Diketoacid inhibitors of HIV-1 integrase: from L-708,906 to raltgravir and beyond

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Abstract: The development of therapeutics targeting the enzyme HIV-1 integrase has been a major focus of AIDS research over the past two decades. Various classes of integrase inhibitors have now been identified, and of these the diketoacids (DKAs) show the greatest promise. This review tracks the development of DKA-based inhibitors from early screening studies through to the release of raltgravir – the first HIV medication targeting integrase to gain FDA approval. SAR data collated from numerous studies has been compared and analysed, shedding light on the geometric and electronic requirements for effective binding to HIV-1 integrase. This information will in turn aid in the rational design of future generations of integrase inhibitors.

KEYWORDS: Diketoacid, DKA, HIV, Integrase Inhibitors, Raltegravir

INTRODUCTION

The condition now known as Acquired Immunodeficiency Syndrome (AIDS) was first described in 1981.[1] Three years later, it's etiological agent, the Human Immunodeficiency Virus (HIV) was identified.[2,3] Today HIV represents an emergency situation and a long-term development issue, a complex scientific puzzle and a global health concern. It is estimated that 33 million people are currently living with HIV/AIDS, with more than 2.7 million new cases *per annum*, and over 2 million AIDS related deaths each year.[4,5] Scientific progress in combating the infection has been remarkable and the prognosis for AIDS patients who have access to a full range of treatment drugs has improved considerably. Mean survival rates (from diagnosis of symptoms) now exceed 8 years, compared to only 6 months prior to the release of AZT.[6]

Before 2003, all FDA approved therapies for HIV patients targeted either HIV reverse transcriptase or HIV protease, two of the three viral enzymes required for HIV replication. Highly Active Antiretroviral Therapy (HAART), which uses carefully tailored combinations of these inhibitors, is now considered the standard of care for AIDS patients.[7] Recent developments have seen the approval of one fusion inhibitor (enfuvirtide) and one entry inhibitor (maraviroc).[8,9] Nevertheless, with the emergence of drug resistant strains of the virus, there is a pressing need for new treatments.[10,11]

Considerable effort has been directed towards the third viral enzyme, HIV integrase, culminating in the release of the first integrase inhibitor, raltegravir, in 2007.[12,13] HIV integrase is responsible for inserting the newly synthesised provirus into the host cell genome. This function is a specific requirement of retroviruses and as such there are no

counterparts to HIV integrase in the host cell. This makes the enzyme an ideal focus for new therapies. A number of potential inhibitors have been identified, initially through the random screening of compound libraries.[14] These include coumarins,[15] phenyldipyrimidines,[16] styrylquinolines[17] and diketoacids (DKAs).[18,19] DKAs are of particular interest as they are the only class of compounds shown to be truly selective for HIV integrase.[6] Extensive lists of inhibitors and an overview of the field can be found in several earlier reviews. [6,11,20-25] However, little effort has been directed to comparing results from the various studies, allowing important trends to be lost in the vast array of data produced. DKAs and structures derived from this chemical motif are the focus of this review, which aims to compare SAR data from compounds in this class rather than provide comprehensive lists of inhibitors. In doing this, we can being to draw a clear picture of the structural and electronic properties required for effective binding to HIV integrase.

1. HIV INTEGRASE

1.1 The HIV Life Cycle

HIV replication begins with the virus recognising and attaching to a host cell through the CD4 glycoprotein receptor.[26] Through a series of biochemical cascades, the viral envelope merges with the cell membrane of the host cell. Partial uncoating of the viral genetic material following this fusion causes RNA and associated enzymes to be released into the cell, ready for replication. The viral RNA then undergoes reverse transcription to give a double stranded DNA copy called a provirus, a process carried out by the viral protein HIV reverse transcriptase and utilising nucleic acids present in the host cell. The viral DNA then binds to a second viral protein, HIV integrase, to undergo 3' processing (3P) which removes the two terminal nucleic acids from the 3' end of each DNA strand. The processed viral DNA remains attached to the integrase enzyme as a multimeric 'pre-integration complex' that includes other viral and cellular proteins. This pre-integration complex is then transported into the nucleus of the host cell where it catalyses a second reaction, strand transfer (ST), in which the viral DNA is inserted into the host DNA. When the cell activates and begins to replicate, the proviral DNA is transcribed into RNA and subsequently translated into viral proteins and polyproteins. These migrate to the host cell membrane, where they are assembled into new virions with the aid of the third viral protein, HIV protease. Virions can then bud off and move on to infect other cells.

1.2 The Structure of HIV Integrase

HIV integrase is a 288 amino acid polypeptide arranged into three domains: the catalytic core domain (CCD), the C-terminal domain and the N-terminal domain.[27] The CCD is comprised of residues 50–212 and contains the active site responsible for catalysing the reactions of HIV integrase.[28] It consists of a central 5-stranded β-sheet with six surrounding helices (**Figure 1**) and is highly flexible. This suggests that the substrate is responsible for the enzyme adopting a precise, catalytically-active conformation. Three amino acids in the CCD, Asp64, Asp116 and Glu152, are highly conserved among retroviral integrases and mutation of any of these residues tends to result in a complete loss of activity.[28] Hence they are thought to be essential components of the active site. It is also believed that one or more divalent metal ions coordinate to these residues and play a key role in catalysis.

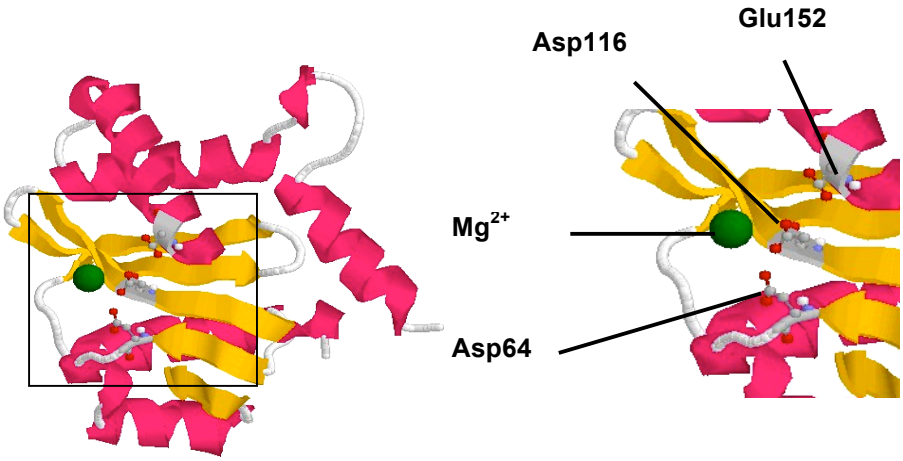


Figure 1: Catalytic core of HIV integrase, showing the three key carboxylate residues[†]

The C-terminal domain is involved in binding both viral and host DNA.[29] It is comprised of five β-strands arranged in a barrel formation. In its dimeric form, this domain forms a large saddle-shaped groove with the appropriate dimensions to bind DNA. The function of the N-terminal domain is unclear. It contains a His-Cys-zinc binding motif which suggests that it also interacts with nucleic acids.[28] Although crystal and solution structures for individual domains have been determined, little is known about the conformation of the active enzyme in solution.[29-33] In the crystal structures, each subunit exists as a dimer, however the spatial arrangement of the active site suggests that at least a tetramer is necessary for activity.[6] The lack of an X-ray crystal structure of the complete, catalytically active enzyme and the highly flexible nature of the CCD make rational design of drugs to target HIV integrase difficult.

A great deal of effort has been made to understand the role of metal cofactors in HIV integrase. Their role is particularly important as many HIV integrase inhibitors are believed to interact with these divalent metal ions.[34,35] Although the exact number and nature of metal cofactors have not been confirmed, the general consensus is that there are two Mg(II) ions in the active site.[32,36] One of these is thought to coordinate Asp64 and Asp116, a proposal supported by X-ray crystallographic data.[37] It has been suggested that the second metal ion is brought into the active site with the DNA substrate and coordinates to Glu152.[38] The distance between the two putative metal ions is a point of contention: figures varying from 3.6 to 7 Å have been reported.[39]

1.3 Mechanism of Strand Transfer

It is thought that the strand transfer reaction occurs via Mg(II)-mediated phosphodiester cleavage, with the two 3'-hydroxyl groups of the viral DNA acting as nucleophiles (**Figure 2**).[40] According to this mechanism, the two viral 3'-hydroxyl groups which were exposed in the 3'-processing step attack phosphodiester bonds on complimentary strands of the host DNA. The resultant pentavalent phosphorus intermediate then collapses, cleaving the host DNA. The site of integration on the two strands is separated by five base pairs, and cellular enzymes such as DNA polymerase are

[†] Image generated in Rasmol using crystallographic data published by Goldgur *et al.* (PDB code 1BIU) (32).

responsible for repairing the integration intermediate.[28] The Mg(II) ions presumably help stabilise the enzyme-DNA complex and may also facilitate charge flow from the viral 3'-hydroxyl to the departing 3'-hydroxyl of the host DNA.[41]

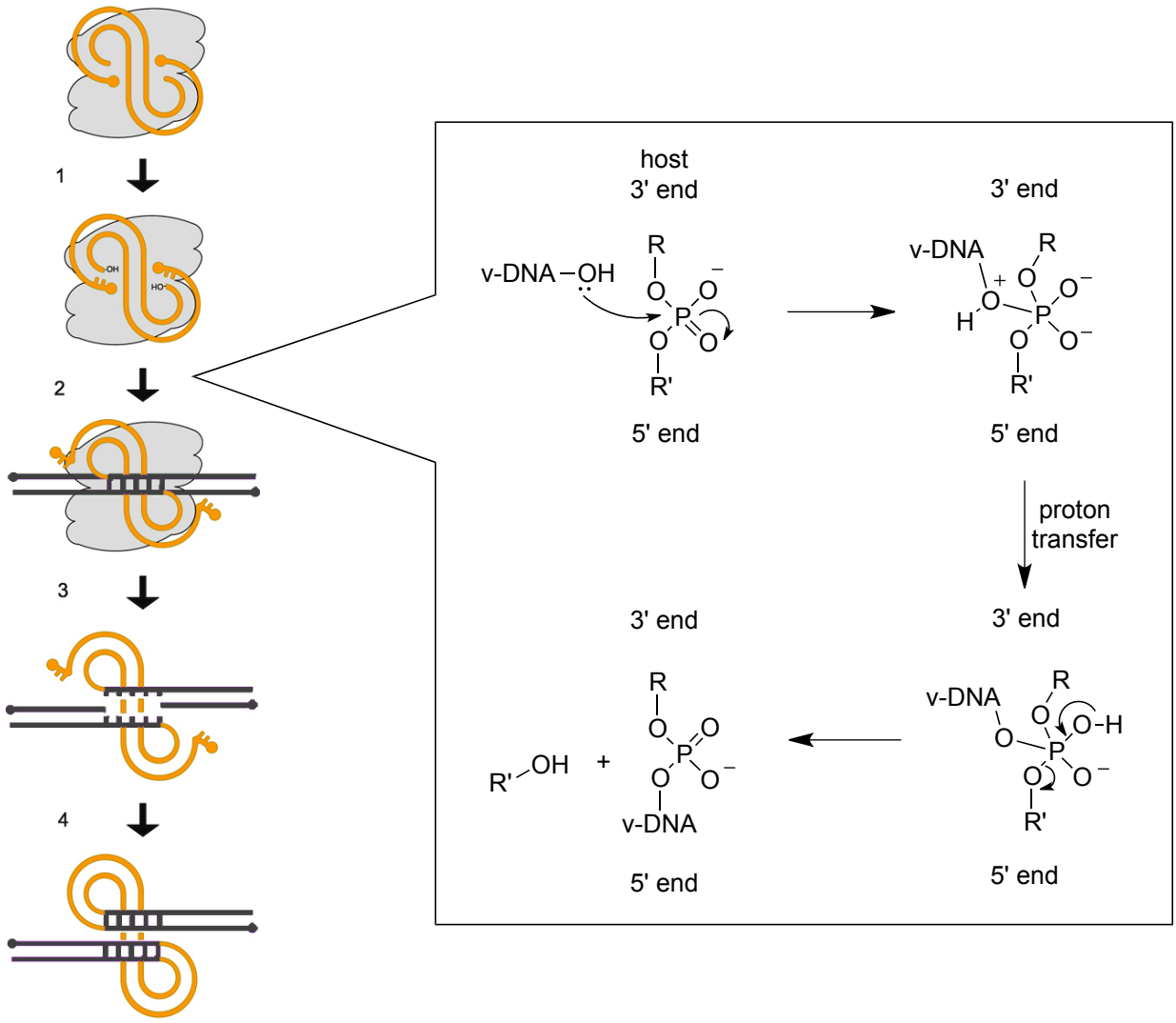


Figure 2: Integration of viral DNA into Host genome[†]

(1) 3' Processing: integrase catalyses the removal of two base pairs from each 3' end of viral DNA. (2) Strand Transfer: 3' ends of viral DNA (v-DNA) attack phosphodiester bonds of host DNA (see inset). (3) Cleavage of host DNA, DNA released from enzyme. (4) DNA repaired by cellular machinery.

2. DIKETO ACID INHIBITORS OF HIV-1 INTEGRASE

In the early 1990s, through the screening of over 250,000 samples, Merck identified a series of diketo acids (DKAs) as potent inhibitors of HIV-1 integrase.[18] These compounds are active both against the purified enzyme and in

[†] Image adapted from that appearing in PLoS Pathogens and is reproduced in accordance with the Creative Commons Attribution Share-Alike licence (42).

antiviral assays and have provided a much-needed platform for the development clinically useful HIV-1 integrase inhibitors. DKAs selectively inhibit the strand transfer reaction of HIV-1 integrase (**Figure 2**, Step 2). Although they also inhibit 3' processing, they are generally 30–70 fold less active against this reaction. Diketo acids tested to date consist of an aromatic region linked to a carboxylate via the conserved β-dicarbonyl moiety (**Figure 3**). Each of these structural elements appears to be integral to the activity and selectivity of the inhibitor, as detailed below.

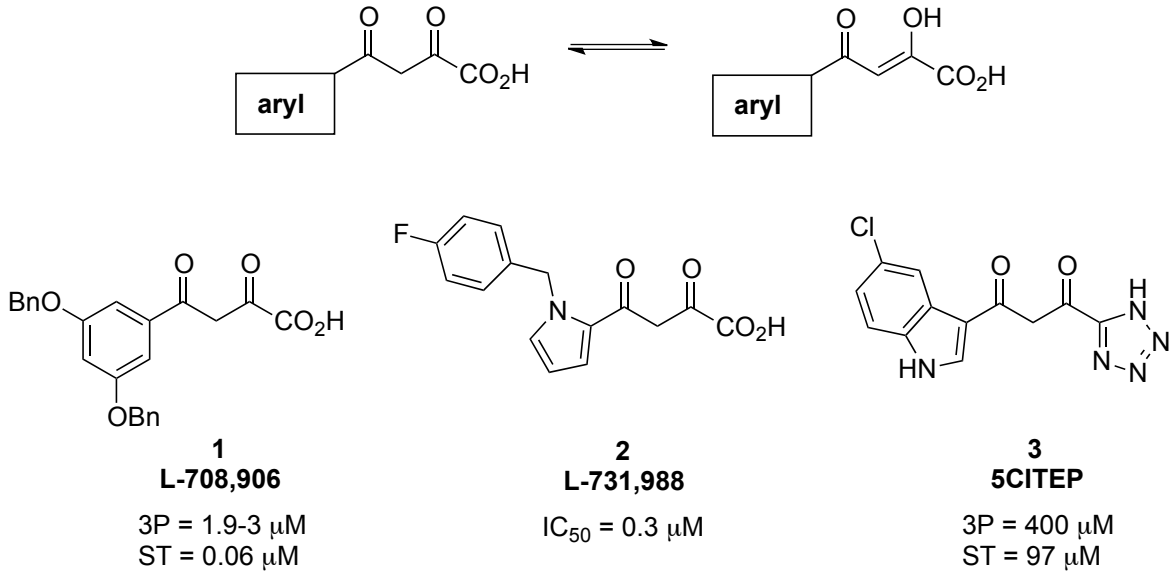


Figure 3: General aryl diketo-acid based inhibitor and three reported examples[18,43]

3P = IC_{50} for 3' processing step; ST = IC_{50} for strand transfer step; IC_{50} refers to whole integration process

2.1 Mechanism of Inhibition

The mechanism by which DKAs inhibit HIV-1 integrase has not been fully elucidated. However it is well documented that β-dicarbonyl compounds in the enolate form complex divalent metal ions. Hence it has been postulated that DKA inhibitors complex one or more divalent metal ions in the provirus-integrase complex, most likely one of the metal ions found at the Asp64, Asp116, Glu152 catalytic triad.[34,35] This coordination would stabilise the complex and inhibit the binding of cellular DNA. This hypothesis is supported by evidence that DKAs bind to integrase only in the presence of such metal ions.[35] There is also evidence to suggest that DKAs are active when introduced as the corresponding metal complexes.[44] This hypothesis is further supported by molecular modelling studies.[45] A related theory suggests that in binding to this central metal ion, the DKA is oriented so as to place its 'diketo tweezers' in position to sequester a second metal ion.[46] This second ion may be introduced by the incoming acceptor DNA.

DKAs specifically recognise assembled strand transfer complexes, which accounts for the observed ST selectivity.[47] In particular the free viral 3-OH end, produced in the 3' processing reaction, is required for DKA binding, suggesting a specific interaction between the DKA and this functional group. This hypothesis is substantiated by a recent crystal

structure in which the inhibitor raltegravir is bound to the structurally related enzyme prototype foamy virus (PFV) integrase.[48] This crystal structure shows the expected two-metal binding mode, as well as a 6 Å displacement of the 3' end of the complexed viral DNA. Van der Waals interactions between the viral DNA and raltegravir are also apparent.

3. STRUCTURE ACTIVITY RELATIONSHIPS

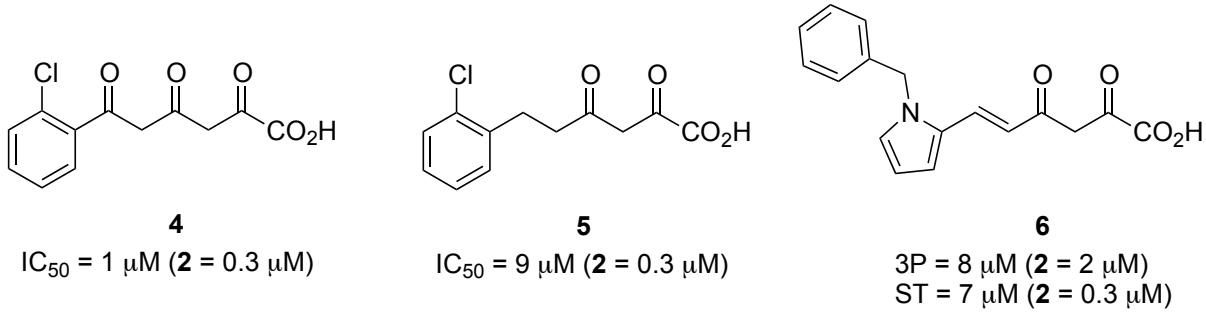
While numerous Structure Activity Relation (SAR) studies have been undertaken, the wide range of assays used makes comparisons difficult. Marchand *et al.* found that using the wild-type enzyme and Mg(II) as the divalent metal ion afforded the most stringent conditions for integrase inhibition assays.[45] Many early studies used Mn(II) as the divalent metal ion, although activity in these assays did not necessarily translate into activity in whole-cell experiments.[35] Various double mutant enzymes have also been employed to improve the solubility of integrase, however these often have resistance profiles that differ significantly from the wild-type enzyme.[49] To allow for some comparisons to be drawn between different studies, the ST IC_{50} values for reference compounds L-708,906 (**1**) or L-731,988 (**2**), has been reported throughout this paper wherever possible. Unfortunately, these two sets of data cannot be compared quantitatively as both reference compounds have not been tested under the same conditions. The standard IC_{50} value for L-708,906 (**1**) is 0.06 μm as reported by Marchand,[44] and for L-731,988 (**2**) is 0.3 μm as reported by Walker *et al.* as these researchers also used wild-type enzyme with Mg(II).[45,50]

3.1 The Carboxylate Moiety

There has been some controversy over the importance of the carboxylate group in DKA inhibitors. The first selective inhibitor of HIV integrase was 5CITEP (**3**) which bears a tetrazole in place of the carboxylate (**Figure 3**).[43] However, it was later found that 5CITEP is only effective in the presence of Mn(II) and is not active in antiviral assays.[35,45] Further studies of tetrazole-containing inhibitors showed that the acid portion of the DKA confers metal selectivity.[45] While both carboxylate and tetrazole derivatives have a high affinity for Mn(II), only the carboxylate-containing compounds were potent in the presence of Mg(II). These results were mirrored in antiviral assays, with only the carboxylate derivatives showing inhibitory activity. It is also interesting to note that blocking the acidic nitrogen in the tetrazole analogues does not necessarily lead to reduced activity.[51] Conversely, masking the carboxyl group with an alkyl group gives compounds which, despite a high affinity for HIV integrase, are inactive.[35] The exception to this is compounds that also incorporate an alternative metal coordinating region.[52] This supports the idea that DKAs bind two metal ions in the active site, one through the dicarbonyl and one through the terminal functionality. It also suggests a different mode of binding between the tetrazole and carboxylate derivatives. Pommier *et al.* have investigated the effect of replacing the carboxylate with a phosphonic acid group on a limited number of substrates.[53] Unlike the change to a tetrazole, this modification appears to be well tolerated in both purified enzyme and whole-cell assays.

3.2 The β-Dicarbonyl Linker

While this moiety is, by definition, conserved across all DKAs, a number of analogues have been made in which this region is extended, either through the addition of a third carbonyl group or with an alkyl linker. The most potent inhibitors in each of these series are **4** and **5** respectively **(Figure 4**).



4
IC_{50} = 1 μM (**2** = 0.3 μM)

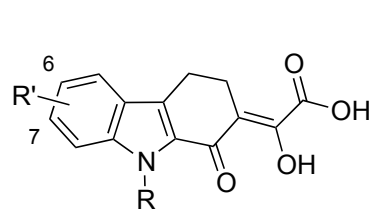
5
IC_{50} = 9 μM (**2** = 0.3 μM)

6
3P = 8 μM (**2** = 2 μM)
ST = 7 μM (**2** = 0.3 μM)

Figure 4: DKA inhibitors with extended diketo chains

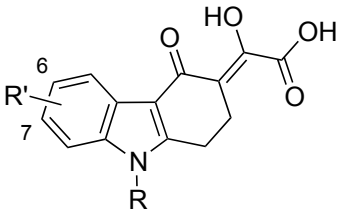
Both the triketo (**4**) and hexanoic acid (**5**) derivates showed activities comparable to the parent dioxybutanoic acid (2-chloro-α,γ-dioxobenzenebutanoic acid, IC_{50} = 17 μM).[50,54] Triketo derivatives in which the carboxylic acid was replaced with a tetrazole were essentially inactive. The authors infer from these results that triketoacids adopt a binding mode comparable to the DKAs and that the third keto-group is not actively involved in binding either of the metal ions. The added rigidity imposed in a hexenoic acid system (**6**) gave compounds which, while active in both purified enzyme and antiviral assays, were not ST selective.[55] There is a single example in which the γ-ketone has been replaced with a cyano group. Activity was comparable with the parent compound, however this type of substitution has not been explored further.[56] Removal of the diketo functionality entirely, by incorporating it into a heterocycle such as an isoxazole, gave compounds that did not bind to HIV-1 integrase.[35,56]

Inhibitors have been synthesised in which the diketo portion was conformationally locked as a dihydrocarbazolone.[57] Both dihydrocarbazol-1-ones (**7**) and dihydrocarbazol-4-ones (**8**) were investigated, allowing comparison of two different conformations of the diketo functionality (**Figure 5**). The dihydrocarbazol-1-ones were consistently more active than the 4-one analogues but both showed lower activity than simple DKA inhibitors such as L-731,988 (**2**). In the 1-one series, alkylation of the carbazolone nitrogen with a 4-fluorobenzyl group (**7b**) resulted in an increase in activity. The reverse selectivity was seen in the carbazol-4-one derivatives (**8a**, **8b**), suggesting substantially different binding orientations for the two sets of compounds. This was further substantiated by the fact that 7-halodihydrocarbazol-4-ones (**8d**) displayed increased activity whereas 6-halo substitution increased the potency of hydrocarbazol-1-ones (**7c**).



7

(**2** = 79% Inhib.)



8

7a: R = H, **R'** = H %inhib. = 37.3
7b: R = 4-FBn, **R'** =H %inhib. = 48.2
7c: R = 4-FBn, **R'** = 6-Cl %inhib. = 54.7
7d: R = 4-FBn, **R'** = 7-Cl %inhib. = 47.1

8a: R = H, **R'** = H %inhib. = 12.9
8b: R = 4-FBn, **R'** =H %inhib. = 0
8c: R = 4-FBn, **R'** = 6-Cl %inhib. = 8
8d: R = 4-FBn, **R'** = 7-Cl %inhib. = 51.3

Figure 5: Carbazolone integrase inhibitors

3.3 The Aryl Portion

This is the most extensively studied aspect of the DKA inhibitor class. It has been found that, like the carboxyl group, the aromatic functionality confers a degree of metal selectivity.[45] For example, indolyl or naphthyl groups confer Mn(II) selectivity while the *bis*-benzyloxyphenyl group of L-708,906 (**1**) results in equal affinity for Mg(II) and Mn(II). The aryl group also has an important influence on selectivity for the ST reaction over 3P.[58]

Two general classes of aryl substituents have been identified, by analysing the lowest energy conformations of various integrase inhibitors.[50] The first class comprises compounds such as **9** in which the aryl group is coplanar with the diketo acid chain (**Figure 6**). In this class, an angle of *ca.* 120^{o} between the diketo and aryl portions appears to be optimal.[34,59] Analogues in which the aryl portion is flexible and not fixed in this plane are considerably less active. In the second class, typified by L-731,998 (**2**), the proximal aromatic group is also coplanar with the diketo functionality but there is a distal aryl portion almost perpendicular to this plane (**Figure 6**). Compounds, such as triketo acids, can potentially adopt either conformation.[50]

The significant difference in the spatial orientation of the aromatic groups in these compounds suggests that HIV-1 integrase possesses two aryl binding pockets. However this analysis has only been preformed on a limited number of inhibitors and a more extensive study is required to confirm the existence of a second aryl binding site. Inhibitors in the second class are consistently less active in the 3P reaction when compared to the indole substituted derivatives, suggesting that the pendant aryl group plays a role in ST selectivity. [58]

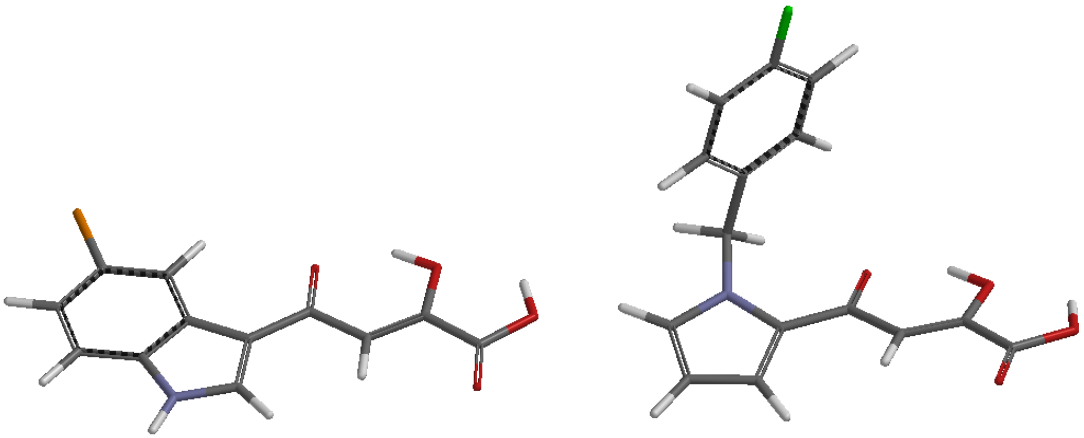


Figure 6: Lowest energy conformations for (a) carboxylate 9 and (b) L-731,988 (2)

Numerous studies have examined the effects of aryl substitution on the inhibitory activity of DKAs against HIV-1 integrase. However, the range of base structures used, as well as the different viral assays employed, makes it difficult to compare these results. Nevertheless, these studies have brought to light several important structural features of the aromatic substituent. Selected aryl-substituted DKA inhibitors are shown in **Figure 7**. Looking first at molecules bearing an indolyl group, such as **9**, it has been found that halogen substitution at the 5-position increases activity.[34] Similar results were obtained for anilide derivatives, typified by **10**, with chloro derivatives more active than their fluoro counterparts.[59] This similarity is not surprising considering that aryl groups of these two compounds adopt a similar orientation in their lowest energy conformations. It is interesting to note that *N*-alkylation of indolyl derivatives with benzyl and 4-fluorobenzyl groups produced compounds that were also potent inhibitors.[60]

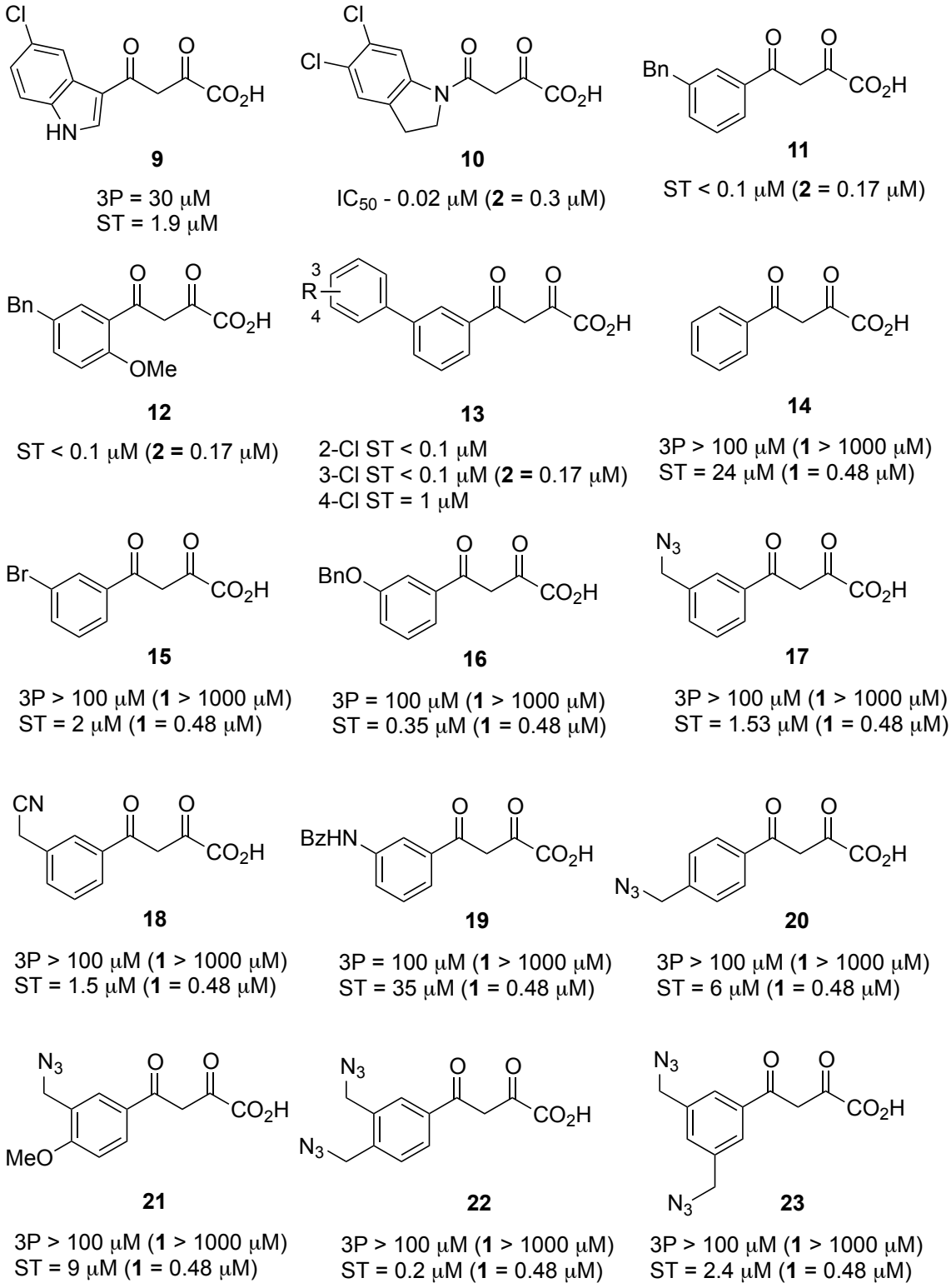


Figure 7: DKA inhibitors with varying aromatic regions

Two studies have used L-731,998 (**2**) as a starting point to probe SARs in the aromatic region. The first of these surveyed the optimum relative orientation of the aromatic and diketo side chains, and found that the 1,3-disubstituted benzene ring, with an angle of 118°, was most active, (**11**).[61] The effect of further substitution on this ring was

generally unpredictable, although substitution at the 6-position was generally well tolerated, for example compound **12**. Halogen substitution at the 2- and 3-positions of the distal aryl ring gave more potent inhibitors, whereas 4-halo derivatives were less active (**13**). In the second study, the dioxybutanoic acid was replaced with its hexenyl counterpart as in compound **6**.[55] Again, 4-substitution of the distal aromatic ring reduced the efficacy of this inhibitor. A number of related derivatives are covered in the patent literature, including examples in which the pyrrole of **2** is replaced by thiophene, thiazole or imidazole, and where the 3-benzyl group of **11** is replaced with various *N*-containing heteroaromatics.[23] Binding data for these compounds is not in the public domain.

Other SAR studies loosely based on L-708,906 (**1**) have been undertaken (**Figure 7**). Generally it has been found that a substitutant at the 3-position is vital to activity. Electron withdrawing groups, such as halides (**15**), benzyl ethers (**16**), azides (**17**) and cyano groups (**18**) at this position all increase potency compared to the unsubstituted aryl.[62,63] However, derivatives containing a cyano group showed decreased antiviral activity. A benzoyl amino substituent (**19**) caused a reduction in potency as did substitution at the 4-position such as in the azidomythel derivative **20**. In some cases, substitution with electron-withdrawing groups at the 3-position in addition to 4-substitution restored activity. As with the L-731,998 analogues, trends in the disubstituted aryls (**21**-**23**) are harder to discern.

In early screening studies, dihydroxylated aromatics such as flavanoids were identified as potential inhibitors of HIV integrase.[46,64] While many of these were subsequently shown to be non-specific for integrase, this work prompted Cotelle *et al.* to synthesise derivatives bearing both diketo acid and catechol functionalities (**Figure 8**).[65]

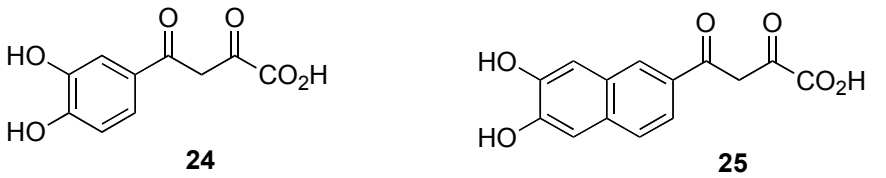
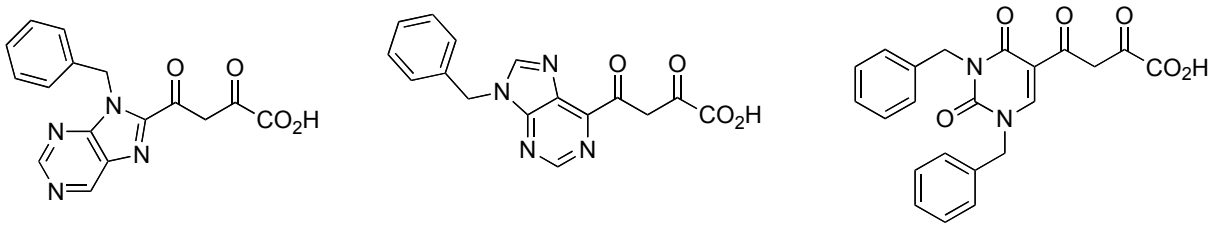


Figure 8: DKA–Catechol hybrid inhibitors

While the benzoyl derivative **24** appears to interact with integrase through both these moieties, the activity of **25** was attributed entirely to the catechol: masking the acid as the corresponding ester did not effect inhibitory activity, whereas removing the dihydroxyl functionality gave compounds that were completely inactive under the assay conditions.[65]

Compounds incorporating purine nucleobases as the aromatic portion have been synthesised and tested against HIV-1 integrase.[66] While these derivatives are highly selective for the ST reaction, the orientation of the purine has a significant effect on potency. For example, simply changing from an 8-substituted purine (**26**) to the 6-substituted analogue (**27**) results in a 10-fold decrease in activity (**Figure 9**). β-Diketo acids with pyrimidine nucleobase

scaffolds, such as **28**, are also potent inhibitors both in purified enzyme and whole-cell assays, but do not display any ST selectivity.[67] This supports the hypothesis that integrase has highly specific aryl binding pockets.



26

IC_{50} = 10 μM (**1** = 0.48 μM)

27

IC_{50} = 100 μM (**1** = 0.48 μM)

28

IC_{50} = 0.2 μM (**1** = 0.48 μM)

Figure 9: Purine and pyrimidine substituted DKAs

3.4 *Bis*-Diketo Inhibitors

A number of research groups have synthesised *bis*-diketo inhibitors, both with and without the acidic functionality (**Figure 10**).[52,62] While such derivatives are active against HIV-1 integrase there is a uniform loss of selectivity for ST inhibition over 3P. Furthermore, these compounds show little antiviral activity. In contrast to the strict geometric requirements of the simple DKAs (**Figure 3**), there seem to be less stringent spatial requirements for these *bis*-diketo compounds. For example, in symmetric *bis*-dioxobutanoic acids (**29**) both 1,3- and 1,4- substituted aromatic linkers are tolerated, indicating that the angle between the two groups is flexible.[62]

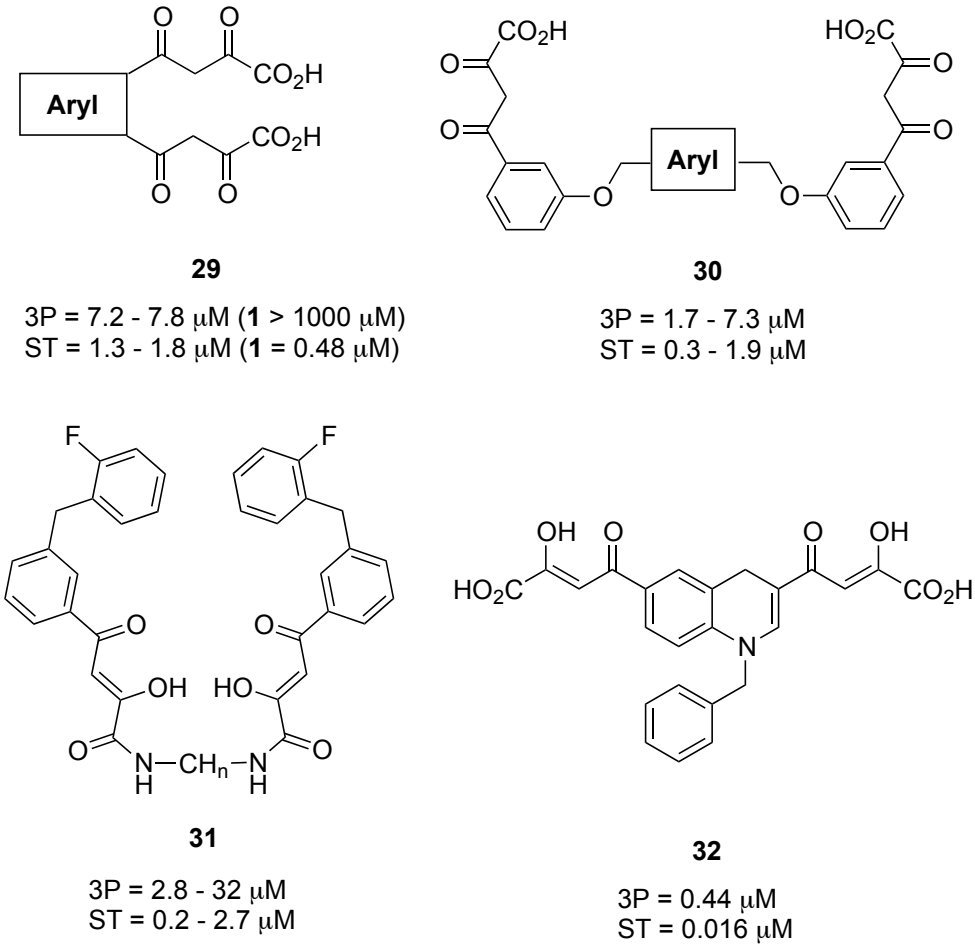


Figure 10: *bis*-Diketo inhibitors

Long *et al.* reported a more extensive study in which two diketo groups were liked via either a disubstituted aromatic ring (**30**) or an amide (**31**).[52] For the first of these, it was found that 1,3-substitution of the aromatic ring gave the most potent inhibitor. Despite promising results in assays with the purified enzyme, none of these compounds exhibited antiviral activity in cell-based assays. Permeability issues were cited as a possible reason for this. However, it is important to note that Mn(II) was the divalent metal ion used in these enzyme assays and, as discussed above, it is well documented that inhibitory activity in the presence of Mn(II) does not necessarily correlate to activity in the presence of Mg(II) or to antiviral activity.[45] For the amide series (**31**), analogues containing a short, rigid linker were the most potent in both assays. Compounds incorporating longer, more flexible chains were highly toxic in cell based assays.[52] The corresponding monomer, bearing a carboxyl group in place of the amide, showed comparable potency against recombinant HIV-1 integrase and was 40 ftimes more active than the dimers in antiviral assays.[52]

More recently, *bis*-diketo acid inhibitors have been reported that do show promising antiviral activity.[68] These compounds consist of two diketo acid functionalities linked by a 4H-quinolonyl group (**Figure 10**). The *N*-alkylated quinolonyl derivative (**32**) was synthesised in an attempt to exploit the high potency of many simple 1,3-substituted diketo acids like **11**. The *bis*-diketo acid **32** bears the same 1,3-substitution pattern as **11** and was active both against purified integrase and in cell based assays. Furthermore, this derivative showed good selectivity for the ST reaction,

unlike previous *bis*-diketo inhibitors, further evidence that access to a second aryl binding pocket is at least partially responsible for ST selectivity.

3.5 Molecular Modelling Studies

Given the flexible nature of integrase, and the absence of a crystal structure of the whole enzyme, it is difficult to ascertain exactly how much information can be gained from computer based docking studies. Indeed, it was shown in studies using GOLD (Genetic Optimisation for Ligand Docking)[69] that the potent inhibitor S1360 (**33**) can be made to bind in three entirely different orientations depending on the protein crystal structure chosen.[70] The crystal structure of the CCD complexed with 5-CITEP (**3**) is often used, replacing 5-CITEP with the inhibitor of interest.[43] This approach appears ill advised as it is believed that crystal-packing constraints influenced the positioning of the original ligand in that crystal structure.[71-73]

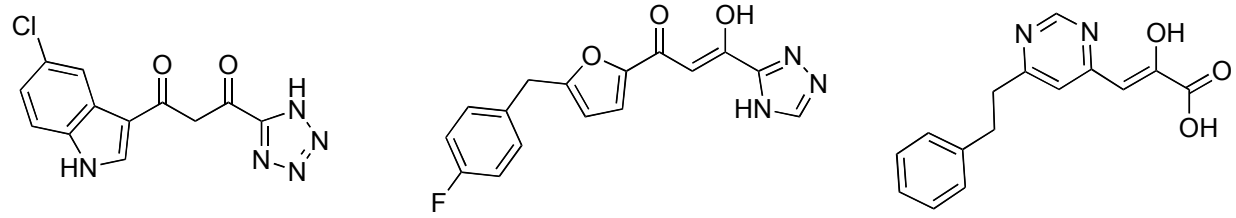
That being said, molecular modelling studies have provided some insight into the variation in activity observed for some inhibitors in the presence of Mg(II) versus Mn(II). Several studies have found that tetrazole- and carboxylate-based inhibitors assume different binding orientations in the presence of Mg(II) whereas they adopt similar binding orientations in the presence of Mn(II).[45,62] All of the carboxylate derivatives studied bind a Mg(II) ion via the diketo functionality and whenever a second ion is present in the model, this is chelated by the carboxylate functionality. In contrast, tetrazole derivatives coordinate Mg(II) with either the diketo group or through the tetrazole functionality. Where coordination occurrs through the diketo group, any additional ions remained uncomplexed by the inhibitor.[62] In the presence of Mn(II), both classes of inhibitors chelate the principle ion through the terminal functional group: either the tetrazole, or through one keto oxygen and one carboxylate oxygen in the case of the carboxylates. This has been explained by considering the more flexible coordination sphere of Mn(II).[45] Other DKA derivatives, such as the *bis*-diketo compounds, generally adopt very different binding modes to the simple DKAs, which may explain the decrease in ST selectivity seen with these analogues.[52]

4 EXPLORING THE DKA PHARMACOPHORE

While DKAs are proving promising drug leads, their likely mode of action (metal ion coordination) is of some concern.[74] Many other enzymes employ divalent metal ions in their active sites and it is probable that DKA-based integrase inhibitors will also have some activity against these enzymes. In particular, some DKAs are known to inhibit RAG1/2, a recombinase enzyme vital in the development of the specific immune system.[75] This enzyme catalyses both a 'nicking' step (analogous to the 3P reaction) and a transesterification step (comparable to integrase ST), both of which are critical to the correct functioning of the enzyme system. Unsurprisingly, this type of enzyme shares many structural features with the integrases, and both 5CITEP (**3**) and L-708,906 (**1**) show inhibitory activity against RAG1/2.[75]

4.1 From Random Screening to Rational Design

In light of this likely lack of selectivity, as well as the known biological lability of the DKA functional group, there has been a push to develop bioisosteres of the DKA portion. This strategy was behind the development of S-1360 (Shionogi and GlaxoSmithKline, **33**) which became one of the first integrase inhibitors to reach clinical trials (**Figure 11**).[76] It was found that the tetrazole moity of 5CITEP (**3**) could be replaced by a triazole without loss of inhibitory activity. Moreover, unlike the parent compound, the triazole derivative was active in anti-viral assays. Extensive screening of diketotriazoles appended to aryl groups identified the substituted furan S-1360 (**33**) as the best candidate for clinical trials. Unfortunately S-1360 did not progress beyond phase II trials due to pharmokinetic issues.[77] Derivatives in which the triazole is replaced with a related heterocycle, for example, a pyridine, thiazole or pyrrole, have also been reported in the patent literature.[78,79]



3
5CITEP
3P = 400 μM
ST = 97 μM

33
S-1360
IC_{50} = 0.04 μM

34
IC_{50} = 0.3 μM

Figure 11: S-1360 and related inhibitors

Researchers at Shionogi also found that the γ-ketone could be replaced with a Lewis basic nitrogen, for example a pyridine or pyrimidine (**34**).[80] This nitrogen atom was key to binding, and derivatives in which the *N*-heterocycle is replaced with a phenyl group are inactive.

Naphthyridine **35** (**Figure 12**) from the Merck laboratories was one of the first rationally designed integrase inhibitors based on the DKA pharmacophore.[81] This compound was designed specifically to afford the required coplanar arrangement of the key functional groups while avoiding the toxicity associated with DKAs and the poor pharmacological profile of polyaromatics. As with S-1360 (**33**), a heteroaromatic nitrogen replaces the carboxylate as the Lewis base. In this series the diketo functionality is also trapped as the α-enol equivalent, thought to be the biologically relevant confomer. The 3-benzyl substituted aromatic region is reminiscent of early DKA analogues such as L-708,906 (**1**) and was designed with the putative secondary aryl binding pocket in mind. Further development lead to L-870,810 (**36**), the first of Merck's integrase inhibitors to enter clinical trials. Although L-870,810 was eventually withdrawn due to acute liver toxicity, analogues of this compound show promising activity.[13]

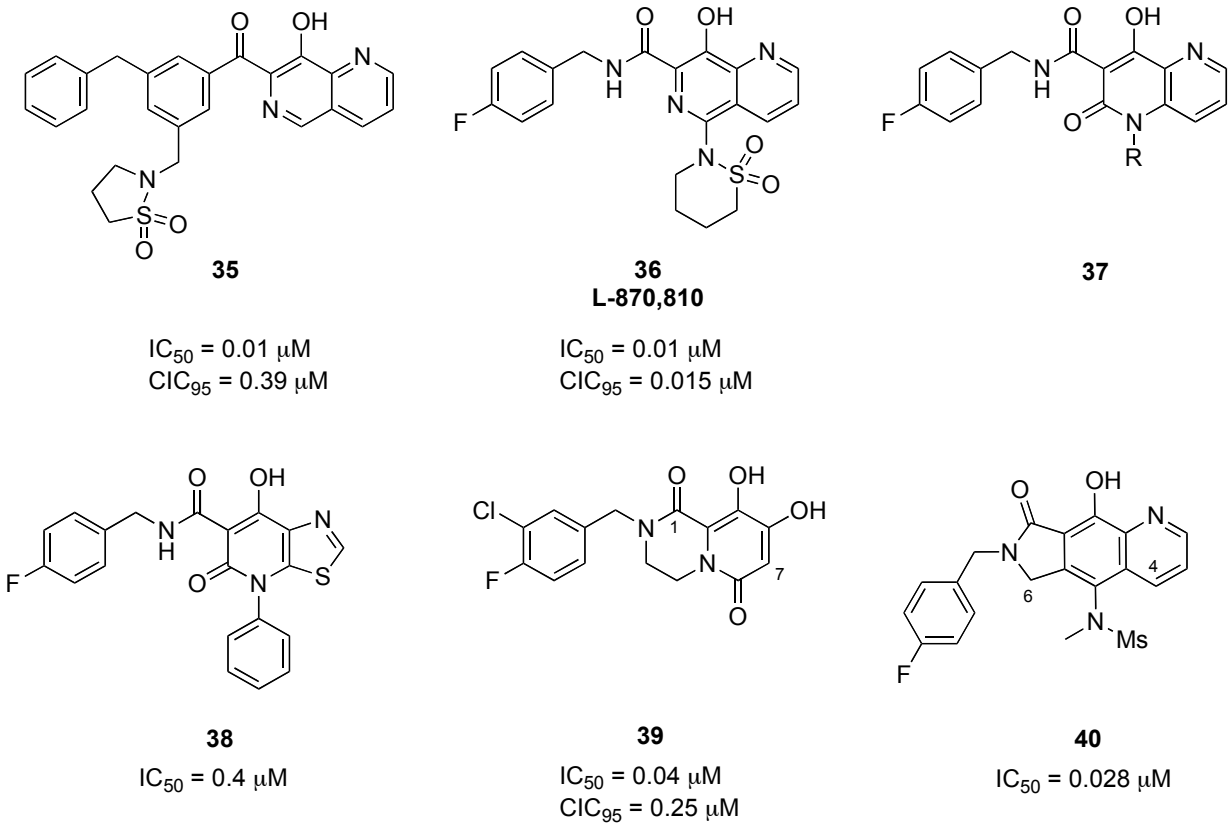


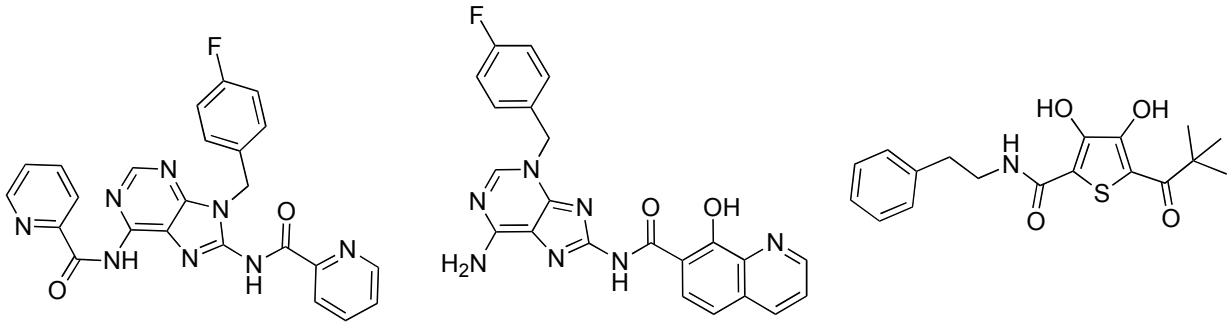
Figure 12: Conformationally locked *N*-heterocyclic DKA derivatives

This work has prompted a number of groups to investigate conformationally locked diketo systems, including the carbazolones[57] discussed previously (Section 3.2). Initially, the focus was on replacing the naphthyridine core with other bicyclic heterocyclic systems including naphthyridinone (**37**), thiazolopyridinone (**38**) and pyridopyrazinedione (**39**) (**Figure 12**). The napthyridinones (**37**) were developed at Merck in conjunction with the naphthyridines (**35**, **36**).[82] *N*-Alkyl substitution is well tolerated, allowing the pharmokinetic properties of this series to be controlled. Boros and co-workers at GlaxoSmithKline replaced the naphthyridine core with a thiazolopyridine (**38**), generating a series of compounds that, while potent inhibitors of HIV-integrase, proved highly toxic in cell-based assays.[83] As with many DKA derivatives, the 4-fluorobenzyl substituent was key to inhibitory activity. The effect of further conformational restraint was investigated using the pyridopyrazinedione skeleton (**39**). Not only would this modification restrict the rotation of the amide (γ-ketone equivalent), it was also expected to bias the orientation of the benzyl side chain through steric interactions.[84] This proved to be a highly effective strategy, giving a series of inhibitors with IC_{50} < 1 μm. Of these the 3-Cl, 4-F derivative **39** was the most potent in both purified enzyme and anti-viral assays (IC_{50} = 0.04 μM, CIC_{95} = 0.25 μM) . Substitution at C-7 of the pyridopyrazinedione with electron donating groups (Br, I, CN) gave compounds with improved activity for the ST reaction, however this did not translate into improved anti-viral activity.

Although developed independently, the tricyclic inhibitors introduced by Kim and co-workers at Gilead Sciences are in effect an amalgamation of L-870,810 (**36**) and the pyridopyrazinedions (**39**), combining the naphthyridine

carboximde core with conformational restraint of the amide system.[85] Compound **40** (**Figure 12**) performed well in assays (IC_{50} = 0.028 μM) and showed reasonable bioavailability, making this an attractive candidate for further studies.[86,87] However this scaffold was not amenable to further substitution, and the introduction of groups at the 4 or 6 positions decreased activity up to 100-fold.[85]

Vince and Li, combined their previous work using purine nucleobases as the aryl portion with the newly developed naphthyridine core to generate either two point (**42**) or three point (**43**) ligands, capable of chelating one and two divalent metal ions respectively (**Figure 13**).[88] Dihydroxythiophenes (DHTs) such as **43** were recently reported to be potent inhibitors of HIV-1 integrase and incorporate a pharmacophore that is analogous to the DKA motif.[89] However, as the two free hydroxyl groups are key to activity, these are perhaps better classed with the dihydroxylated aromatics.



41
%inhib. = 72% (**2** = 79%)

42
%inhib. = 41% (**2** = 79%)

43
IC_{50} = 0.03 μM

Figure 13: Selected DKA analogues

4.2 The Development of Raltgravir

The potential of DKA-inspired inhibitors was cemented with the development of the pyrimidinone carboxamide skeleton by Merck, leading to the first USFDA approved HIV-1 integrase inhibitor, raltegravir (MK-0518, Merck, **44**). The original breakthrough came with the identification of dihydroxypyrimidine-4-carboxamide **45** by screening inhibitors of the structurally related enzyme Hepatitis C Virus (HVC) polymerase against HIV-1 integrase (**Figure 14**).[90,91] This compound was highly active against purified integrase, but this did not translate into activity in cellular assays on account of the inhibitor's high binding affinity for human plasma. This problem could be attenuated through *N*-methylation to give the *N*-methylpyrimidone skeleton (**46**, **47**).[92] A range of groups at the 2-position were tolerated and could thus be used to fine-tune pharmokinetic properties, eventually giving rise to MK-0518 (**44**). MK-0518, later renamed raltegravir, showed nanomolar activity against both the purified enzyme and HIV-infected cells, and displayed a good pharmokinetic profile.[12] Raltegravir was subsequently fast-tracked through clinical trials and approved for use in treatment-experienced patients in 2007. This was considered a major breakthrough, particularly for patients infected with multiple-drug resistant strains of HIV. So far, raltegravir is living up to its potential, effectively controlling HIV load in patients with limited treatment options.[93] Furthermore, preliminary studies suggest

raltegravir displays less CNS toxicity than efavirinez, currently the frontline NNRTI for HAART in the UK.[94] Several recent reviews summarise the data generated in clinical trials in more detail and readers are directed to these for more information.[95,96]

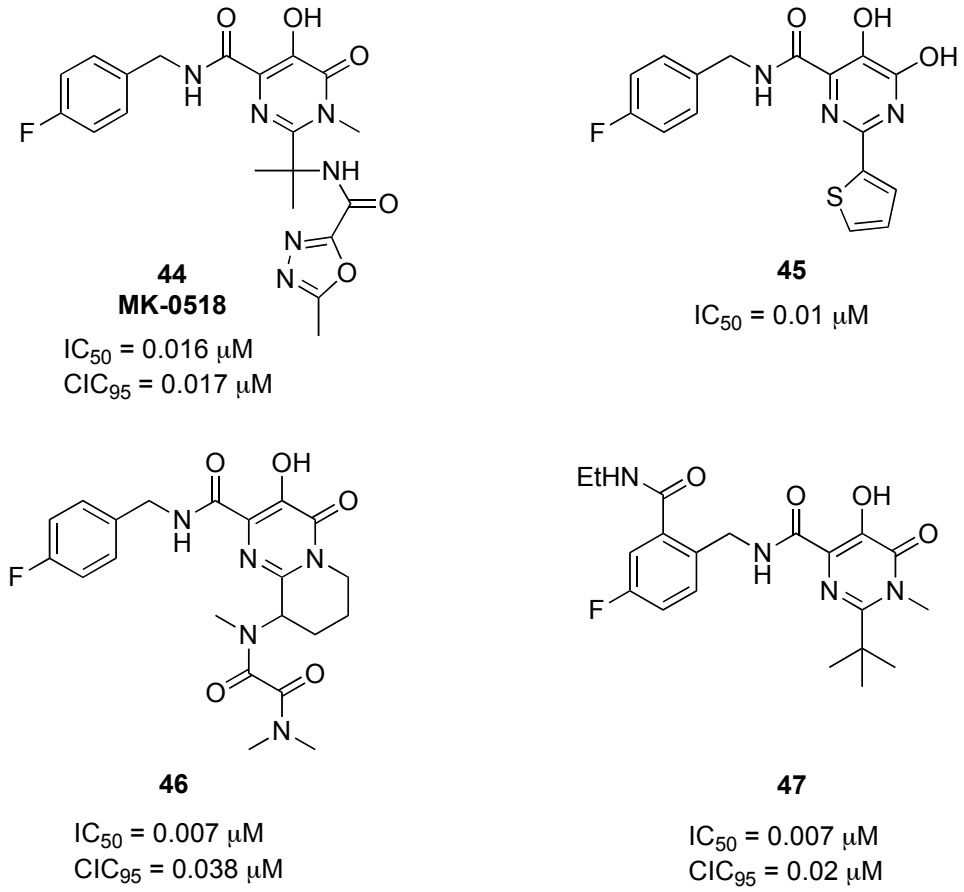


Figure 14: *N*-Methylpyrimidone inhibitors

Later studies at Merck have looked at the effect of further rigidification of this skeleton[84,97] and at substitution of the pendent benzylamine group.[98] The most potent inhibitors in each of these series are **46** and **47** respectively (**Figure 14**).

4.3 Future Directions

A number of related compounds are now in clinical trials, including Elvitegravir (GS-9137, Gilead Sciences, **48**) and MK-2048 (Merck, **49**) (**Figure 15**).[13] Elvitegravir is derived from quinolone antibiotics and is currently in Phase III clinical trials.[99] Unlike previous examples, elvitegravir does not contain a γ-ketone or Lewis basic equivalent. Despite this significant difference, the resistance profile of **48** appears to show moderate overlap with raltegravir. MK-2048 (**49**), a second-generation inhibitor developed at Merck is also progressing well through clinical trials and is touted to be even more potent *in vivo* than raltegravir.[100] Naphthyridine GSK-364735 (GlaxoSmithKline, **50**), which made it to Phase II trials, was developed from **37** (Section 4.1).[101] Substitution of both the core and amide portions produced a

compound that retain the activity of the early derivatives without the toxicity issues. A second such inhibitor, GSK-572 (Shionogi & Co. Ltd and ViiV-Heathcare, **51**) is now in Phase II clinical trials.[102]

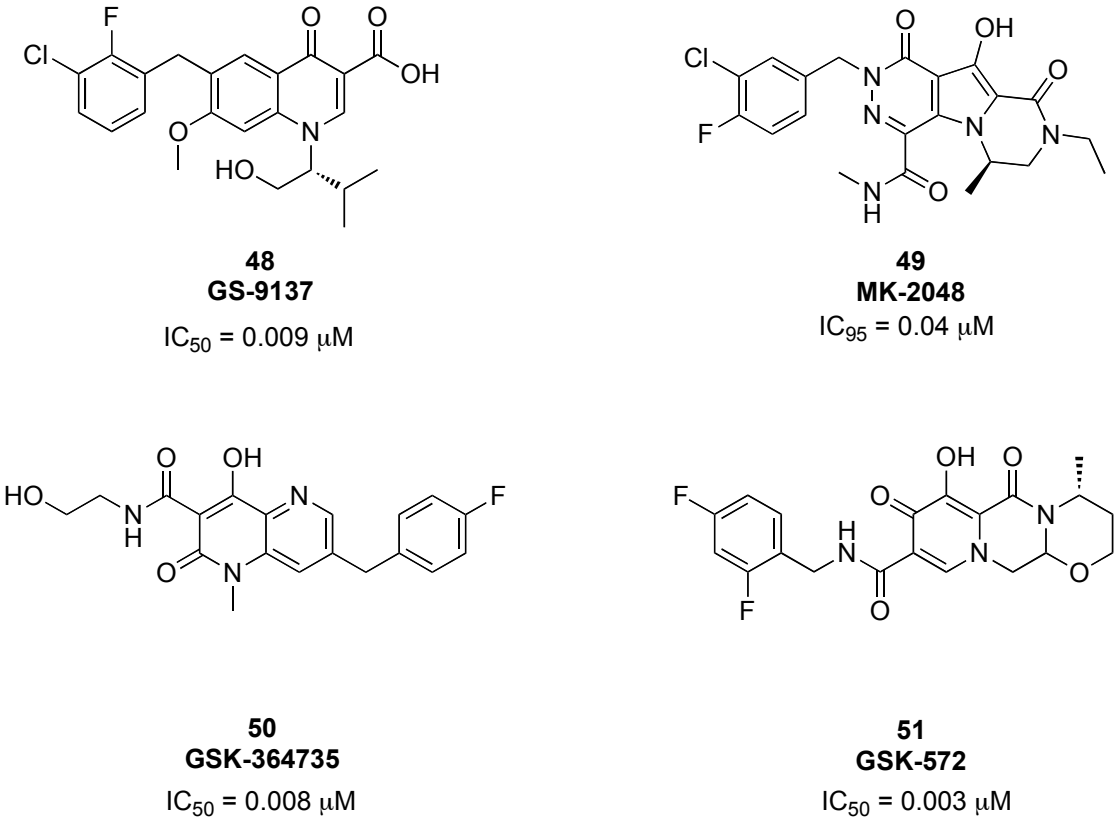


Figure 15: Integrase inhibitors currently in clinical trials

Fuelled by the success of raltegravir and now elvitegravir, the development of DKA-like integrase inhibitors continues apace. Of particular interest is recent work by Deadman and co-workers, who have incorporated the *N*-methylpyrimidone motif of raltegravir into polycyclic scaffolds, giving compounds such as **52** and **53** (**Figure 16**).[103] Compound **52** was the most potent inhibitor identified in this series, and became the subject of further investigation. Replacing the pendant amide functionality with various heterocyclic isosteres lead to **54**, a potent inhibitor of both wild-type and raltegravir-resistant strains of HIV.[104,105] This unexpected resistance profile makes **54** a promising lead compound for clinical development.

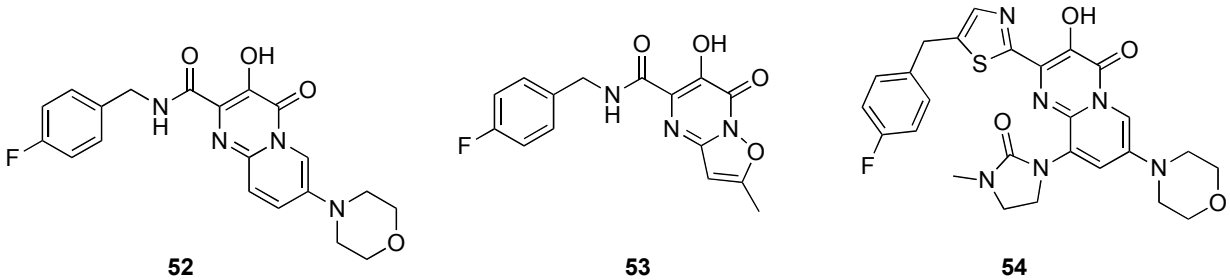


Figure 16: Recently Reported Integrase Inhibitors

Natural products are another important source of drug leads and a number of compounds isolated from fungi and bacteria have shown activity against HIV integrase.[106] These include polyhydroxylated aromatics, terpenes, polyketides and cyclic peptides.[21,22] A number of these, including integramycin (**55**) and phomasetin (**56**), show some resemblance to the DKA pharmocaphore (**Figure 17**).[107,108] Similarities between the native targets of these mycotoxins and retroviral enzymes makes this an interesting area for further study.

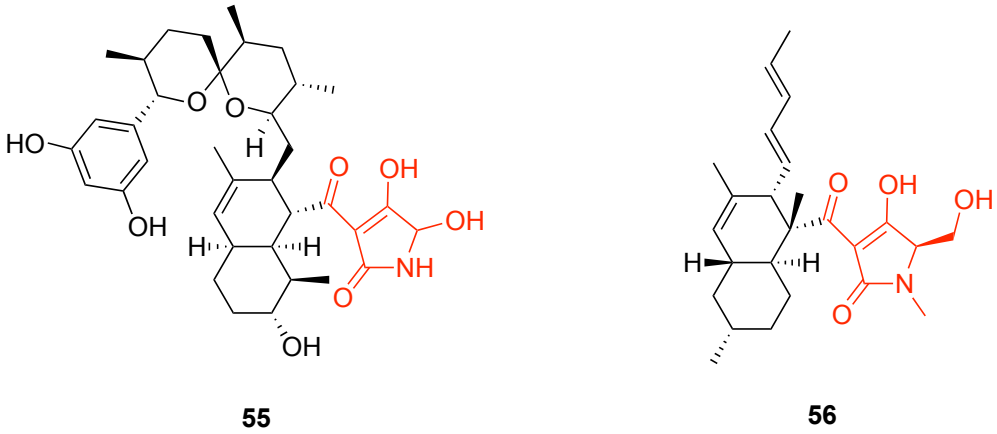


Figure 17: Natural product derived inhibitors of integrase

5 Resistance and Binding Modes

Already numerous strains of HIV resistant to raltegravir and the DKAs have emerged.[109] Given that all the DKAs and derivatives discussed here are believed to have a common mode of activity, considerable cross-resistance between these compounds is expected. Indeed, this is frequently the case. However a small handful of DKA-derived inhibitors retain activity against these resistant strains, suggesting a more complex mode of binding interaction than originally proposed.[105,110]

5.1 Cross-Resistance in HIV Integrase

Viral strains resistant to the DKA-type inhibitors arise through mutations in the integrase coding sequence (which is further proof that this enzyme is the primary target of such drugs).[109] Many of these mutations have been characterised and most negatively affect integration and viral activity.[111] Generally, one primary mutation is associated with a series of specific secondary mutations, leading to double- and triple-mutant strains.

The resistance profile for raltegravir (**44**) is the most thoroughly studied and is comprised of three independent primary mutations (N155H, Q148H/K) and several secondary mutations.[112,113] Both the traditional DKAs and elvitegravir (**48**) show high levels of cross-resistance with raltegravir.[18,114,115] GSK-572 (**51**) is still in early development and highly resistant viruses have been found but as yet not fully characterised. However, preliminary data implies surprisingly little cross-resistance with either raltegravir or GS-9137.[102] GSK-572 **(51**) is believed to act at the interface of the enzyme-DNA complex, in the same manner as raltegravir and elvitgravir, however the lack of overlap between their resistance profiles suggests a different binding orientation for GS-572. The existence of multiple binding modes was proposed by Hazuda and co-workers, who found that L-870,810 (**36**) retained activity against several DKA-resistant strains of HIV, and *vice versa*.[110] The mutations responsible were characterised and mapped to a crystal structure of the integrase CCD, showing that while both sets of mutations surrounded the active site, they were clustered on different sides for L-870,810 and the DKAs. The only mutation to confer cross-resistance was N155S. This residue points directly into the active site and is involved in metal binding. The recently reported inhibitor **54** (Section 4.3) also shows a resistance profile that is distinct from raltegravir.[105] Whether this translates into activity against GS-9137 resistant HIV, or conversely cross-resistance with GSK-572 or L-870,810 has not yet been reported.

5.2 Two Binding Modes for DKA-Based Inhibitors

It is possible that some integrase inhibitors, typified by GSK-572, bind to HIV-1 integrase with a reversed orientation relative to raltegravir. This would indicate a second possible binding mode in which overlap of the metal-binding pharmacaphores is retained but not that of the pendant substituents. This model seems more consistent with the observed divergent patterns of resistance, and explains the observation that inhibitors with substituents extending in both directions from the diketo core show increased affinity for HIV-1 integrase.[110] It also concurs with molecular dynamics simulations, which predict both flexible binding modes and also multiple ligand binding regions adjacent to the active site. Modelling studies using the recently published PFV integrase structure suggest that at least one of these pockets is induced by inhibitors like raltegravir, and that the aryl binding in this pocket is responsible for the observed displacement of viral DNA from the enzyme-inhibitor-DNA complex.[116] However, further studies are required to confirm the existence of these putative binding regions

CONCLUSIONS

DKA-derived inhibitors of HIV integrase have now been in development for over ten years, with one reaching FDA approval and several more in advanced clinical trials. The absence of a crystal structure for the complete protien has made the rational design of inhibitors difficult, although situation is changing as a crystal structure for structurally related PFV integrase has recently been published. The picture is further complicated by the apparent existence of

multiple ligand binding regions around the enzyme active site. However, through a combination of SAR studies, the characteristion of mutant strains, and molecular modelling experiments, a picture of the active site is beginning to take shape. It has been shown that an ability to bind divalent metal ions is essential for inhibitory activity and that this role can be filled by a wide array of metal-chelating heterocycles. Co-planarity of these metal chelating functionalities is key to activity and trapping the α-ketone equivalent as the corresponding enol is advantageous. The region opposite the metal-chelating pharmacophore does not appear to be involved in enzyme binding and a wide range of substituents is tolerated here. Substitution at this position has been used extensively to tune the pharmokinetic properties of promising inhibitors. Pendant aromatic groups confer selectivity and appear to dictate the binding orientation of inhibitors to integrase. The majority of the inhibitors reported to date are typified by L-731,988 (**2**) and appear to access an aromatic pocket in a different plane to the DKA-functionality. In some models, this pocket is shown to accommodate the 3' end of viral DNA in the absence of a suitable inhibitor. Recent results imply that there is at least one more binding pocket, potentially on the opposite side of the active site to that accessed by L-731,988. Exploting alternative binding pockets, binding modes and orientations will be key to the development of inhibitors with complimentary resistance profiles, and thence the future of integrase inhibitors in the treatment of HIV.

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