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HIV-1 Integrase Strand Transfer Inhibitors Stabilize an Integrase-Single Blunt-Ended DNA Complex

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Summary

Integration of HIV (human immunodeficiency virus) cDNA ends by integrase (IN) into host chromosomes involves a concerted integration mechanism. IN juxtaposes two DNA blunt-ends to form the synaptic complex (SC) which is the intermediate in the concerted integration pathway. SC is inactivated by strand transfer inhibitors (STI) with IC₅₀ values of ~20 nM for inhibition of concerted integration. We detected a new nucleoprotein complex on native agarose that was produced in the presence of STI >200 nM, termed IN-single DNA (ISD) complex. Two IN dimers appear to bind in a parallel fashion at the DNA terminus producing a ~32 bp DNaseI protective footprint. In the presence of Raltegravir, MK-2048 and L-841,411, IN incorporated ~20 to 25% of the input blunt-ended DNA substrate into the stabilized ISD complex. Seven other STI also produced the ISD complex (≤ 5% of input DNA). The formation of the ISD complex was not dependent upon 3' OH processing and the DNA was predominately blunt-ended in the complex. Raltegravir-resistant IN mutant N155H weakly form the ISD complex in the presence of Raltegravir at ~25% level of wild type IN. In contrast, MK-2048 and L-841,411 produced ~3 to 5-fold more ISD than Raltegravir with N155H IN, which is susceptible to these two inhibitors. The results suggest STI are slow binding inhibitors and the potency to form and stabilize the ISD complex is not always related to inhibition of concerted integration. Rather, the apparent binding and dissociation properties of each STI influenced the production of the ISD complex.

Keywords

HIV-1; integrase; slow-binding inhibitor mechanism; integrase-single DNA complex

Introduction

The retrovirus integrase (IN) is responsible for integration of the linear cDNA into the host genome. Human immunodeficiency virus type 1 (HIV) IN binds at the terminal DNA sequences within the cytoplasmic preintegration complex (PIC) and cleaves a dinucleotide from the 3' OH blunt-ended termini 1; 2. Upon nuclear transport, IN inserts the two recessed viral DNA ends by a concerted mechanism into cellular DNA 3. The 3'-OH processing and strand transfer reactions are catalyzed through the use of divalent metal ions coordinated by

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at

the conserved D,D,-E motif within the catalytic core domain (CCD) of IN 4. Strand transfer inhibitors (STI) bind in the CCD of IN bound to viral DNA that prevents integration of HIV DNA into the host genome 5; 6. Raltegravir (RAL) received FDA approval as the first IN strand transfer inhibitor to treat HIV infected individuals 7; 8.

Efficient reconstitution of the HIV concerted integration reaction requires IN, a linear DNA substrate (~1 to 1.6 kb) with a long terminal repeat (LTR) end, and supercoiled DNA as target 9; 10; 11; 12; 13. We and others have developed methods to investigate nucleoprotein complexes in vitro to understand the molecular mechanisms associated with concerted integration and strand transfer inhibition. IN noncovalently juxtaposes two LTR blunt-ends producing a nucleoprotein complex termed the synaptic complex (SC) identified on native agarose gels (Fig. 1, *upper*) 14. SC is a transient intermediate in the concerted integration pathway and possesses biochemical properties associated with the PIC 14; 15; 16; 17; 18. Concerted integration requires an active IN tetramer at the LTR ends 16; 19; 20. The 3' OH processing of both DNA ends by IN within SC is slow 14. Upon capture of the target DNA by SC and the subsequent concerted integration reaction, the strand transfer complex (STC) is produced 16. STI binding to IN within SC renders it inactive and thus prevents target DNA binding 14; 16; 21. Recently, we established that the physical trapping of the HIV-1 SC at physiologically low nM concentrations using different structural classes of STI (Fig. 1, *lower* and Fig. 2) correlate with their potency for inhibition of the concerted integration reaction, defined by IC₅₀ values of each inhibitor 21.

The crystal structures of the prototype foamy virus (PFV) intasome without and with STI have been resolved 20; 22. The PFV intasome was formed with 3' OH recessed LTR oligonucleotides (17/19 nucleotides) and upon crystallization, the crystals were soaked with STI (1 mM) to allow binding of the inhibitors. RAL, MK-2048, elvitegravir (EVG), and other STI displaced the terminal nucleotide on the catalytic 3' OH end thus demonstrating a precise mechanism for inactivation of the intasome thereby preventing concerted integration. Structure-based modeling of the functional HIV intasome further supported the idea that the STI displaced the terminal reactive adenosine at the 3' OH end 23.

IN bound to a single viral DNA end is capable of inserting a 3' OH recessed DNA end into a supercoiled DNA target producing a circular half-site (CHS) product 9; 12. HIV IN associated with a single U5 DNA molecule possessing a recessed 3' dideoxyadenosine end was suggested to be a transient intermediate to the stable synaptic complex by atomic force microscopy, but the intermediate was not observable upon agarose gel electrophoresis 24. A single 3' OH recessed 5' thiolated U5 oligonucleotide covalently linked to IN was capable of single-ended strand transfer activity and binding a STI 25. Scintillation proximity assays (SPA) employing IN, again bound to a single 3' OH recessed end, demonstrated that the terminal (catalytic) adenosine on the 3' OH recessed end controls the kinetics of association and dissociation of a ³H-labeled STI 26. A time-dependent association of six different STI using SPA with either blunt or recessed ended DNA substrates suggested that a particular conformation of IN induced by 3' OH processing was not necessary for STI binding and subsequent strand transfer inhibition 27. These latter two studies suggested that STI were capable of efficient binding, in a slow time-dependent manner, to IN bound to a single viral DNA end.

In this report, we determined that several STI were capable of efficiently trapping a HIV IN-single DNA complex detected on native agarose gels. The ability of STI to induce the formation of a stable nucleoprotein complex was tested using U5 blunt-ended DNA under catalytic 3' OH processing conditions. Upon incubation at 37°C, an STI-induced IN-single DNA complex (ISD) (Fig. 1, *lower*) that represented ~20 to 25% of the input LTR DNA substrate was identified by native agarose gel electrophoresis. Out of ten inhibitors

investigated, RAL28 MK-204829, and diketo acid L-841,411 efficiently formed the stable ISD complex. The other STI were capable of forming the ISD complex to lesser degrees. Production of the ISD complex was time, temperature, and inhibitor concentration dependent. Relatively higher concentrations of the above STI were required to produce the ISD complex than the trapped SC mirroring the necessity of higher STI concentrations to inhibit the CHS reaction than the concerted integration reaction [15, 21]. The formation of the stable ISD complex was not dependent on 3' OH processing activity. The ISD complex was more efficiently produced (~2 fold) when the 5'-LTR end of the DNA substrate was labeled with a Cy3 fluorophore. RAL-resistant IN mutant N155H [31, 32] formed the ISD complex at ~25% level of wild type (wt) IN produced in the presence of RAL. In contrast, MK-2048 and L-841,411 efficiently produced the ISD complex with N155H. The results suggest that STI are slow binding inhibitors, bind to an IN-single DNA complex containing a blunt-end, modify IN-DNA interactions, and dissociate from the ISD differentially.

RESULTS

Different STI produce distinct IN-LTR DNA complexes identified by native agarose gel electrophoresis

Assembly of HIV SC (Fig. 1) using IN and blunt-ended LTR DNA substrates is a time-dependent process with maximum formation occurring between 30 to 45 min incubation at 37°C, followed by its near disappearance on native gel after ~120 min [14, 15]. The majority of DNA blunt ends in SC are not immediately processed by IN (~85% remain after 20 min and ~50% after 50 min) [14, 17]. Concurrently, upon the 3' OH processing of both DNA ends in SC and binding to supercoiled target DNA, the concerted integration reaction occurs, producing the STC [14, 16, 18]. HIV IN must be assembled on an LTR end prior to STI binding within the active site of IN [33, 34].

HIV IN was assembled on a blunt-ended U5 substrate to investigate the capabilities of different STI (Fig. 2) at varying concentrations to either produce or prevent the formation of nucleoprotein complexes, identified by native agarose gel electrophoresis. IN (60 nM) and 1.6 kb Cy3:U5 DNA (3 nM) were pre-incubated for 15 min at 14°C prior to the addition of target DNA and either L-870,810 or L-841,411, followed by incubation for 30 min at 37°C. With both inhibitors, increasing inhibitor concentrations (>0.2 µM) resulted in an accumulation of trapped SC [17] with the subsequent disappearance of the STC on the native agarose gel (Fig. 3, lanes 4 to 6 and 7 to 11, respectively), compared to reactions without inhibitors (Fig. 3, lanes 2 and 3). H-SC is a nucleoprotein complex that contains multimeric forms of SC on native agarose gels [14]. Surprisingly, diketo acid L-841,411 (Fig. 3, lanes 7 to 11) produced a new "trapped" nucleoprotein complex termed ISD which migrated slightly slower than the input 1.6 kb Cy3:U5 DNA. Naphthyridine carboxamide L-870,810 produced a smaller quantity of the ISD complex (Fig. 3, lanes 4 to 6). Similar data with a 1.1 kb Cy3:U5 DNA were obtained using L-841,411 which demonstrated assembly of the complex was independent of DNA size (data not shown). In summary, the efficient formation and stabilization of the ISD complex upon gel electrophoresis was dependent upon the concentration and structure of the inhibitor.

Two-dimensional gel electrophoresis [35] of the ISD complex formed in the presence of L-841,411 (data not shown) or MK-2048 showed the presence of only free 1.6 kb Cy3:U5DNA (Fig. S1), ruling out strand transfer activity within the ISD complex. In order to confirm that the ISD complex was composed of only a single DNA molecule, we perform a mixing experiment using 1.1 kb and 1.6 kb U5 DNAs. We observed only two ISD bands corresponding to the two different size DNAs (data not shown) further suggesting that the ISD complex contained only a single DNA molecule. In summary, the results showed that the ISD complex formed in the presence of inhibitors was devoid of strand transfer activity.

The slower migration of the ISD complex relative to the input DNA substrate was due to non-covalent association with IN.

Structurally different STI produce the ISD complex with widely varying efficiencies

We performed several screens to determine the capability of structurally different STI to produce the ISD complex using either blunt-ended U5 (Fig. 4) or Cy3:U5 (Table 1) DNA substrates. No target DNA was present. The ISD was detected by SYBR Gold staining (Fig. 4A, lanes 1–20), including a control reaction with Cy3:U5 for comparison to U5 (Fig. 4A, lanes 21–23).

With U5 DNA, the initial screen for forming the ISD complex with different STI was performed at either 5 μ M (Fig. 4A, odd lanes 3–19) or 100 μ M (Fig. 4A, even lanes 4–20) with incubation for only 30 min at 37°C. For quantitative measurements, the STI concentrations were set at 5 μ M and 200 μ M and incubation was extended to 2 h (Fig. 4B). L-841,411, RAL, and MK-2048 were capable of producing the highest quantities of the ISD complex. EVG, naphthyridine carboxamide L-870,810 and L-870,812 and diketo acids L-731,988 and 118-D-24, produced smaller quantities of the ISD complex (Fig. 4B). The monofunctional quinolonyl diketo acid inhibitor RDS 2197 and bifunctional RDS 1997 were also capable of producing medium quantities of the ISD complex. Notably, RDS 1997 at the higher concentration (Fig. 4A, lane 18)(Fig. 4B) essentially disrupted most IN-viral DNA interactions (Table 1).

Table 1 illustrates the ability of these inhibitors at a wider range of concentrations to produce the ISD complex using Cy3:U5 blunt-ended DNA upon incubation for 2 h for 37°C. The results suggest that there were no major differences in the overall qualitative pattern for formation the ISD complex with all STI using either U5 DNA (Fig. 4B) or Cy3:DNA (Table 1). The ISD complex formed with L-841,411 and RAL, starting from 0.25 μ M up to 100 μ M for 2 h at 37°C, revealed that Cy3:U5 DNA (Fig. 5A) is a better substrate than U5 DNA (Fig. 5B) by ~ 2-fold (Fig. 5C). As a control for inhibitor binding to IN, we observed that no ISD complex was produced by L-841,411 using a 1.5 kb Cy3: non-LTR DNA substrate (Fig. 2S), demonstrating LTR DNA sequences were necessary to form this nucleoprotein complex. In summary, all of STI were capable of forming the ISD complex to varying degrees demonstrating that an IN-single DNA complex can be stabilized in the presence of an appropriate STI.

Cy3 fluorophore at the 5' DNA end does not affect enzymatic properties of IN

The presence of Cy3 on the 5' end of the nontransferred DNA strand did not affect the assembly of HIV SC nor its concerted integration activity 17 L-841,411 and MK-2048 similarly inhibited the concerted integration and CHS reactions using either the 1.6 kb Cy3:U5 DNA (Fig. S3) or U5 DNA (Table 2)15' 21. The 3' OH processing activity of IN using either DNA substrate was also not affected (see below). The results suggest that the fluorophore at the 5' end does not affect strand transfer or 3' OH processing activities of IN but may enhance the stability of the ISD complex upon native gel electrophoresis.

Biochemical properties of the ISD complex

We further characterized other functional properties of IN within the ISD complex. The efficient assembly and maximum formation of HIV SC and trapped SC required incubation at 37°C 14. Efficient formation of the ISD complex also required incubation at 37°C. For example at 28°C and 21°C, only 54% and 30% of the ISD was formed in comparison to that produced at 37°C in 30 min with 1 μ M L-841,411. The production of the ISD was independent of pH between 6.8 and 7.5 under standard assay conditions at 37°C and, required Mg⁺⁺ and PEG. The optimum NaCl concentration necessary to produce the ISD

complex was 0.1 M NaCl, similar to SC without inhibitor present 14; 17. HIV SC is stable to salt treatment (0.5 M NaCl) prior to native agarose gel electrophoresis at 4°C 16; 17. The ISD complex was also stable to treatment at 0.5 M NaCl prior to electrophoresis at 4°C, but was destabilized when exposed to 1 M urea in the gel. The results suggest that similar components and conditions are required to form the ISD complex and SC.

Common functional mechanisms associated with the formation of both the ISD complex and trapped SC by inhibitors

Earlier SPA studies displayed a time-dependent inhibition of integration by STI using either blunt or 3' OH recessed ended substrates suggesting that STI are slow-binding inhibitors 26; 27 RAL displayed a time-dependent mechanism for inhibition of HIV concerted integration 21. The formation of the ISD complex was also a time-dependent process with L-841,411 and RAL at 1 μ M (Fig. 6A). The formation rate of the ISD complex (Fig. 6B) and SC (Fig. 6C) showed that L-841,411 produced both complexes faster than RAL. The higher quantities of the ISD complex produced in comparison to trapped SC suggest that the ISD complex was not derived from SC. The data suggests that slow-binding of STI to different IN-DNA complexes is common.

Production of the ISD complex by STI was not dependent on 3' OH processing

STI selectively inhibit concerted integration activity of IN at low nM concentrations but also inhibit 3' OH processing at higher inhibitor concentrations 5; 36; 37. We determined the IC₅₀ values for 3' OH processing with nine STI (Table 2), of which six STI inhibited reactions are shown in Fig. 7 (top shows each STI reaction). The ISD complex was formed in the presence of increasing concentrations of STI for 2 h at 37°C using an unlabeled 1.6 kb blunt-ended U5 DNA substrate. The U5 DNA was extracted, digested with *Hind*III, and the catalytic strand was labeled on the 5' end with ³²P 14. The unprocessed and processed catalytic strands are 105 and 103 nucleotides in length, respectively (Fig. 7A and 7B) 14. With IN only, significant half-site strand transfer activity was detected as DNA bands above the 105 nucleotide catalytic strand (Fig. 7A and 7B, lane 6). Minimum strand transfer activities were detectable at 1 μ M with all of the STI (Fig. 7A and 7B, lanes 7, 12, 17). The disappearance of the 103 nucleotide fragment with increasing inhibitor concentration measured the inhibition of the 3' OH processing reaction (Fig. 7A and 7B, lanes 7–11, 12–16, 17–21).

Inhibition of the 3' OH processing reaction is quantified with U5 DNA (Fig. 7C) (Table 2) and Cy3:U5 DNA (Fig. 7D). All of the inhibitors displayed similar kinetics for inhibition of 3' OH processing with IC₅₀ values of ~7 to 9 μ M except L-870,812, L-731–988, and RDS2197 that possessed IC₅₀ values of ~70 to 80 μ M (Table 2). The 3' OH processing reaction progresses slowly with time and the rate was dependent on the presence of the inhibitor (Fig. S4). At 1 μ M RAL (Fig. S4B) and the other STI (Fig. 7A, 7B, and 7C), 3' OH processing appears to be higher because the strand transfer reaction is preferentially inhibited that results in a higher yield of cleaved DNA. Significant processing (~20%) was still occurring at 5 μ M inhibitor (Fig. 7C and 7D)(Fig. S4B) even though a majority of the ISD is formed at ~2 μ M (Table 1)(Fig. 5). At very high concentrations of STI ($\geq$ 50 μ M) (Fig. 7)(Table 1), no processing is occurring where the maximum quantity of the ISD complex was detected on agarose gels. In summary, the data suggests that the formation of the ISD complex was not dependent on 3' OH processing.

The ISD complex predominately contains blunt-ended DNA

Using a U5 blunt-ended substrate, we confirmed that the ISD complex contained blunt-ended U5 DNA by extraction of the isolated complex from an agarose gel. The amount of 3' OH processing was determined in the extracted DNA when the ISD complex was formed at

1 μ M, 5 μ M, and 10 μ M MK-2048 (Fig. 8A and 8B). In-solution reactions were performed in parallel. At 1 μ M inhibitor, ~90% of the DNA in the extracted ISD complex and the in-solution samples was blunt-ended. At 5 μ M and 10 μ M MK-2048, both treated samples had paralleled increasing amounts of blunt-ended DNA with less 3' OH recessed ended DNA present. At the lower concentrations of STI, we cannot preclude minor processing activity is still proceeding within the ISD complex. The results suggest that the ISD complex predominately contains blunt-ended DNA. We confirmed that a Cy3-U5 DNA substrate possessing a 3' OH recessed end was capable of forming the ISD complex in the presence of MK-2048 (data not shown).

IN dimers are associated with the ISD complex

The majority of HIV IN multimeric species observed in SC and STC are either dimers, tetramers, or a larger-size multimer 16⁺ 17, although only a tetramer is necessary for concerted integration (Fig. 1) 16⁺ 19⁺ 20. We determined the multimeric status of IN in the ISD complex. The complex was formed with 1.6 kb Cy3:DNA in the presence of L-841,411 (100 μ M) for 1 h at 37°C. The complex was cross-linked with BS3 for 1 h at 14°C in solution and isolated on a native 0.7% agarose gel. IN was extracted from the ISD complex and the samples were subjected to SDS-PAGE and Western Blot analysis 17. The vast majority of IN multimers detected by the C-terminal rabbit antiserum were dimers with a minor population of tetramers and a larger-size multimer (Fig. S5, lane 3). The N-terminal antiserum only detected dimers (Fig. S5, lane 6). As a control, both antisera were capable of detecting monomers and other multimers when only purified IN was cross-linked with BS3 (Fig. S5, lanes 2 and 5). The results suggest the ISD complex contains only a majority of IN dimers. But, we cannot exclude the possibility that a larger portion of IN may exist as a tetramer in the ISD complex that cannot be identified because of ineffective crosslinking by BS3.

L-841,411 and RAL disrupt binding of IN on the noncatalytic strand of U5 near position 9-A in the ISD complex but do not disrupt the overall ~32 bp DNaseI protective footprint

DNaseI footprint analysis of HIV SC, H-SC, and STC showed that wt IN protects ~32 bp at the U3 and U5 DNA termini and in the presence of either 0.75 μ M L-870,810 17 or RAL 21. The same size ~32 bp DNaseI footprint is also observed with the nucleoprotein complex that catalyzes the insertion of a single DNA end by HIV IN into target DNA17

The ISD complex was formed with IN (60 nM) and 1.1 kb 5' ³²P-U5 DNA (3 nM) in the presence of either 100 μ M L-841,411 or RAL for 2 h at 37°C. A ~32 bp DNaseI protective footprint was observed with the isolated ISD complex formed in the presence of either L-841,411 (Fig. 9A, lane 6) or RAL (Fig. 9B, lane 6) in comparison to digested naked U5 DNA (Fig. 9A and 9B, lanes 5 and 7). A DNaseI enhanced cleavage was observed near nucleotide position 9-A with both inhibitors as well as significant enhanced cleavages near ~32 bp (Fig. 9A and 9B, lane 6) in comparison to control DNaseI digestions of naked DNA (Fig. 9A and 9B, lanes 5 and 7). The DNaseI enhanced cleavages near and at ~32 bp suggests that IN distorts these nucleotides in this region, similar to that observed in SC, H-SC, trapped SC, and STC 17⁺ 21. The DNaseI footprint between nucleotides 22 to 29 are modified and some bands are not completely protected by IN in the ISD complexes suggesting some DNA molecules may not necessarily have IN stably bound in this region. For example, the DNA band migrating near position 28-A was 84% protected relative to the same band in the digested naked U5 DNA control (Fig. 9, compare lanes 6 to 7, respectively). The results suggest IN maintains its multimeric structure on the U5 LTR end in the ISD complex similarly as observed in SC, without or formed in the presence of 0.75 μ M RAL or L-870,810 21.

As a control, a very similar ~32 bp DNaseI protective footprint was observed with trapped SC using L-841,411, isolated in the same experiment as the ISD complex. But, the enhanced cleavage observed in the ISD complex near 9-A (Fig. 9A, lane 6) was absent in the trapped SC (Fig. 9A, lane 8). The result suggests that the interactions of IN with the U5 end in the ISD complex are slightly modified in comparison with trapped SC in the presence of L-841,411.

Finally, DNaseI footprint analysis of the ISD complex produced with 100 μ M L-841,411 using a 1.2 kb 5'-³²P-U3 DNA produced a ~32 bp DNaseI protective footprint (data not shown). The results indicate that two IN dimers are aligned in a parallel fashion on U3 or U5 ends and remained associated with the ISD complex produced with L-841,411 or RAL (Fig. 1, lower panel).

Formation of the ISD complex with RAL resistant IN mutant N155H depends upon the susceptibility to a particular STI

There are several primary drug-resistant IN pathways which appear upon treatment of HIV infected individuals with RAL, followed by secondary mutations that further increase drug resistance [31, 32]. One primary resistant mutation occurs through the N155H pathway [32]. We introduced the N155H mutation into the wt IN NY clone which does not contain any natural polymorphism for RAL resistance (data not shown) as observed in IN inhibitor-naïve patients [38]. The specific activity of N155H for concerted integration is ~70% of wt IN using a 1.6 kb blunt-ended U5 DNA substrate [15, 21]. The IC₅₀ values for RAL to inhibit wt IN and N155H IN concerted integration activities were 21 $\pm$ 4 nM and 68 $\pm$ 15 nM, respectively; the IC₅₀ values for MK-2048 were 42 $\pm$ 5 and 42 $\pm$ 3 nM, respectively [21].

We determined that IN containing the N155H mutation had a decreased capacity to form the ISD complex in the presence of RAL as compared to wt IN. Under non-saturating (0.5 μ M) and saturating (5 μ M) concentrations of RAL for 2 h at 37°C, N155H was not able to efficiently form the ISD complex (Fig. 10A and 10B) in comparison to wt IN (Fig. 10C). There was a ~60% reduction in the formation of ISD with N155H in comparison to wt IN, both at 60 nM. A similar reduction was obtained with N155H even when incubation was increased to 3 h (data not shown). In contrast, both L-841,411 and MK-2048 effectively formed the ISD complex with N155H at various inhibitor concentrations (Fig. 10B). A similar reduction in the formation of the ISD complex with RAL was obtained if wt IN and N155H IN were used from the HIV IIIB strain (data not shown) [15]. In summary, the results suggest that IN possessing the N155H mutation lacks the capacity to efficiently form the ISD complex in the presence of RAL but efficiently forms the ISD with MK-2048 [21] and L-841,411 [15] to which N155H IN is susceptible.

Discussion

We have identified a new HIV IN-single DNA complex on native agarose that was stabilized in the presence of different structural classes of STI. The ISD complex possesses biochemical properties associated with both SC and trapped SC. Inhibitor screening and titration experiments identified that RAL, MK-2048, and L-841,411 were the most effective inhibitors for producing the ISD complex using either blunt-ended U5 or Cy3:U5 DNA substrates. The formation of the ISD at 37°C was time-dependent suggesting that slow binding of STI is common. The STI-induced assembly of the ISD complex was not dependent upon 3' OH processing and the DNA in the isolated ISD complex was essentially blunt-ended. RAL-resistant IN mutant N155H had a ~70% decreased capacity to produce the ISD complex in the presence of RAL in comparison to wt IN suggesting that the IN-single DNA complex has biologically relevant properties.

A tetramer of IN is responsible for concerted integration (Fig. 1, upper panel). The ISD complex appears to have two parallel aligned IN dimers at the DNA terminus (Fig. 1, lower panel) that is responsible for the ~32 bp DNaseI protective footprint (Fig. 9), similar to the protection pattern associated with SC and trapped SC 17: 21. The IC₅₀ values to inhibit the single-ended strand transfer reaction by HIV IN are significantly higher than for inhibition of concerted integration catalyzed by SC (Table 2). The physiologically low nM concentrations of STI to inhibit concerted integration suggests that STI binding to the active tetramer within trapped SC is far more efficient and effective than binding to an IN dimer located at the DNA terminus in the ISD complex (Fig. 1, lower panel)(Fig. 4 and Fig. 5).

With SPA, extended pre-incubation of STI (60 min at 37°C) was necessary for effective binding and inhibition at low nM concentrations prior to initiation of strand transfer 26: 27. The formation of the ISD complex was also time-dependent (Fig. 6) and did not require 3' OH processing of blunt-ended DNA (Fig. 7 and Fig. 8)(Table 2). After 2 h of incubation of IN with blunt-ended U5 DNA at 1, 5, and 10 µM of MK-2048, the majority of DNA ends in the isolated ISD were ~90, 96, and 98% blunt-ended, respectively (Fig. 8A and 8B). In addition, the majority of DNA blunt-ends were not processed at higher STI concentrations (Fig. 7 A–D,) where the highest amounts of the ISD complex was formed and isolated on native agarose (Table 1). In summary, the results suggest production of the ISD complex by STI favors DNA with blunt-ends.

The detection of SC and ISD on native gels may be related to the ability of the STI to remain stably associated with each IN-DNA complex as well as the intrinsic stability of each complex without inhibitor upon gel electrophoresis. Titration experiments demonstrated that the majority of trapped SC occurs by ~0.25 µM with RAL, EVG, and MK-2048 with detectable quantities occurring by ~0.02 µM 21. The reason why EVG effectively traps SC and inhibits concerted integration at low nM concentrations like MK-2048 and RAL 21 but fails to effectively form the ISD complex (Fig. 4B)(Table 1) is unknown. Two possibilities appear apparent. First, the interactions of IN with a single DNA blunt-end for EVG binding may not be optimal for formation of the ISD complex in contrast to the other STI although, this possibility appears least likely. The simplest explanation may be the dissociation of EVG is significantly faster from the ISD complex than with SC resulting in its instability upon gel electrophoresis. In contrast, L-841,411 effectively forms the ISD complex similar to MK-2048 with wt IN (Fig. 10C) but has a ~2-fold higher IC₅₀ value to inhibit concerted integration 15 (Table 2). The N155H mutation in HIV IN decreased the capacity of RAL and MK-2048 to form the ISD complex but did not modulate L-841,411 ability to form and stabilize this complex (Fig. 10B and 10C). The N155H mutation in HIV IN causes an increase susceptibility to L-841,411 15. The results suggest that the initial slow binding of an STI to an IN-DNA complex may be universal but dissociation of the STI may vary significantly with the different complexes. In fact, the efficacy of different STI in clinical settings may be related to inhibitor dissociation rates as measured by the use of wild type and drug-resistant IN mutants 39:40

The formation of the ISD complex is enhanced ~2.0 fold when the 5' end of the HIV U5 DNA is labeled with Cy3 (Fig. 5). The profiles for production of the ISD complex using various concentrations of STI with either blunt-ended U5 (Fig. 4) or Cy3:U5 DNA substrates appear similar (Fig. 5 and Table 1). These data suggest Cy3 does not affect the ability of a specific STI to produce the ISD complex but rather enhances the stability of the ISD complex upon electrophoresis. Cy3:U5 and Cy5:U5 DNAs are productive substrates for assembly studies of SC and the concerted integration reaction with HIV 17 and RSV 41 IN. HIV IN is capable of 3' OH processing of viral DNA ends within the PIC that contain an additional nucleotide added by reverse transcriptase 42: 43 again suggesting flexibility in the active site, possibly through the flexible loop (residues 140 to 149)44. Finally, the IC₅₀

values for inhibiting wt HIV IN concerted and CHS integration reactions with L-841,411 and MK-2048 (Fig. S2) and, RAL or EVG (data not shown) using Cy3:U5 DNA substrate, were nearly identical to IC_{50} values obtained with U5 DNA without the fluorophore present (Table 2) 14; 15; 17. Inhibition of 3' OH processing with both DNA substrates by multiple STI are equivalent (Fig. 7C and 7D). These above results suggest that the active site of IN is amenable to the placement of fluorophores at the 5' DNA ends without measurable effects on activities in vitro. IN is required to "melt" the ends of viral DNA for 3' OH processing 45 which ultimately results in the extension of the 5' end of the DNA outside the PFV intasome 20 and, as modeled in the HIV intasome 23. It appears likely that Cy3 attached at the 5' end of the DNA outside the HIV SC may help stabilize the nucleoprotein complex. In summary, further investigation is necessary to understand what mechanism is responsible for the enhanced formation or stability of the ISD complex by the presence of Cy3 at the 5' end of U5 DNA.

RAL resistance primarily occurs through several independent pathways containing mutations in IN (N155H and Q148H/K/R), with secondary mutations generally producing larger reductions in RAL susceptibility 31; 32. The replication capacity of HIV containing the N155H mutation is ~70% of wt HIV 32; 46 which is similar to the specific activity for concerted integration activity of IN containing the N155H mutation in comparison to wt IN 15; 21. The IC_{50} value to inhibit concerted integration catalyzed by IN containing the N155H mutation with RAL is ~3-fold higher than observed with wt IN 21. Production of the ISD complex with the N155H mutant in the presence of RAL was reduced to approximately one-third the level of wt IN (Fig. 10A and 10B) while the reduction with MK-2048 was less. MK-2048 inhibits both wt IN and N155H concerted integration activity with an IC_{50} value of $42 \text{ nM} \pm 3$ 21. The results suggest that a subtle structural change has occurred in IN via the N155H mutation affecting binding of RAL 22 but did not significantly affect the ability of IN to promote concerted and CHS integration 15; 21, or the replication capacity of the virus containing this mutation 32; 46.

HIV SC is the transient intermediate formed with U5 and U3 blunt-ended substrates that are slowly processed at the 3' OH ends by IN 14. SC represents the precursor to the intasome containing two 3' OH recessed ends that is capable of concerted integration 47. Besides displacing the catalytic 3' OH terminus of U5 in the PFV intasome co-crystal 22, STI modify the binding of IN on the internal sequences of the noncatalytic strand at the U5 and U3 LTR termini in trapped SC 17; 21. Modification of IN binding to the noncatalytic strand by RAL and L-841,411 is also observed within the ISD complex (Fig. 9). Our results support the idea that certain STI can efficiently produce an IN-single DNA complex (Fig. 4 and Table 1) containing either a blunt or recessed DNA end (Fig. 8). In summary, the results suggest that STI modify IN interactions with the DNA in SC, the precursor to the HIV intasome.

Materials and Methods

Purification of HIV IN

Recombinant wt HIV IN (pNY strain) 9; 48 and IN possessing the single N155H drug-resistant mutation were used in this study. Proteins were expressed in *Escherichia coli* BL21(DE3) cells and purified to near-homogeneity 48. Purified IN (pNY) was used unless indicated. Protein concentrations were determined by absorbance using $50400 \text{ M}^{-1}\text{cm}^{-1}$ at 280 nm. Molar concentrations of IN were expressed as a dimer.

Viral DNA substrates

HIV 1.1 kb and 1.6 kb single ended U5 and 1.2 kb single ended U3 LTR DNA substrates were prepared as described 14. The LTR blunt ended DNA substrates were 5'-end labeled

using [γ - ^{32}P] ATP and T4 polynucleotide kinase 14. The 5' end labeled Cy3 1.6 kb U5 DNA substrates were produced by PCR 17.

IN inhibitors

The strand transfer inhibitors L-870,810, L-870,812, L-731,988, L-841,411, RAL, and MK-2048 were generously supplied by Merck Research Laboratories and 118-D-24 by NIH AIDS Reagent Program. EVG, RDS1997, and RDS 2197 were generously supplied by Drs. Y. Pommier and C. Marchand. EVG was also obtained from Selleck Chemicals. Stocks (10 mM) of each inhibitor were made in 100% dimethyl sulfoxide and stored in small aliquots at -70°C for single use.

Assembly of nucleoprotein complexes and the concerted integration reaction

Assembly of HIV SC and the concerted integration assay were performed as described 14; 17 In brief, specified concentrations of IN were pre-incubated with 1.6 kb blunt-ended U5 DNA (3 nM)(unlabeled, 5' ^{32}P or Cy3 labeled) at 14°C for 15 min in 20 mM HEPES buffer (pH 7.0) containing 10 mM MgCl_2 , 5 mM dithiothreitol, 100 mM NaCl, 25 μM ZnCl_2 , and 10% polyethylene glycol (6000 Da). The assembly of nucleoprotein complexes was initiated without or with inhibitors as described in each experiment and, either in the absence or presence of supercoiled target DNA (3 nM). Samples were incubated in the presence of STI at 37°C for 30 min or as described to produce the ISD complex. The reactions were stopped by the addition of EDTA to a final concentration of 25 mM. Nucleoprotein complexes were separated on 0.7 % native agarose gels at 4°C and identified by laser scanning with a Typhoon variable imager for Cy3 fluorophore or SYBR Gold. Measurements for Cy3:U5 DNA and SYBR Gold stained DNA were always within the linear range of detection. Cy3 fluorophore (580 nm) and SYBR Gold (520 nm) emission patterns do not overlap which allowed a quantitative evaluation between the two DNA substrates on the same gel. The DNA products were quantified using ImageQuant 5.2 software.

DNaseI protection analysis

The 1.1 kb ^{32}P -U5 and 1.2 kb ^{32}P -U3 blunt-ended DNA substrates were used for DNaseI protection analysis. After incubation of the IN-viral DNA-target mixture for 2 h at 37°C in the presence of RAL or L-841,411, samples were equilibrated at 14°C . DNaseI (0.3 units per 100 μl) was added and incubated for 2 or 3 min at 14°C 17 Reactions were stopped by addition of EDTA and the samples were subjected to native agarose gel electrophoresis. The ISD complex was excised and the DNA purified. Samples were analyzed on denaturing 15% PAGE to determine which terminal LTR sequences were protected by IN.

3' OH processing analyses

The 3' OH processing activity of IN using U5 blunt-end DNA substrate in solution was previously described 14 DNA was also isolated from the ISD complex and analyzed in a similar fashion.

Chemical cross-linking of IN subunits

Chemical cross linker bis(sulfosuccinimidyl)suberate (BS3) was used to crosslink IN in the ISD complex. The complex was formed as described above in the presence of L-841,411 and chemical cross-linking was performed with 25 μM BS3 at 14°C for 60 min 17 The ISD complex was isolated from a native gel, cross-linked IN was extracted from the complex, and subjected to Western Blot analysis using rabbit antisera directed against peptides derived from the N-terminus or C-terminus of IN17.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations used

HIV	human immunodeficiency virus type 1
IN	integrase
LTR	long terminal repeats
PIC	preintegration complex
SC	synaptic complex
H-SC	higher-order synaptic complex
STC	strand transfer complex
ISD	integrase-single DNA complex
STI	strand transfer inhibitors
RAL	raltegravir
EVG	elvitegravir
FS	full-site
CHS	circular half-site
D-D	donor-donor
wt	wild-type
PFV	prototype foamy virus

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Fig. 1.

Differential formation of HIV nucleoprotein complexes by strand transfer inhibitors. The top panel depicts the assembly of the synaptic complex (SC) using HIV IN and a LTR DNA substrate. IN is displayed as a dimer with the catalytically active tetramer outlined by a red oval. The lower panel shows the effects of low and higher nM concentrations of strand transfer inhibitors (STI) that differentially produce the different nucleoprotein complexes. In the presence of different STI (red triangle), the "trapped" SC contains two LTR ends juxtaposed by IN while the bottom nucleoprotein complex contains a single LTR blunt-ended DNA molecule. The latter complex is called an IN-single DNA ("ISD") complex.

Fig. 2.

Structures of different STI. The name of each STI is indicated below the inhibitor. The diketo acid, naphthyridine carboxamide, and quinolinonyl diketo inhibitors are grouped together.

Fig. 3.

Different STI produce distinct IN-LTR DNA complexes detected on native agarose gel. Assembly of 1.6 kb 5'-Cy3:U5 blunt-ended DNA (3 nM) and IN (60 nM) was performed at 14°C for 15 min followed by the addition of supercoiled target DNA (3 nM) and either L-870,810 or L-841,411 at various concentrations (top) for 30 min at 37°C. Samples were subjected to native 0.7% agarose gel electrophoresis at 4°C. The Typhoon laser scan was set for excitation and emission bands for the Cy3 fluorophore. Lane 1 is without IN, marked C. Lanes 2 and 3 were duplicate experiments with IN and no inhibitor, marked 0. Lanes 4 to 6 were with L-870,810 while lanes 7 to 11 were with L-841,411 (concentrations at the top). The identities of the different IN-DNA complexes without inhibitors are indicated on the

left. They are: higher-order synaptic complex (H-SC), strand transfer complex (STC), circular half-site (CHS) complex, and synaptic complex (SC). On the right with STI present, the trapped H-SC and SC along with the inhibitor induced IN-single DNA (ISD) complex are shown. The input Cy3:U5 DNA substrate is indicated.

Fig. 4.

Structurally different STI produce the ISD complex with widely varying efficiencies. A. The ISD complex was formed with 1.6 kb blunt-ended U5 DNA (3 nM) and IN (75 nM) in the presence of each STI for 30 min at 37°C. No target DNA was present. Lanes 1 and 21 were without IN and lanes 2 and 22 were with IN but without inhibitor. Lanes 1 to 20 were with U5 DNA and lanes 21 to 23 were with Cy3:U5 DNA. Lanes 3 to 19 (odd numbered lanes) had 5 μ M and the even number lanes had 100 μ M of each inhibitor. Lane 23 was with 5 μ M MK-2048. The ISD complex was identified on the native agarose gel by scanning for SYBR Gold stain on a Typhoon variable imager. The ISD complex, trapped SC and H-SC, are identified on the right. The band that appears just below the trapped SC (also in control lane

1) represents a doublet of the input substrate apparently due to nonspecific interactions between DNA ends (see Fig. 3 and Fig 5 also). B. With three independent experiments, the percent of ISD produced with U5 DNA at 5 μ M and 200 μ M of each inhibitor were determined and plotted. The formation of the ISD complex with STI was described in A except incubation was for 2 h.

Fig. 5.

Specificity of 5' end-labeled DNA substrate to produce the ISD complex. A. The Cy3 labeled 1.6 kb Cy3:U5 DNA (3 nM) was incubated with 60 nM HIV IN with increasing concentrations of either L-841,411 or RAL for 2 h at 37°C. The assays were performed as described in Fig. 4. Lane 1 was DNA and 100 μM inhibitor only. Lane 2 was an assembly control with no inhibitor. Lanes 3 to 9 and lanes 10 to 16 were with 0.25, 0.5, 1, 2, 5, 10, and 100 μM of L-841,411 and RAL, respectively. The gel was laser scanned for Cy3 fluorophore on the Typhoon variable imager. B. Unlabeled 1.6 kb U5 DNA (3 nM) was incubated with 75 nM HIV IN with increasing concentrations of either L-841,411 or RAL for 2 h at 37°C. The assays were performed as described in A. The gel was laser scanned for

SYBR Gold stain on the Typhoon variable imager. C. The percentages of each ISD complex (calculated from A and B) were plotted against inhibitor concentrations from three independent experiments. Solid symbols (● and ▼) represent L-841,411 and open symbols (○ and △) represent RAL. Circles (● and ○) represent Cy3:U5 DNA and triangles (▼ and △) represent U5 DNA.

Fig. 6.

Formation of the ISD complex and trapped SC was time-dependent with RAL and L-841,411. A. The SC and ISD complexes were formed in the presence of 1 μ M inhibitor at 37°C as described in Figure 3 except in the absence of target DNA. At various times, individual reactions were stopped with EDTA and stored on ice until completion of the experiment (3 h). Lanes 1 and 11 were DNA only while lanes 2 and 12 were with IN which produced SC and H-SC after 3 h without inhibitor. Lanes 3 to 10 and 13 to 20 were 0, 15, 30, 45, 60, 90, 120, and 180 min, respectively, for L-841,411 and RAL. The samples were subjected to electrophoresis on a 0.7% native agarose gel. The quantities of the ISD complex

and trapped SC produced were determined from three independent experiments, shown in B, and C, respectively.

Fig. 7.

Inhibition of HIV IN 3' OH processing by STI. A. IN (60 nM) and unlabeled 1.6 kb blunt-ended U5 DNA (3 nM), in the presence of increasing concentrations of STI, were incubated for 2 h at 37°C. The extracted DNA was digested with *Hind*III and the 5'-end of the catalytic and non-catalytic strands were labeled with ^{32}P 14. Samples were subjected to denaturing 6% PAGE gels. Lanes 1–4 contain the sequence ladder generated using the non-catalytic strand sequence of U5 LTR. Lane 5 was without IN and lane 6 was without inhibitor. Lanes 7 to 11, 12 to 16, and 17 to 21 contained increasing concentrations (top) of RAL, MK-2048, and L-841,411, respectively. The 103 nt band represents the processed DNA and the 105 nt band unprocessed catalytic strand DNA. The non-catalytic strand is 101

nt. The 3' OH processing reaction was determined from the ratios of processed and unprocessed DNA strand. B. The same protocol was used as described in A except EVG, L-870,810 and L-870,812 were used. C. Graphical representation of the 3'OH processing reaction mediated by IN as described in A and B. D. Graphical representation of 3' OH processing reaction mediated by IN using the 1.6 kb blunt-ended Cy3: U5 DNA in presence of increasing concentration of STI as described in A and B.

Fig. 8.

The ISD complex formed in presence of MK-2048 contained predominately blunt-ended DNA. A. The ISD complex was formed with IN (60 nM) and 1.6 kb blunt-ended U5 DNA (3 nM) with MK-2048 (1, 5 and 10 μM) for 2 h at 37°C. The ISD complex was excised from the native agarose gel. The DNA was purified from the ISD, digested with *HindIII*, and labeled with ³²P as described in Fig. 7. An aliquot was removed from each sample before agarose electrophoresis and processed in parallel to provide net 3' OH processing (in-solution). The 3' OH processing of the LTR ends was analyzed on denaturing PAGE. Lane 1 was without IN and lane 2 was without inhibitor. Lanes 3 to 5 contained MK-2048 at concentrations of 1, 5 and 10 μM, respectively. Lanes 6 to 8 contained the DNA purified from the ISD complex at the same concentrations of MK-2048. Lanes 9–12 contained the sequence ladder generated using the non-catalytic strand sequence of U5 LTR as the primer. B. Graphical representation of net 3'-processing in-solution and within the isolated ISD complex. The 3' OH processing activity was determined from the data shown in panel A.

Fig. 9.

DNaseI protection analysis of the ISD complex. A. The ISD complex was produced with 60 nM IN and 1.1 kb ^{32}P - 5' U5 (3 nM) in the presence of 100 μM L-841,411 for 2 h at 37°C. The samples were cooled to 14°C and subjected to DNaseI digestion (0.3 units) and stopped with EDTA. The ISD complex was isolated from a native agarose gel after staining with SYBR Gold. The DNAs were extracted and subjected to denaturing PAGE (15%). Lane 1 contained input DNA; lanes 2 to 4 were chemical markers, lanes 5 and 7 were naked DNA digested with DNaseI for 2 and 3 min, respectively; and lanes 6 and 8 contain the ISD complex and trapped SC, respectively. The vertical bar defined the area of protection by IN in comparison to naked DNA. The stars represent positions of DNaseI enhanced cleavages

in the ISD complex and trapped SC. B. The ISD was produced with 100 μ M RAL and DNaseI protection analysis was described in A. The lanes were described in A except no trapped SC was isolated. The 3 min and 2 min (lanes 5 and 7) digestion times of naked DNA were switched related to A.

Fig. 10.

RAL-resistant HIV IN mutant N155H failed to efficiently form the ISD complex in the presence of RAL in comparison to wt IN. A. The ISD complex was formed using equivalent concentrations (60 nM) of purified N155H and wt IN with 1.6 kb Cy3:U5 DNA (3 nM). The ISD complex was formed with N155H and varying quantities of STI for 2 h at 37°C. Lane 1 was with IN only. Lanes 2 to 8, 9 to 15, 16 to 22 were with L-841,411, RAL, and MK-2048, respectively. The STI concentrations were 0.25, 0.5, 1, 2, 4, 6, and 10 μ M, respectively. B. The quantities of ISD produced by N155H were determined from three independent experiments and plotted as percentages of each ISD against inhibitor concentrations up to 10 μ M. Closed circle (●), closed triangle (▼), and open circle (○) represent L-841,411,

MK-2048, and RAL, respectively. C. The quantities of ISD produced by wt IN were determined from three independent experiments and plotted as described in B. For easier evaluation, the data shown in Fig. 5C with wt IN using L-841,411 and RAL were plotted again along with the new MK-2048 data.

Table 1

STI specificity for producing the ISD complex.

		ISD Complex (%) ^a									
		Inhibitors									
μ M		L-841, 411	RAL	MK-2048	EVG	L-870,810	L-870,812	L-731,988	118-D-24	RDS 1997	RDS 2197
1	10.6 $\pm$ 1.0	6.8 $\pm$ 1.4	11.9 $\pm$ 1.4	1.0 $\pm$ 0.6	1.6 $\pm$ 1.0	2.4	2.7	ND	ND	5.2	6.6
2	13.9 $\pm$ 0.4	8.3 $\pm$ 1.8	14.9 $\pm$ 2.0	1.2 $\pm$ 0.8	2.6	2.7	2.0 $\pm$ 1.0	ND	ND	6.7	9.0
5	17.1 $\pm$ 4.2	10.6 $\pm$ 1.8	20.5 $\pm$ 0.5	1.2 $\pm$ 0.5	2.84 $\pm$ 0.7	3.6 $\pm$ 0.4	1.3	3.0	3.2 $\pm$ 1.0	8.1	11.1
10	20.1 $\pm$ 2.9	12.9 $\pm$ 2.1	21.2 $\pm$ 2.2	1.8 $\pm$ 0.6	4.6	5.9	5.6 $\pm$ 0.9	1.8	3.1 $\pm$ 2.4	0.5 $\pm$ 0.4	13.4 $\pm$ 0.8
100	24.4 $\pm$ 3.3	17.1 $\pm$ 2.7	24.5 $\pm$ 2.8	2.5 $\pm$ 0.6	5.9	5.6 $\pm$ 0.9	1.8	3.1 $\pm$ 2.4	0.5 $\pm$ 0.4	13.4 $\pm$ 0.8	

^aReactions were with 60 nM wt HIV IN and 3 nM Cy3:U5 DNA in presence of different concentrations of STI for 2 h at 37°C. The assay details for formation of the ISD complex were described in the text. The numbers under each STI represents the percent of input DNA substrate incorporated into the ISD complex at a given concentration (μ M) of STI (left most column). In most cases, at least three independent experiments were performed to determine the reported SD values.

Table 2

IC₅₀ values (μM) to inhibit the 3'OH processing and strand transfer reactions of HIV IN

Inhibitors	Concerted Integration ^a	CHS ^b	3'OH Processing ^c
RAL	0.021 *	0.25 *	7.0 ± 0.3
MK-2048	0.042 *	0.365 *	7.5 ± 1.3
EVG	0.008 *	0.145 *	7.89 ± 0.34
L-841,411	0.110 **	1.0 **	9.1 ± 1.0
L-731,988	0.385 ± 0.1	1.1 ± 0.2	78.7 ± 8.7
L-870,810	0.055 **	0.245 **	8.5 ± 2
L-870,812	0.102 **	0.7 **	72.5 ± 12
RDS 1997	0.09 *	0.35 *	8.8 ± 0.4
RDS 2197	0.495 *	1.75 *	80.2 ± 20.9

^a IC₅₀ to inhibit the formation of concerted or FS products;

^b IC₅₀ to inhibit the formation of CHS products.

^c IC₅₀ to inhibit the 3'OH processing activity. SD was determined from at least three independent experiments.

* IC₅₀ values for concerted and CHS products with RAL, MK-2048, EVG, RDS 1997 and RDS 2197 were published previously 21.

** IC₅₀ values for concerted and CHS products with L-841,411, L-870,810 and L-870,812, were determined in an earlier study 14