products (two fluorescent labels are removed during DEB generation, thus normalization required multiplication by 2). Panel D presents the production of ST products (normalized by multiplying by 0.67). Two compounds, B and F, inhibit the transition from PEC to SEB and SEB to DEB with similar efficacies. They act differentially, however, on DEB and ST formation. The DEB product formed in the presence of compound B goes quickly to ST, whereas compound F by inhibiting DEB formation also inhibits the formation of the ST product. Compounds E and D moderately inhibit DEB formation but do not inhibit the production or loss of any other product of the reaction. Compounds A and C show no influence on any of the cleavage steps.

parative purposes. In general, the efficiencies of PEC formation for the mutant Tnps are lower than those for E54K/L372P Tnp, consistent with the results previously reported by Naumann and Reznikoff (23). The relative inhibitory effects for compounds B, C, and F are generally comparable for E54K/L372P Tnp, D97A Tnp, and D188A Tnp. However, for mutant Tnp E326A, the inhibitory effects of compounds B, C, and F are about half of that observed for E54K/L372P Tnp. This observation suggests that the E326A mutation in the transposase active site has impaired DKA B, C, and F inhibition of PEC formation.

Significant differences in PEC amounts were observed when pre-cleaved DNA substrates were used. Tnp E326A formed 6 to 7 times more PECs than the E54K/L372P protein with 0.1 mM and 0.2 mM concentrations of compound B. In the presence of 0.1 mM compounds C and F, 3.5 and 2.5

times more PECs were formed, respectively, with E326A Tnp than with E54K/L372P. Increases in PEC formation with Tnp E326A was observed for most tested concentrations of the compounds mentioned above (Table 1B). Interestingly, the abundance of PECs exceeded the level achieved with the DMSO control for most concentrations of compounds B, C, and F. These results indicate that the presence of DKA compounds in the reaction has not inhibited but rather enhanced PEC formation for the mutant Tnp E326A (Table 1B).

Impaired inhibition of PEC formation with pre-cleaved DNA substrates was also noticed for the D97A Tnp mutant. Most concentrations of compounds C and F gave an increase of 1.5–2-fold in PEC formation. Compound B quadrupled the efficiency of PEC formation by D97A Tnp (Table 1B). The enhancement of PEC formation with respect to the

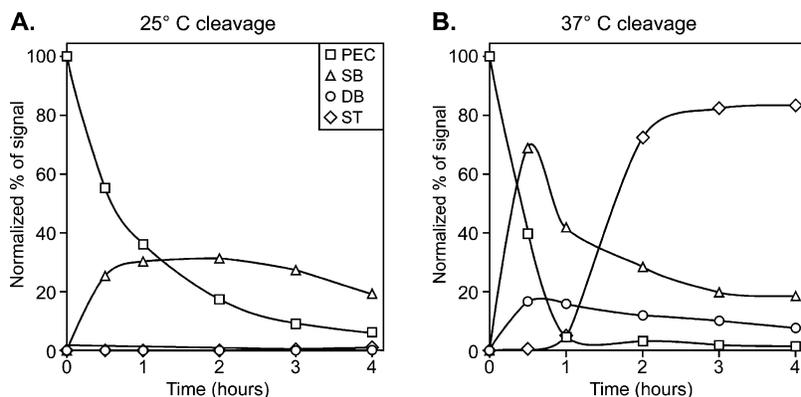


FIGURE 7: SEB to DEB transition is impaired at 25 °C. At 25 °C (panel A), the Tn5 Tnp catalyzed cleavage reaction was impaired relative to that catalyzed at 37 °C (panel B). The production of SEB products is moderately impaired at 25 °C, while generation of DEB products (and subsequent ST products) is almost completely blocked. This result is compatible with a dependent, sequential model of transposon–DNA cleavage. It supports the hypothesis that the Tn5 PEC changes its conformation between the first and the second DBB–transposon boundary cleavage.

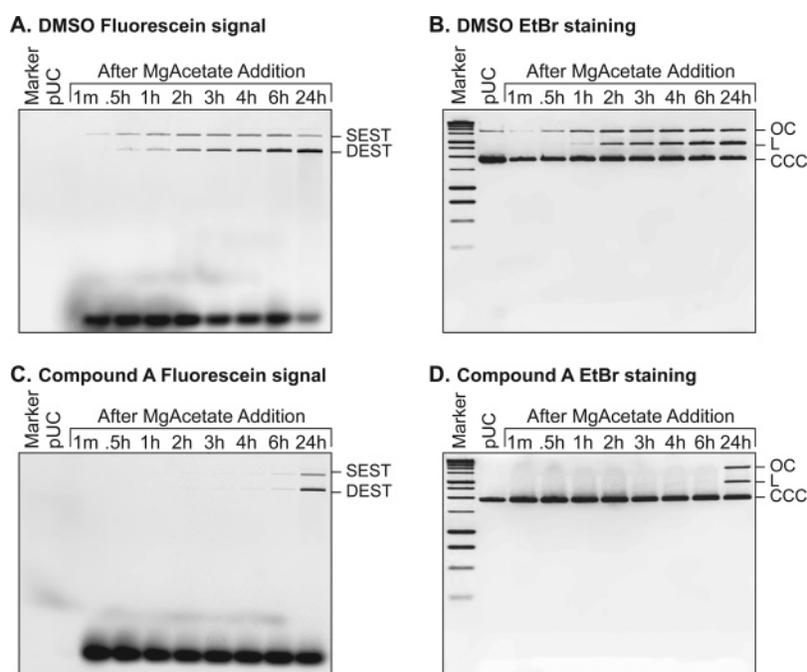


FIGURE 8: DKA inhibition of strand transfer. The PECs formed with fluorescein labeled, pre-cleaved DNA containing ES and 5'-6-FAM of the non-transferred strand were incubated with 1 mM compound A (panels C and D), supercoiled plasmid DNA (CCC), and  $Mg^{++}$  acetate for various times and analyzed by agarose gel electrophoresis. The location of the fluorescein signal that was introduced by strand transfer of the labeled DNA from the PEC was determined. Staining the gels with EtBr tracks the reaction through the formation of nicked open circles (OC) (one strand transferred) and linear molecules (L) (both strands transferred). The single-end strand transfer (SEST) and double-end strand transfer (DEST) products were identical to the ethidium bromide (EtBr) detected OC and L bands. Panels A and B represent the results for the DMSO control and panels C and D for compound A. Compound A, a potent inhibitor of this reaction for ES DNA containing a 5'-phosphate on the non-transferred strand, caused inhibition of SEST and DEST formation for several hours. For this particular DNA substrate, compounds D and E block strand transfer with modest efficiency (data not shown), while compounds B and F block strand transfer totally for 24 h (data not shown). Abbreviations: marker lane = linear size standards; the pUC lane = unreacted pUC19.

DMSO control occurred at the lowest tested concentrations of compounds B, C, and F.

No differences in PEC formation in the presence of compounds A, D, and E was observed for both full-length and pre-cleaved DNA substrates when the Tnp active site mutants were used.

*Presence of  $Mg^{++}$  Alters DKA Inhibition of PEC Formation.* Assuming DKA compounds contact the active site of Tnp, binding any other molecule in the same position should perturb the binding of the compounds. Thus, we expected an altered inhibition of PEC formation when pre-cleaved PEC formation was performed in the presence of  $Mg^{++}$ . To

evaluate this hypothesis, we have assessed PEC formation assays with  $Mg^{++}$  acetate added to the reaction prior to the addition of the inhibitors. The  $Mg^{++}$  molecules are relatively big and positively charged; therefore, their binding limits the space and would change the charge in the Tnp active site cavity. Additionally,  $Mg^{++}$  binding is coordinated by transposase's active site DDE residues, and the above experiments with DDE Tnp mutants indicated that some of these residues were potentially involved in Tnp–DKA contacts.

In order to avoid catalysis, only the pre-cleaved DNA substrates were used to investigate the effect of  $Mg^{++}$  on

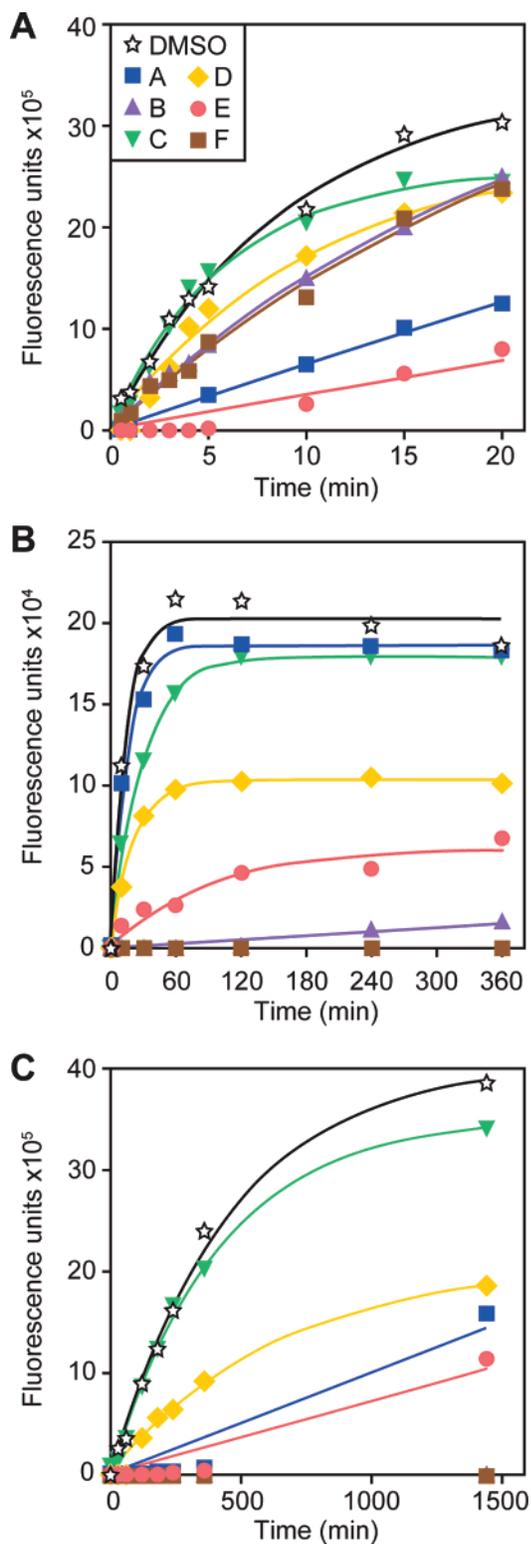


FIGURE 9: Dependence of DEST formation inhibition on the non-transferred strand 5'-structure. Inhibition of the reaction by some DKA compounds depends on the nature of the ES DA 5'-end. Panels A, B, and C represent results for the ES substrates with 5'-phosphate, 5'-OH and 5'-6-FAM on the non-transferred strand respectively. Compounds B and F, the most effective in inhibition of DEST formation with 6-FAM and OH substrate, show moderate inhibition of the reaction in the presence of the phosphate group on the 5'-end of the non-transferred strand. Compound A does not inhibit DEST formation when the OH substrate is present, while it is quite effective in the presence of two other types of the DNA substrate. Compounds C, D, and E show the same potency of inhibition with all three DNA substrates.

DKA compound inhibition of PEC formation. The results of experiments were normalized in the same manner as that described previously. The presence of  $Mg^{++}$  in the reaction prior to the addition of compounds B, C, or F prevented the inhibition of PEC formation. Twice as much of the PECs were formed in the presence of  $Mg^{++}$  at 0.05 mM concentrations of inhibitors B, C, and F than in the absence of added  $Mg^{++}$  (Table 2). In the reactions with  $Mg^{++}$ , the increase in PEC formation over the DMSO control was observed at most concentrations tested (Table 2). For DKA compounds A, D, and E, PEC formation reaches the same level in the presence or absence of  $Mg^{++}$  (data not shown). Similar results were obtained for substrates either with or without phosphate on the 5'-end of non-transferable strand.

## DISCUSSION

The DKA compounds used in this study were identified from a library of analogues synthesized as inhibitors of HIV-1 IN. It has been demonstrated that DKA compounds bind to the IN active site and preferentially inhibit the strand transfer reaction between the viral and chromosomal DNAs (12, 15). Strand transfer inhibition prevents integration and replication of the HIV-1 virus (24, 25). It has also been reported that compounds that inhibit HIV-1 IN or Tn5 Tnp can cross-react between the two proteins (Stillmock and Hazuda, unpublished data, (17)). On the basis of the similarities of IN and Tnp active site structures and their catalytic mechanisms, we propose that the information obtained for one of these proteins could be adopted with caution for the other. Because Tn5 Tnp is technically easier to work with than HIV-1 IN, we used Tn5 Tnp as a surrogate model for studying the modes of action of the IN inhibitors. In addition, this study should directly provide us with tools to better define the Tn5 transposition process. There are two surprising results from our studies on DKA compound inhibition of Tn5 transposase. The first notable result was that the six tested DKA compounds demonstrated a striking variety of effects on transposase mediated activities. The second notable result is that DKA compound inhibition of PEC formation activity did not require the presence of  $Mg^{++}$ . Neither of these observations were expected on the basis of a simple extrapolation of models for DKA inhibition of HIV-1 integrase. These observations suggest that the DKA compounds will be extremely powerful tools for studying the functioning of Tn5 transposase but that either the DKA mediated inhibition of HIV-1 integrase is potentially more complicated than we had previously thought was the case or that the comparison of DKA inhibitor activity between the two systems is more complicated than our initial assumption.

**Inhibition of PEC Formation.** All six tested DKA compounds inhibited PEC formation, although with widely varying efficacies. The PEC formation inhibition results for both full-length (containing DBB sequences) and pre-cleaved DNA substrates demonstrate that the most potent inhibition of PEC formation is caused by compounds B, C, and F (with apparent  $K_I$  values of  $\sim 10\text{--}15\ \mu\text{M}$ ). Compound E inhibited PEC formation least effectively. Enhanced inhibition was noticed for all DKA compounds in the presence of the pre-cleaved DNA substrate; however, the effect of DBB DNA absence was most striking for compounds A and D. The simplest interpretation of these results is that different DKA

Table 1: Results of PEC Formation Assays, with E54K/L372P, D97A, D188A, and E326A Transposase<sup>a</sup>

A. Full Length DNA										
	DMSO	compound B			compound C			compound F		
		0.05 mM	0.1 mM	0.2 mM	0.01 mM	0.05 mM	0.1 mM	0.01 mM	0.05 mM	0.1 mM
EKLP	100	51 ± 4	32 ± 4	10 ± 2	69 ± 6	32 ± 4	8.0 ± 1	61 ± 4	42 ± 1	10 ± 3
D97A	100	44 ± 4	36 ± 5	17 ± 4	54 ± 5	20 ± 9	6.0 ± 3	57 ± 3	45 ± 3	17 ± 3
D188A	100	53 ± 9	32 ± 3	27 ± 7	58 ± 2	27 ± 1	2.5 ± 1	52 ± 6	32 ± 2	15 ± 3
E326A	100	80 ± 15	65 ± 5	38 ± 12	97 ± 1	78 ± 5	11 ± 6	87 ± 10	76 ± 7	30 ± 10

B. Pre-Cleaved DNA										
	DMSO	compound B			compound C			compound F		
		0.05 mM	0.1 mM	0.2 mM	0.01 mM	0.05 mM	0.1 mM	0.01 mM	0.05 mM	0.1 mM
EKLP	100	48 ± 3	18 ± 2	12 ± 4	83 ± 6	25 ± 4	11 ± 2	77 ± 3	39 ± 4	23 ± 4
D97A	100	118 ± 3	96 ± 3	62 ± 10	127 ± 8	41 ± 7	6.0 ± 1	115 ± 2	74 ± 9	35 ± 5
D188A	100	43 ± 1	35 ± 2	25 ± 2	74 ± 2	26 ± 2	2.6 ± 1	90 ± 5	46 ± 8	22 ± 3
E326A	100	203 ± 9	194 ± 4	155 ± 5	155 ± 15	145 ± 15	69 ± 5	125 ± 1	158 ± 9	98 ± 8

<sup>a</sup> Panels A and B represent data for full-length and pre-cleaved DNA substrates respectively. For each transposase the values were normalized to % signal shifted in the DMSO control for E54K/L372P Tnp. The alteration of the PEC inhibition in the presence of compounds C, B, and F indicate that the Tnp active side domain is a target for DKA compound binding.

Table 2: Results of PEC Formation Assays with Pre-Cleaved DNA Substrates Performed in the Presence or Absence of Mg<sup>++</sup> Ions<sup>a</sup>

	DMSO	compound B			compound C			compound F		
		0.05 mM	0.1 mM	0.2 mM	0.01 mM	0.05 mM	0.1 mM	0.01 mM	0.05 mM	0.1 mM
-Mg <sup>++</sup>	100	61 ± 10	48 ± 8	27 ± 5	84 ± 3	50 ± 4	17 ± 7	70 ± 10	58 ± 7	32 ± 8
+Mg <sup>++</sup>	100	127 ± 2	120 ± 4	117 ± 7	105 ± 5	107 ± 9	55 ± 7	130 ± 7	128 ± 8	116 ± 2

<sup>a</sup> The amount of PECs formed in the presence of DMSO was equal in both experiments and was calculated as 100%. The presence of Mg<sup>++</sup> ions in the reaction prevents the inhibition of PEC formation, which would occur in the absence of Mg<sup>++</sup> for B, C, and F compounds.

compounds have differential inhibitory effects on PEC formation because of their differing contacts with Tnp and that for some compounds such as A and D the inhibitor and DBB DNA share some Tnp contacts. Notice that the PEC formation assay is likely to study a somewhat different set of inhibitor contacts than that examined for many other assays in this study (and that examined in HIV-1 IN inhibition assays) because the latter studies were performed with pre-made protein-DNA complexes in which the DNA may play a role in inhibitor binding. In addition, these assays, for the most part, were performed in the absence of Mg<sup>++</sup> as apposed to the catalytic assays.

*Inhibitor Induced Dissociation of PECs.* The ability of some DKA compounds to induce PEC disassembly indicates that these compounds, when present at high concentrations (~1 mM), affect the stability of complexes that normally exist for hours. PEC induced dissociation was observed for compounds B, C, D, and F, although only compound F caused dissociation when the DBB was covalently linked to the substrate DNA (Figure 4). The results from the dissociation assays again suggest that in the absence of DBB DNA the nucleoprotein complexes are more accessible to the inhibitors, especially for compounds B, C, and D. Since PEC dissociation requires substantially higher concentrations of inhibitors than does the inhibition of catalysis, we propose that there are two different DKA binding sites in the Tn5 PEC with the lower affinity site being related to the dissociation phenomenon. The two sites DKA binding model has been proposed previously by Pommier and Marchand (25). Alternatively, each compound may bind to a single site, but the contacts and therefore the affinities may differ depending on the molecular constituents (i.e., DNA, Mg<sup>++</sup>)

and the precise protein conformation present in the complexes for the different assays.

*Inhibition of Transposon-DBB DNA Cleavage.* After PEC complexes form, Tn5 Tnp activated by Mg<sup>++</sup> initiates cleavage of the DNA boundaries between the transposon and the DBB through two catalytic steps: 3'-transferred strand nicking and hairpin formation (9, 22). This catalysis occurs at both transposon-DBB DNA boundaries in the PEC. Pilot experiments demonstrated that four of the six DKA compounds are capable of inhibiting DNA cleavage catalysis at concentrations lower than those required for causing the dissociation of pre-formed PEC complexes. Therefore, we chose to study inhibition of cleavage using 0.125 mM of the various DKA compounds in order to avoid the complication of PEC dissociation. At 0.125 mM, all DKA compounds (except compounds A and C) delayed DNA cleavage indicating that they were inhibitors of this process.

As shown in Figures 5 and 6, transposon-DBB DNA cleavage can be seen to occur in two general steps giving rise to PECs in which one cleavage event has occurred (single-end break PECs (SEB) and double-end break PECs (DEB)). The appearance of an intermediate SEB product is compatible with two models. Either the two cleavage events are independent of each other or else they are dependent, sequential events likely to require a conformation change in the PEC between the SEB and DEB events. A dependent, sequential event implies that an asymmetry exists in the PEC prior to DNA cleavage. Because the X-ray crystallographic structure of the Tn5 PEC containing pre-cleaved DNA is symmetrical (5, 8), we had not entertained the possibility of asymmetry despite the fact that the Tn10 PEC has an IHF imposed asymmetry (26, 27). Compounds B and F have comparable delaying effects on SEB formation, but F has a

stronger effect on DEB formation than does B. Compound F's effect on DEB formation is also manifested in the DEB dependent step of STP formation. Because both SEB formation and DEB formation involve exactly the same catalytic processes, the selective effect of compound F on DEB formation suggests that F acts, in part, by inhibiting a required conformational change between SEB and DEB formation.

In order to study the possible existence of a required conformational change occurring between SEB formation and DEB formation, we also examined the effect on SEB and DEB formation of lowering the reaction temperature to 25 °C (Figure 7). The SEB to DEB cleavage reaction is dramatically inhibited at 25 °C, supporting the hypothesis that an important temperature sensitive conformational change occurs at this step. We have recently reported that a selective delay in DEB formation occurs as a consequence of mutations in Tnp residues 244 and 253 in a  $\beta$ -clamp that is folded over the ES in the PEC (28). Perhaps the  $\beta$ -loop contacts to the ES play a role in facilitating the required conformational change. The existence of a required conformational change in the SEB to DEB process suggests that the Tn5 synaptic complex in the presence of covalently linked DBB DNA (that is, prior to cleavage) is asymmetrical in contrast to the solved PEC structure containing pre-cleaved ES DNA. The determination of precisely which step in transposon-DBB DNA cleavage (3'-transferred strand nicking or hairpin formation) is blocked by the inhibitors is an important question that will be addressed in future studies.

The conclusion that the Tn5 transposase-DNA synaptic complex undergoes a conformational change as it proceeds from the first transposon-DBB DNA cleavage to the second transposon-DBB DNA cleavage is reminiscent of the isomerization event that is proposed to occur in site specific recombination by systems such as Cre-*loxP* (29). These systems utilize a different form of chemistry in their DNA breakage and reunion events and have tetrameric rather than dimeric complexes. Nonetheless, catalysis is first performed by two CRE protomers arranged diagonally, and then the second set of catalytic events is performed by the second pair of CRE protomers. In between these two sets of events, the overall protein-DNA complex undergoes a conformational change.

Compounds A and C appear to have no effects on transposon-DBB DNA cleavage. There are two obvious explanations for this observation. Either compounds A and C have their access to Tnp blocked by the presence of DBB DNA or compounds A and C bind to the PEC but have no effect on DNA cleavage. A possible negative effect of DBB DNA on the binding of compound A is consistent with the observation that DBB DNA decreases the efficacy of compound A in blocking PEC formation and the hypothesis that compound A and DBB DNA share some contact sites on Tnp.

*Inhibition of the Strand Transfer.* The final steps in Tn5 transposition involve target capture and strand transfer. Because we know that strand transfer is most efficient when the target is supercoiled (30, 31) and because we wished to avoid complications that would result from differing inhibitions of DNA cleavage, we used an assay for target capture/strand transfer that examined the transfer of pre-cleaved transposon DNA into supercoiled plasmid DNA. Compound

A inhibited strand transfer most severely when examined with the biologically relevant substrate (containing a phosphate group at the 5'-end of the non-transferred strand) (Figure 8A). Notice that compound A was effective at inhibiting strand transfer even though it demonstrated no potential for blocking DNA cleavage. The selectivity of compound A for strand transfer inhibition is surprising since the hairpin formation step (which is required for cleavage and thus is not inhibited by compound A) also utilizes a 3'-OH as the nucleophile, as does strand transfer (8). Alternative explanations can be posited for compound A's differential effectiveness against strand transfer versus cleavage. It could be that A is ineffective at inhibiting cleavage because its activity is sensitive to the presence of DBB DNA (see the PEC formation assays) or, alternatively, that compound A is really inhibiting the target capture step and not strand transfer.

*Inhibitors Bind in or Near the Active Site of Tn5 Tnp.* It has been shown that IN inhibitors, similar to those analyzed in this article, bind within the IN active site (14, 32). Because of the structural similarities between IN and Tn5 Tnp, we assumed that the Tnp active site domain would also be a target for DKA compound binding. PEC formation and dissociation data in the presence of the DKA inhibitors demonstrated an impaired inhibition for some DKA compounds when the DNA substrates contained DBB DNA. This observation suggests that DBB DNA may compete with the inhibitors for binding to Tnp consistent with the active site domain being the location for inhibitor binding.

Additional data consistent with the inhibitors binding in or near the active site of Tn5 Tnp came from our examination of inhibitor effects on strand transfer. The biologically relevant substrate for strand transfer is the product of the transposon-DBB cleavage process, that is, an ES in which the non-transferred strand contains a 5'-phosphate. Such a substrate was used in our studies. However, we also studied inhibitor effects using a substrate with a 5'-OH on the non-transferred strand. Surprisingly, this substrate demonstrates quite different sensitivities to some inhibitors. Specifically, compounds B and F were quite potent inhibitors of strand transfer for the 5'-OH substrate but had quite modest inhibition in the presence of a 5'-phosphate. In other words, the phosphate impaired inhibition in these cases. In addition, the presence of the 5'-phosphate (or a 5'-6-FAM fluorescein) enhances compound A activity. One explanation for the results is that the 5'-phosphate sterically blocks DKA B and F binding but provides a contact for compound A binding. This analysis is complicated by the crystallographic observation that the presence of the 5'-phosphate not only places a bulky group in the active site but also alters the placement of the non-transferred strand placement (Czyz et al., unpublished work). In this light, it is interesting to note that the efficacy of inhibition by compound E is insensitive to the 5'-end non-transferred strand structure.

More direct data supporting active site contacts for some inhibitors comes from our analysis of the effects of the active site mutations E326A and D97A. Mutation E326A impairs the PEC formation inhibition properties of DKA compounds B, C, and F both in the presence and the absence of DBB DNA, although the impairment is more severe in the absence of DBB DNA. The same compounds are also impaired in their PEC formation inhibition activity when using the D97A

mutant protein for PEC formation with pre-cleaved DNA. The observation that active site mutations E326A and D97A impair the inhibitory activity of compounds B, C, and F clearly indicates that these three inhibitors bind at or near the Tnp active site domain. The Tnp active site mutations either inhibit DKA–Tnp binding or alter the nature of DKA binding such that the Tnp–DKA complex will allow DNA binding and PEC formation to occur.

*Mg<sup>++</sup> Impairs the Inhibition of Pre-Cleaved PEC Formation.* To study the effect of Mg<sup>++</sup> on the PEC formation reaction, we used pre-cleaved DNAs to avoid any complication occurring because of catalysis or lack thereof. We discovered that in the presence of DKA compounds B, C, and F Mg<sup>++</sup> impairs the inhibition of PEC formation. It is possible that the presence of Mg<sup>++</sup> ions in the Tn5 Tnp active site changes the protein topology and makes the Tnp catalytic site less accessible for DKAs. Recently, the Pommier group reported that the metal dependent folding of the IN active site could influence the binding of  $\alpha$ -hydroxytropolones, other selective strand transfer HIV-1 IN inhibitors (33).

Because the presence of Mg<sup>++</sup> was proposed to coordinate the DKA compounds binding to IN (15) to explain our results, we note the following. The first studies examined only Mg<sup>++</sup>'s role in DKA (or 5CITEP) binding to IN and not its effects on DNA binding (14, 15). Next, the molecular modeling and docking studies suggested that the DKAs seem to bind to the active site of IN differently in the presence of Mg<sup>++</sup> than in the presence of Mn<sup>++</sup> (34). It was noticed that some DKA's show less potency in the presence of Mg<sup>++</sup> than in the presence of Mn<sup>++</sup>. The HIV-1 IN strand transfer inhibition assay results indicate that the DKA dependent sequestration of divalent ions in the IN active site strongly depends on the chemical structure of the DKA compound (34). Therefore, it is possible that the compounds we tested in this study do not chelate the Mg<sup>++</sup> ions, although they bind the Tnp active site. The high inhibition rate of PEC formation in the absence of Mg<sup>++</sup> for most DKA compounds tested in this work does not support the hypothesis that for PEC formation there is metal dependent DKA inhibitory binding. This observation, of course, does not rule out an important role for Mg<sup>++</sup> in DKA mediated inhibition of strand transfer or other steps in transposition.

Our observation that DKA compounds caused PEC dissociation at much higher concentrations than is sufficient for catalysis inhibition is consistent with the actual proposed mechanism of action of DKA's with HIV-1 IN (13, 24). In this model, the IN donor DNA site binds the donor DNA and starts to catalyze 3'-processing. Then a structural change of IN allows the binding of target DNA in the acceptor site and strand transfer occurs (24). It is known, that some DKA compounds (L708.906) at lower concentrations preferentially inhibit the strand transfer reaction (12). However, it has been suggested that bifunctional DKAs could bind not one but two discrete parts of the IN active site, the donor DNA site and acceptor DNA site, depending on compound concentration. According to the scheme proposed by Pommier and Marchand, divalent metal coordination is crucial for DKA binding only to the acceptor site, where the strand transfer reaction occurs. This model could explain why we do not observe DKA compounds inhibition of PEC formation in the presence of Mg<sup>++</sup> in our Tn5 Tnp reaction.

Table 3: Summary of DKA Compound Inhibitory Effects on Tn5 Transposition Reactions<sup>a</sup>

DKA compounds	PEC		cleavage inhibition	5'P strand	5'OH strand
	formation inhibition	PEC dissociation		transfer inhibition	transfer inhibition
A	+++*	No (60,40)	No	+++	No
B	++++	+++ (40)	++++	++	++++
C	++++	++++ (40)	No	+	+
D	+++*	++ (40)	+	++	++
E	++	No (60,40)	+	+++	+++
F	++++	++++(60,40)	++++	++	++++

<sup>a</sup> The data summarizes the results from PEC formation, PEC dissociation, transposon – donor DNA cleavage, and strand transfer assays. Abbreviations: “NO” indicates no inhibition, (40) indicates that dissociation occurs for pre-cleaved but not full-length substrate, (60, 40) indicates that dissociation occurs for both pre-cleaved and full-length substrates, \* indicates that compounds A and D are more potent inhibitors of PEC formation in the absence of dbb DNA (17-fold for A and 9-fold for D). Strand transfer experiments were performed in the presence of either a phosphate or an OH group at the 5'-end of the non-transferred strand. The number of “+”s reflects the degree of inhibition by a specific DKA compound.

## CONCLUSIONS

The present article provides an initial analysis of how a set of DKA compounds known to inhibit HIV-1 IN affect the activity of Tn5 Tnp. HIV-1 IN and Tn5 Tnp are members of the same super family of proteins sharing the same active site architecture, the same active site residues, and similar catalytic mechanisms. Thus, it was not surprising that in two screens a subset of the small molecule inhibitors of one protein demonstrated cross-inhibition versus the other protein. We propose that analyzing the mode of action of dual inhibitors using the technically easier to use Tn5 transposition system might have two benefits: it may help to elucidate how the inhibitors work against HIV-1 IN, and it may offer potent tools for studying the mechanism of Tn5 transposition. We do not know yet whether the first goal will be realized; however, it is clear from our analysis of just 6 HIV-1 IN DKA inhibitors that these compounds will be quite useful in further dissecting the mechanism of Tn5 transposition. For instance, compound A appears to selectively inhibit strand transfer or target capture, likely making it a useful tool for studying these steps in Tn5 transposition. The preferential inhibition of the SEB to DEB cleavage event by compound F elucidated the sequential, dependent nature of the two cleavage events. The ability of compounds B, C, D, and F to cause disassembly of the PEC may provide a useful tool for analyzing this complex. The effects of the six tested DKA compounds on different steps in Tn5 transposition are summarized in Table 3. Finally our studies revealing that the efficacy of compounds B, C, and F was impaired by active site mutation E326A (and to a lesser extent D92A) agrees with the proposal that compounds B, C, and F bind to the active site and specifically supports a preferential role for E326 (equivalent to HIV-1 IN E152) in this process, which is exactly the model proposed from the cocrystallographic examination of HIV-1–5CITEP complexes, and supports proposals based upon the location of DKA resistant mutations in HIV-1 IN.

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