INTEGRASE INHIBITORS TO TREAT HIV/AIDS

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Abstract | HIV integrase is a rational target for treating HIV infection and preventing AIDS. It took approximately 12 years to develop clinically usable inhibitors of integrase, and Phase I clinical trials of integrase inhibitors have just begun. This review focuses on the molecular basis and rationale for developing integrase inhibitors. The main classes of lead compounds are also described, as well as the concept of interfacial inhibitors of protein–nucleic-acid interactions that might apply to the clinically used strand-transfer inhibitors.

HIGHLY ACTIVE ANTIRETROVIRAL THERAPY (HAART). A therapeutic regime that consists of a protease inhibitor (PI) or a non-nucleoside reverse transcriptase inhibitor (NNRTI) in combination with two nucleoside reverse transcriptase inhibitors.

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Acquired immunodeficiency syndrome (AIDS) is one of the greatest challenges to humankind. AIDS and HIV infection represent global health hazards, complex scientific puzzles, obvious targets for drug discovery and vaccination, and both have enormous social, economical and ethical ramifications. First reported in 1981 in a small number of patients[1–3], AIDS has now become a major epidemic with more than 38 million people infected worldwide, including approximately 1 million in the United States (for details see REF. 4), 580,000 in Western Europe and more than 25 million in Sub-Saharan Africa (www.unaids.org). Since AIDS was first clinically identified, scientific and therapeutic progress has been extraordinary. It took less than 6 years to identify the pathogenic virus, HIV, that caused AIDS, develop sensitive tests to detect infected people during the latency period and to introduce the first rationally designed effective therapy, AZT. However, AIDS remains out of control[4], especially in developing countries where societal factors are a major hurdle to combating the epidemic.

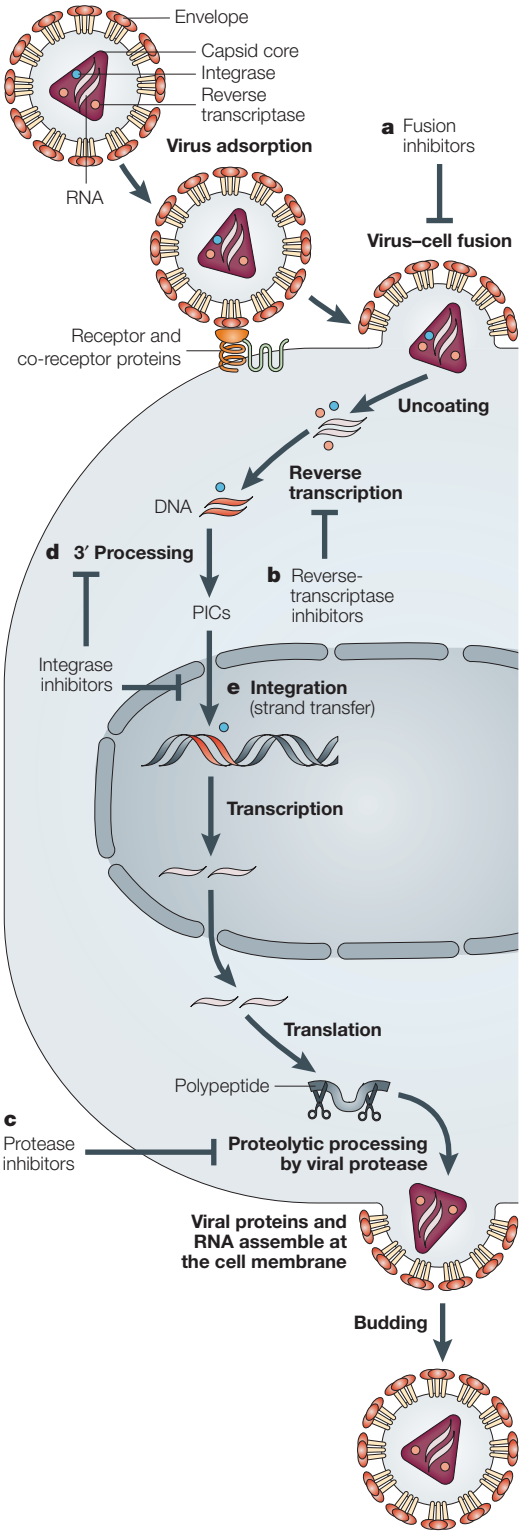
The prognosis of AIDS patients who have full access to current therapies has completely changed since the first cases of AIDS were reported. Today, the median survival for HIV-positive patients receiving treatment exceeds 8 years. The median survival for current combination therapies has not been determined, because these combinations were only introduced 8 years ago. The life expectancy for AIDS patients was less than 1 year before AZT was introduced in 1987. This dramatic change is due to the development of effective therapies, to early detection of HIV-positive individuals, and to a sustained effort to analyse and understand viral-resistance mechanisms, which can be overcome by rational drug development and combination therapy. The story of the development of anti-HIV therapies is a good example of rapid drug development and for turning a dreadful disease into a manageable chronic infection. There have been six major factors that have led to effective HIV therapies: first, early recognition of the severe population health problem posed by AIDS; second, adequate government prioritization and funding for basic research; third, elucidation of the genetics and life cycle of HIV; fourth, identification of viral-specific drug targets and the development of screening assays for drug discovery; fifth, the development of clinical tests for measuring viral load and therefore evaluating therapeutic efficacy; and sixth, drug combinations to overcome innate and acquired drug resistance resulting from the high-mutator phenotype of retroviruses.

FDA-approved therapies target three steps of the HIV life cycle: reverse transcription, proteolytic maturation and fusion (FIG. 1; TABLE 1). Triple therapy, commonly referred to as HIGHLY ACTIVE ANTIRETROVIRAL THERAPY (HAART), is now the standard for treatment. It consists of a protease inhibitor (PI) or a non-nucleoside reverse transcriptase inhibitor (NNRTI) in combination with two nucleoside reverse transcriptase inhibitors (NRTI; TABLE 1) (for details see REF. 5). HAART, however, is often not well-tolerated by the patients. It requires discipline, is expensive and leads to multidrug resistance[6]. Therefore, additional therapeutic approaches are warranted. One such approach is to target the third viral enzyme,

integrase, and recent studies from the Merck group have demonstrated the feasibility and efficacy of integrase inhibitors in animal models[7].

Harnessing the anti-HIV drug market with new therapeutic approaches could also be lucrative. In 2003, the estimated worldwide sales for each of the single reverse transcriptase inhibitors stavudine (Zerit; Bristol-Myers Squibb), lamivudine (Epivir; GlaxoSmithKline), tenofovir (Viread; Gilead) and efavirenz (Sustiva; Bristol-Myers Squibb) was between US$400 and $600 million. Sales for Trizivir and Combivir, which consist of three and two NRTIs, respectively, combined in the same formulation, were between US$680 and $880 million (TABLE 1) (for details see REF. 8).

The first integrase inhibitors were reported approximately 10 years ago[9–11]. Integrase inhibitors are now reaching early clinical development. The most advanced compounds from Merck and GSK–Shionogi belong to a class of compounds known as strand-transfer inhibitors (diketo aryl (DKA) and DKA-like inhibitors). These compounds share mechanistic and structural features that will be reviewed.



Requirement of integrase for HIV replication

Approximately 40–100 integrase molecules are packaged within each HIV particle. The primary role of integrase is to catalyse the insertion of the viral cDNA into the genome of infected cells, although integrase can also act as a cofactor for reverse transcription[12,13]. Integration is required for viral replication, because transcription of the viral genome and the production of viral proteins requires that the viral cDNA is fully integrated into a chromosome[14]. Following reverse transcription, the viral cDNA is primed for integration in the cytoplasm by integrase-mediated trimming of the 3′-ends of the viral DNA (FIG. 1d). This step is referred to as 3′-PROCESSING. It requires both fully functional integrase and the integrity of the last 10–20 base pairs at both ends of the viral cDNA (the *att* sites; see FIG. 2a and BOX 1, figure part a). 3′-processing consists of the endonucleolytic cleavage of the 3′-ends of the viral DNA (green arrows in FIG. 2a and BOX 1, figure part a). This cleavage occurs immediately 3′ to a conserved CA dinucleotide motif (underlined in BOX 1, figure part a). Alterations of this sequence prevent integrase from catalysing 3′-processing. This reaction generates CA-3′-hydroxyl DNA ends, which are the reactive intermediates required for STRAND TRANSFER (see below; FIG. 2c and BOX 1, figure part a).

Following 3′-processing, integrase remains bound to the viral cDNA as a multimeric complex that bridges both ends of the viral DNA within intracellular particles called PRE-INTEGRATION COMPLEXES (PICs). Isolated PICs contain both viral and cellular proteins in addition to

Figure 1 | **The HIV replication cycle and drug targets.** Current therapies target attachment/fusion of HIV to the host cell outer membrane (**a**) and the viral enzymes reverse transcriptase (**b**) and protease (**c**). Integrase, the third viral enzyme, catalyses two steps in the viral replication cycle. First, integrase catalyses the processing of the 3′-ends of the viral cDNA (3′-processing step) (**d**); integrase then remains bound in a complex with the viral cDNA ends in the pre-integration complexes (PICs). Following nuclear translocation of the PICs, integrase catalyses the insertion (strand-transfer step) of the viral cDNA ends into host chromosomes (**e**) (FIG. 2). The diketo aryl (DKA) integrase inhibitors preferentially block the strand-transfer step, whereas other inhibitors (FIG. 4) block both the 3′-processing and strand-transfer steps.

3′-PROCESSING
Integration requires two consecutive steps that are catalysed by integrase: 3′-processing and strand transfer. 3′-processing corresponds to an endonucleolytic cleavage of the 3′-ends of the viral cDNA. This cleavage is sequence-specific and occurs immediately 3′ to a conserved CA dinucleotide motif.

STRAND TRANSFER
The second step of the integration reaction, which corresponds to the ligation of the viral 3′-OH cDNA ends (generated by 3′-processing) to the 5′-DNA phosphate of an acceptor DNA (physiologically a host chromosome).

PRE-INTEGRATION COMPLEX (PIC). A macromolecular complex formed during and after 3′-processing and carrying the 3′-processed viral cDNA ends with viral and cellular proteins to the nucleus, prior to integration.

Table 1 | **Anti-AIDS therapies approved by the FDA**

FDA approval	Brand name	Generic name	Manufacturer
Fusion inhibitors			
2003	Fuzeon	Enfuvirtide (T-20)	Roche Pharmaceuticals & Trimeris
Nucleoside reverse transcriptase inhibitors (NRTIs)			
1987	Retrovir	Zidovudine (AZT)	GlaxoSmithKline
1991	Videx	Didanosine (ddI)	Bristol-Myers Squibb
1992	Hivid	Zalcitabine (ddC)	Roche Pharmaceuticals
1994	Zerit	Stavudine (d4T)	Bristol-Myers Squibb
1995	Epivir	Lamivudine (3TC)	GlaxoSmithKline
1997	Combivir	Lamivudine+ Zidovudine	GlaxoSmithKline
1998	Ziagen	Abacavir	GlaxoSmithKline
2000	Trizivir	Abacavir + lamivudine + zidovudine	GlaxoSmithKline
2000	Videx EC	Didanosine (ddI)	Bristol-Myers Squibb
2001	Viread	Tenofovir disoproxil	Gilead Sciences
2003	Emtriva	Emtricitabine (FTC)	Gilead Sciences
2004	Epzicom	Abacavir+ Lamivudine	GlaxoSmithKline
2004	Truvada	Emtricitabine+ Tenofovir	Gilead Sciences
Non-nucleoside reverse transcriptase inhibitors (NNRTIs)			
1996	Viramune	Nevirapine	Boehringer Ingelheim
1997	Rescriptor	Delavirdine (DLV)	Pfizer
1998	Sustiva	Efavirenz	Bristol-Myers Squibb
Protease inhibitors (PIs)			
1995	Invirase	Saquinavir	Roche Pharmaceuticals
1996	Norvir	Ritonavir	Abbott Laboratories
1996	Crixivan	Indinavir (IDV)	Merck
1997	Viracept	Nelfinavir	Pfizer
1997	Fortovase	Saquinavir Mesylate	Roche Pharmaceuticals
1999	Agenerase	Amprenavir	GlaxoSmithKline
2000	Kaletra	Lopinavir+ Ritonavir	Abbott Laboratories
2003	Reyataz	Atazanavir	Bristol-Myers Squibb
2003	Lexiva	Fosamprenavir	GlaxoSmithKline

the integrase–DNA complexes. The viral proteins reverse transcriptase (RT), matrix (Ma), nucleocapsid (Nc) and Vpr can contribute to the transport of PICs through the nuclear envelope. Some cellular proteins packaged within PICs can bind to integrase (for review see REF. 15) and stimulate the enzymatic activities of integrase. These proteins include interactor 1 (INI1)[16] (the first integrase-binding protein discovered), lens epithelium-derived growth factor (LEDGF, also known as p75)[17], embryonic ectoderm-development protein[18] and heat-shock protein 60 (HSP60)[19]. Promyelocytic leukaemia (PML) protein also colocalizes and co-migrates with PICs[20]. Two cellular proteins, high-mobility group protein A1 (HMGA1, also known as HMG1(Y)) and barrier to auto-integration factor (BAF), regulate integration by binding to DNA directly. HMGA1 stimulates integrase activity[21,22] (for a review see REF. 15); BAF stimulates intermolecular integration and suppresses auto-integration. By contrast to other lentiviruses, such as the oncoretroviruses murine Moloney virus and Rous sarcoma virus, which require mitotic nuclear-envelope breakdown to access the chromosomes of infected cells, HIV-1 PICs are able to cross the nuclear envelope. The karyophilic property of the PICs enables HIV to replicate in non-proliferative cells, such as macrophages[23].

Once in the nucleus, integrase catalyses the insertion of the viral cDNA ends into host chromosomes (FIG. 1). This 'strand transfer' reaction (FIG. 2c,d) consists of the ligation of the viral 3′-OH DNA ends (generated by 3′-processing) to the 5′-DNA phosphate of a host chromosome. Integrase can also catalyse the reverse reaction, referred to as DISINTEGRATION[24]. Physiological integration requires the concerted joining of both ends of the viral cDNA on opposite DNA strands of the target (ACCEPTOR DNA) host chromosome with a canonical five-base-pair stagger (FIG. 2d). The five-base stagger indicates that each viral cDNA end attacks the chromosomal DNA across its major groove (FIG. 2d). Completion of integration requires ligation of the 5′-end of the viral DNA. This last step of integration can only take place after trimming of the last two nucleotides at the proviral DNA 5′-ends and extension (gap filling) from the 3′-OH

DISINTEGRATION
The reverse of the strand transfer reaction catalysed by the integrase catalytic core.

ACCEPTOR DNA
The DNA into which the donor DNA is integrated, which physiologically is host chromosomal DNA. Also termed 'target DNA'.

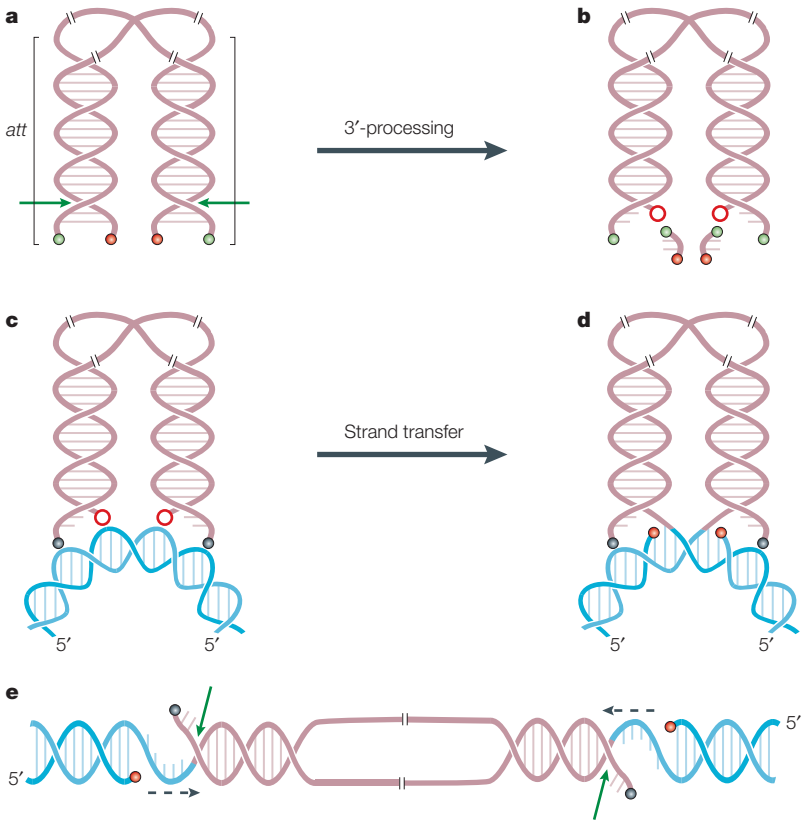


Figure 2 | **The two integrase catalytic reactions (3′-processing and strand transfer).** The figure shows the viral DNA recombination (*att*) sites. 3′-processing takes place in the cytoplasm following reverse transcription (FIG. 1). It is a water-mediated endonucleolytic cleavage (green arrow in **a** and BOX 1, figure part a) of the viral DNA immediately 3′ from the conserved CA dinucleotide (BOX 1, figure part a). 3′-processing generates reactive 3′-hydroxyls at both ends of the viral DNA (red circles (**b**); other 3′-hydroxyl ends and 5′-phosphate ends are shown as red and green dots, respectively). Integrase multimers (not shown) remain bound to the ends of the viral DNA as the pre-integration complexes (PICs) translocate to the nucleus. The second reaction (**c** to **d**) catalysed by integrase is strand transfer (3′-end joining), which inserts both viral DNA ends into a host-cell chromosome (acceptor DNA in blue). Strand transfer is coordinated in such a way that each of the two 3′-hydroxyl viral DNA ends (red circles) attacks a DNA phosphodiester bond on each strand of the host DNA acceptor with a five-base-pair stagger across the DNA major groove (**d**). Strand transfer leaves a five-base, single-stranded gap at each junction between the integrated viral DNA and the host acceptor DNA, and a two-base flap at the 5′-ends of the viral DNA (**d** and **e**). Gap filling and release of the unpaired 5′-ends of the viral DNA (arrows in **e**) are carried out in coordination with cellular repair enzymes.

end of the genomic DNA (FIG. 2e). It is likely that cellular enzymes/ pathways are involved in this 5′-processing, although their identity remains uncertain[25,26].

By contrast to the strict DNA-sequence requirement for the viral DNA ends (see the *att* sites in BOX 1, figure part a), HIV integration-site selection shows minimal sequence selectivity with regard to the chromosomal sequence ('target' or 'acceptor') in which integration takes place[27,28]. This is also the case with recombinant integrase or PICs in the presence of pure acceptor DNA[29] or nucleosomes[30]. Nevertheless, HIV integrates preferentially inside transcribed genes[28], whereas murine leukaemia virus (MLV) integrates preferentially in transcription start regions[31]. This difference might contribute to the greater oncogenicity of MLV compared with HIV. It is plausible that cellular factors, such as chromatin remodelling, and transcription complexes, such as those bound to integrase in the PICs (and described above), are implicated in the selection of the HIV integration sites within transcribing genes. The fully integrated viral genome is also referred to as the provirus.

DDE MOTIF
Catalytic triad consisting of two aspartate (DD) amino-acid residues and one glutamate (E). DDE motifs are conserved among integrase, transposase and phosphoryltransferase enzymes. The HIV-1 integrase DDE motif consists of residue D64, D116 and E152.

HIV integrase structure and functional domains

Retroviruses encode their three enzymes (protease, reverse transcriptase and integrase) within the *POL* gene, which is translated as the Pol polyprotein. Protease is at the 5′-end and integrase at the 3′-end of the *POL* gene (BOX 1). Integrase is generated during virus maturation (FIG. 1) by cleavage of the Pol polyprotein by HIV protease. HIV integrase is a 32-kDa protein comprising three structural domains: the amino-terminal domain (NTD), the catalytic core domain (CCD) and the carboxy-terminal domain (CTD) (BOX 1, figure part b). The atomic structure of each of these domains has been determined by X-ray diffraction or solution nuclear magnetic resonance (NMR) (for review see REF. 32). FIGURE 3 shows the structures of the CCD in association with the CTD[33], and of the NTD with the CCD[34].

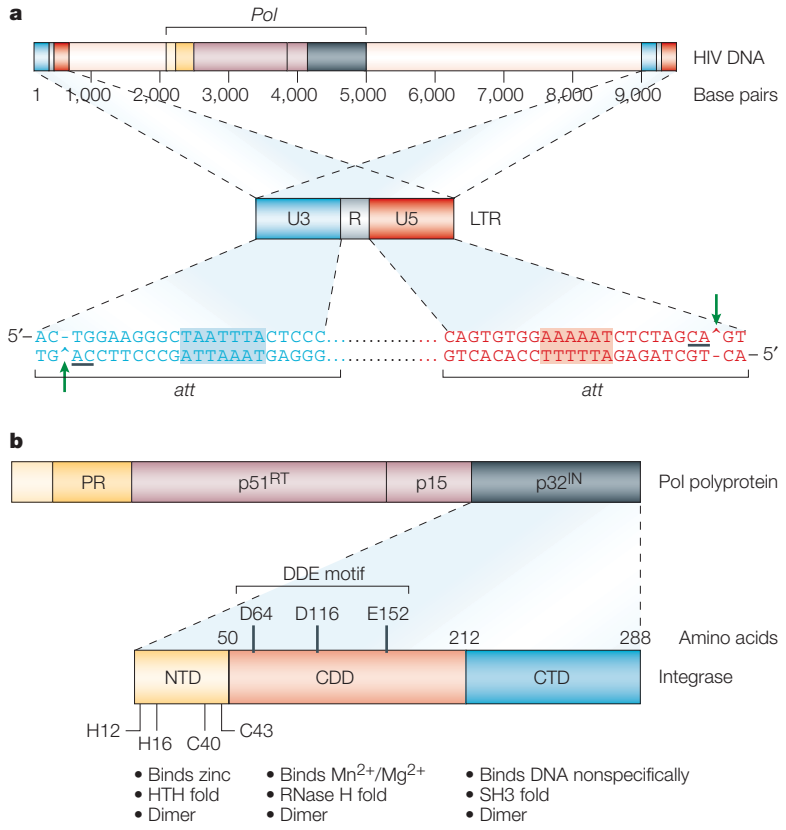
CCD, which encompasses residues 50–212, forms a dimer in all the structures examined. It is, structurally, remarkably similar to other retroviral integrases (MLV and avian sarcoma virus (ASV)), to the Tn5 and mu transposases, to RNase H, to the Holiday junction recombinase RuvC[35] and to the PIWI domain of Argonaute[36], the RNase associated with Dicer in RNA interference (for review on structural similarities see REF. 37). This family of DNA-processing enzymes (polynucleotide transferases) contains a canonical three-amino-acid DDE MOTIF formed in HIV-1 integrase by the catalytic triad D64, D116 and E152 (BOX 1; FIG. 3). These residues are highly conserved in all integrases and retrotransposases (DD[35]E motif). Mutation of any of these three acidic residues abolishes integrase's enzymatic activities and viral replication. The two D64 and D116 residues form a coordination complex (chemical bonds) with a divalent metal (Mg^{2+} or Mn^{2+}). Because a second metal has been observed in an ASV integrase crystal structure[38], and because of the two-metal structure for polynucleotide transferases[35,39], it has been proposed that a second metal (Mg^{2+} or Mn^{2+}) can be coordinated between D116 and E152 once HIV-1 integrase binds its DNA substrate(s)[40,41]. It is therefore likely that the metal(s) coordinate(s) integrase and the phosphodiester backbone of the DNA substrate(s) during the 3′-processing and strand-transfer steps. In most structures, the CCD contains a short disordered loop (encompassing residues 141–150), the structure of which can be stabilized by DNA. Although the CCD contains the enzyme catalytic site(s), in the absence of the NTD and CTD it can only catalyse the disintegration reaction — the reverse of the strand-transfer reaction — *in vitro*[24,42]. Although the physiological significance of this reaction is not known. Disintegration is the only reaction catalysed by the isolated CCD[24]. To catalyse 3′-processing and strand transfer, the CCD needs both the NTD and CTD in a dimeric complex[43].

The NTD encompasses residues 1–50 and contains an HHCC motif (BOX 1, figure part b) that is common to all retroviral integrases. Binding of one Zn^{2+} atom to the HHCC motif stabilizes the folding of the NTD domain and is required for integrase activity. Single mutations of any of these four residues reduce integrase enzymatic activity[44]. The NTD dimer interface is different in the crystal structures (FIG. 3) and the solution NMR structure (not shown), which is indicative of multiple arrangements of the integrase multimers (for review see REF. 32). The NTD is the preferential binding region for two cellular transcription factors in the PICs, INI1 and LEDGF/p75[16,17].

The CTD, which encompasses residues 212–288 (BOX 1, figure part b), has an overall SH3 fold (FIG. 3). It binds DNA nonspecifically and is required for integrase 3′-processing and strand-transfer activities. The CTD binds the cellular embryonic ectoderm development protein[18] as well as RT, and this interaction seems to be required for reverse transcription[13]. FIGURE 3 shows the structures of the CCD both with the NTD and the CTD. Together these two structures indicate the possibility that the NTD is positioned between the CCD and CTD, next to the extended α-helix joining the CCD and the CTD. The solution NMR dimer interfaces for the NTD and the CTD, which are different from those observed in the crystallographic structure (as in FIG. 3) might be used in higher-order complexes (tetramers and/or octamers), which have been proposed to correspond to the active enzyme.

Box 1 | The HIV provirus gene structure and integrase domains

Part a of the figure shows HIV viral cDNA containing the *pol* gene. The long terminal repeats (LTRs) at both ends of the viral cDNA consist of three consecutive elements, U3–R–U5, repeated in the same orientation. The sequence of the tips of the U3 (in blue) and U5 (in red) repeats at the ends of the viral cDNA following reverse transcription. The 3′-ends can extend further following reverse transcription (not shown)[96]. Integration requires correct sequences at both ends of the proviral DNA (*att* sites). 3′-processing catalyses the resection (green vertical arrows) of the viral DNA ends immediately 3′ from the conserved CA dinucleotide (underlined), thereby generating two dinucleotides (5′-GT) (one from each viral 3′-end) and reactive 3′-hydroxyl DNA ends (FIG. 2). The shaded A/T sequences, which start approximately ten base pairs from the viral DNA ends, are also conserved across HIV strains[77]. Integrase ($p32^{IN}$) is encoded at the 3′-end of the *pol* gene. Part b shows the product of the *pol* gene as a long polypeptide precursor consisting of the three viral enzymes (protease (PR), reverse transcriptase (RT) and integrase (IN)). Integrase is generated by cleavage of the Pol polyprotein by HIV protease during maturation and is packaged within new viral particles. HIV-1 integrase consists of three structural and functional domains. The amino-terminal domain (NTD) contains four essential and conserved amino-acid residues (two histidines (H12 and H16) and two cysteines (C40 and C43)) that coordinate one zinc atom. The catalytic core domain (CCD) contains the acidic catalytic triad (DDE motif: D64, D116 and E152) coordinating one or possibly two divalent metals (Mn^{2+} or Mg^{2+}) (FIG. 3; BOX 3). The carboxy-terminal domain (CTD), like the other two domains, forms homodimers and participates in DNA binding.



Integrase inhibitors: 10 years to trials

The therapeutic rationale for developing integrase inhibitors has been clear for many years, even before the recent pharmacological validation of integrase[7]. Indeed, integrase is essential for retroviral replication, and the absence of a host-cell equivalent of integrase means that integrase inhibitors do not interfere with normal cellular processes, and therefore have a high therapeutic index. This claim, however, requires a caveat: the DKAs L-708,906 and 5CITEP as well as blocking integrase also block — albeit at 10–20-fold higher drug concentrations — the activity of the V(D)J RAG1/2 recombinases that generate the normal antibody repertoire[45]. Some DKAs can also inhibit RNase H[46]. This cross-reactivity probably results from the mechanistic and structural similarities between recombinases, RNases and integrases[37], although the structure–activity relationship (SAR) for integrase and other phosphotransferases are clearly distinct[46].

New drugs and novel targets are also needed, because it is well established that anti-HIV drug combinations are much more effective than monotherapies, which is why HAART regimens have become the standard of care for AIDS patients (for details see REF. 5). Combination therapy also reduces the emergence of drug-resistant viruses, as the multiple mutations that are needed to overcome drug resistance decrease viral fitness. Hopefully, integrase inhibitors will become a potential additive to HAART or a salvage therapy for patients resistant to currently available anti-HIV drugs.

The screening and discovery of integrase inhibitors generally relies primarily on simple assays that use recombinant integrase and short oligonucleotide substrates that mimick the viral DNA ends[47–50] (BOX 2). Inhibitors of recombinant integrase can be subdivided according to whether they are antiviral, cytotoxic or inactive in cell culture, and whether they target other viral processes besides integration (FIG. 4,5). Extensive lists of inhibitors can be found in several reviews[51–55].

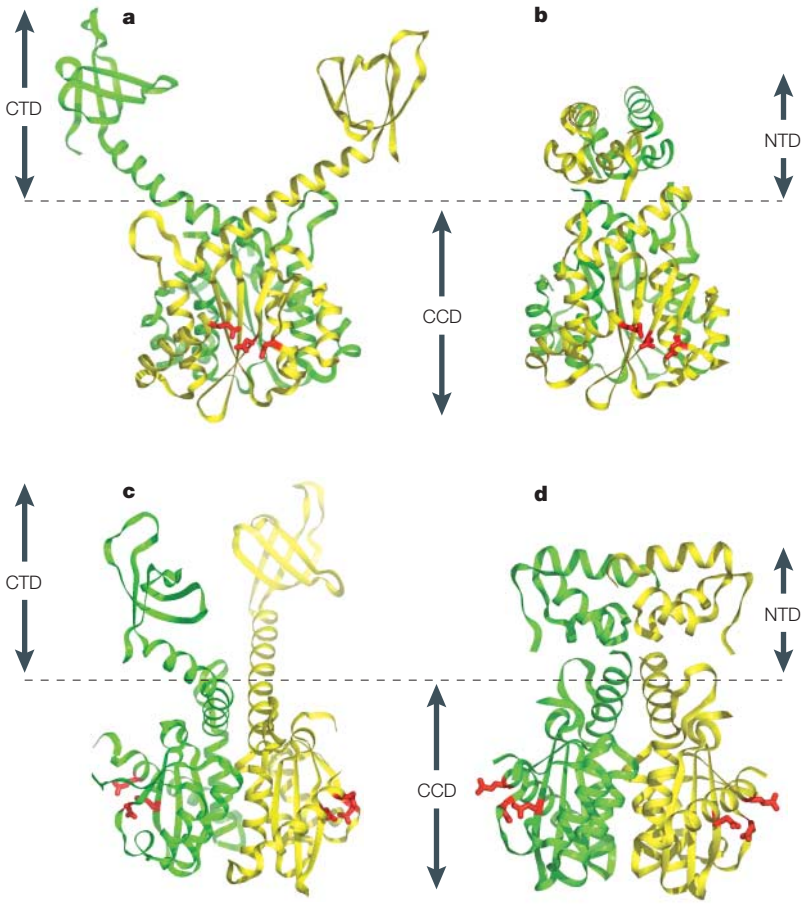


Figure 3 | **HIV-1 integrase dimer crystal structure. a** and **b** | Side views showing the catalytic acidic triad in red (the canonical DDE motif consisting of residues D64, D116, E152; BOX 1, figure part a) in the catalytic core domain (CCD) of integrase. The two subunits of the dimer are shown in yellow and green. **c** and **d** | Front views of the same structures (after 90° anticlockwise rotation of panels **a** and **b**, respectively). **a** and **c** | Structure of the CCD–carboxy-terminal domain (CTD) dimer[33]; (PDB codes: 1EXQ & 1EX4). **b** and **d** | Structure of the amino-terminal domain (NTD)–CCD[34] (PDB code: 1K6Y). Combining the structures (**a** with **b**; **c** with **d**) indicates the positioning of each NTD into the cavity between the CCD and CTD in the full-size integrase dimer[34]. The functional structure of integrase is probably tetrameric, and would therefore involve another dimer interface (unknown, and therefore not represented here).

At least four criteria need to be met to conclude that integrase is the cellular target of an antiviral inhibitor found to be active against recombinant integrase. First, time-of-drug-addition experiments must show drug efficacy consistent with the integration phase — that is, following reverse transcription and before maturation (between 4 and 16 hours following infection)[56–59] (FIG. 1). Second, infected cells treated with the drug must show an accumulation of 2-long terminal repeat (LTR) circles[56,58,60] and decreased HIV integration into host chromosomes[57–60]. The 2-LTR circles result from the accumulation of viral cDNA and its circularization by cellular enzymes. Third, integrase mutations must be found in drug-resistant viruses[56,59–62]. And fourth, the drug should be inactive (or markedly less active) in biochemical assays against recombinant integrases bearing the mutations identified in the drug-resistant viruses[56,60,62].

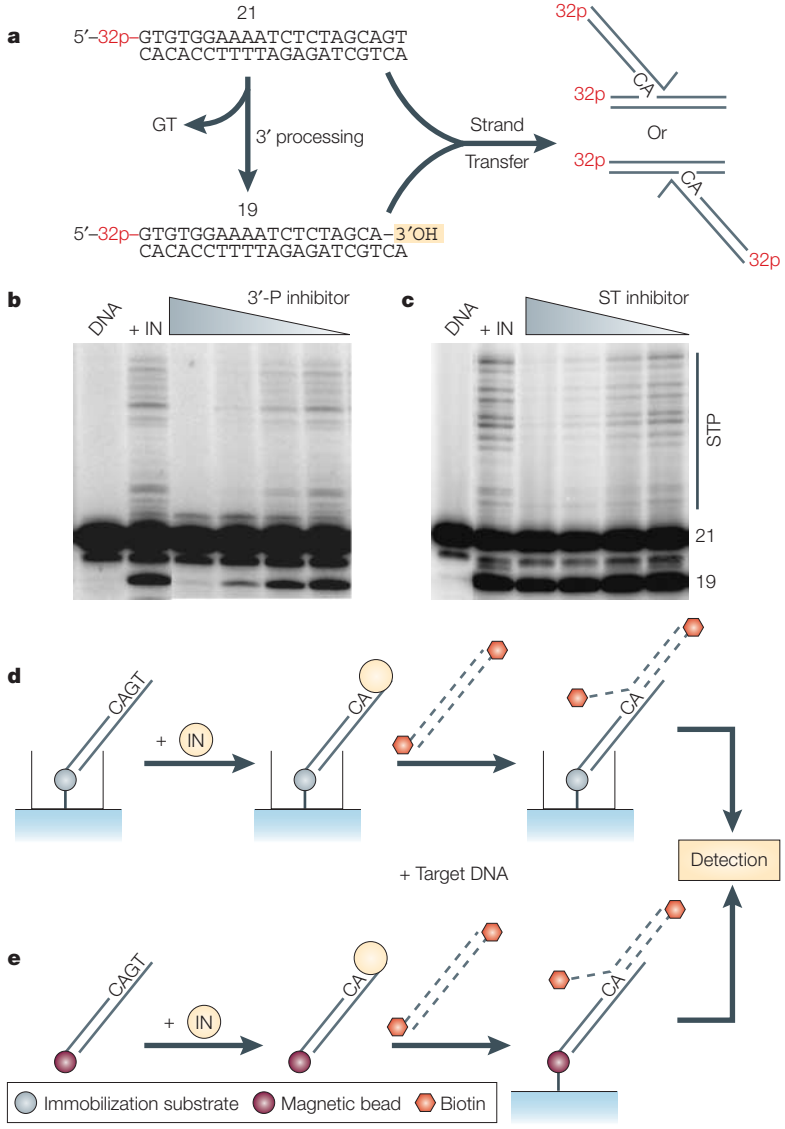
Presently, the DKAs (discussed separately in the next section) meet all four of these criteria[56,58,60,62]. Studies are ongoing to determine whether the antiviral activity of the phenyldipyrimidines (PDPs), such as V-165, and styrylquinolines (SQLs), such as FZ41, (FIG. 4b) is directly linked to integrase inhibition. Phenyldipyrimidines (for example, V-165) are synthetic antiviral compounds active in the nanomolar range against both viral replication and recombinant integrase[57]. In contrast to DKAs, V-165 inhibits 3′-processing at least as effectively as it inhibits strand transfer because it blocks the formation of integrase–DNA complexes[57]. V-165 might therefore need to be present in the infected cells prior to the formation of PICs (FIG. 1). Time-of-addition experiments and data demonstrating inhibition of cellular integration and the accumulation of 2-LTR circles (BOX 2) are indicative of integrase inhibition by V-165 in infected cells. Integrase mutations leading to resistance to V-165 have also recently been identified (M. Witvrouw, personal communication), which indicates that integrase is targeted by V-165 in infected cells. However, it should be noted that V-165 and other PDPs inhibit RT in the same concentration range as integrase[57], indicating that this family of integrase inhibitors might have more than one target.

SQLs (FZ41) (FIG. 4b) are synthetic compounds like PDPs and DKAs. SQLs were designed[63] to chelate the divalent metal (Mg^{2+} or Mn^{2+}) in the integrase CCD. In common with PDPs, SQLs compete for the binding of viral DNA to integrase and inhibit 3′-processing as well as strand transfer[64]. They also block 3′-processing equally well in Mg^{2+} and Mn^{2+}[63]. A recent report showed that SQLs prevent the nuclear import of recombinant integrase[65]. However, it is not clear whether this effect is relevant for the anti-integrase activity of SQLs. Interestingly, the most active antiviral SQLs are catechols[66] matching the pharmacophore[67,68] obtained from the first integrase catechol inhibitors identified 10 years ago[9,69]. SQL-resistant viruses contain integrase mutations (C280Y, V165I and V249I), indicating that integrase is targeted by SQLs in cells. These mutations also reduce viral replicative fitness[59]. Although these resistance mutations do not confer cross-resistance to a DKA[59], it has not been determined whether recombinant integrase containing the mutations is resistant to SQLs. SQLs are on the list of interesting leads for the development of therapeutic integrase inhibitors.

Some integrase inhibitors with antiviral activity target other steps in the HIV life cycle, and therefore cannot be considered integrase-specific inhibitors (FIG. 5a). For instance, guanosine quartet oligonucleotides (AR177 (Zintevir; Aronex)), which are among the most potent inhibitors of recombinant integrase[70], select for gp120 rather than integrase mutations in cell culture[71]. The same is true for L-chicoric acid (LCA) and its derivatives for which the resistance phenotype can be completely rescued by recombination in the *gp120* wild-type gene[72]. Coumarin derivatives inhibit HIV-1 protease in the same concentration range as integrase[73]. These examples underline the importance of thorough cross-studies to validate integrase as the antiviral drug target,

Box 2 | **Examples of biochemical assays for integrase inhibitor screening**

Parts a and b show the short oligonucleotides derived from the U5 long terminal repeat (LTR) DNA ends (BOX 1, figure part a) that are generally used. The choice of divalent metal — Mg^{2+} or Mn^{2+} — is important, because antiviral inhibitors are generally active either in Mg^{2+} or Mn^{2+}, whereas compounds active only in Mn^{2+} are generally cytotoxic and not antiviral[41,97]. In part a, the 21-mer oligodeoxynucleotide is radiolabelled with ^{32}P (in red) at the 5′-terminus. Recombinant integrase can use the same oligonucleotide species as both donor and acceptor. Release of the GT dinucleotide (BOX 1, figure part a and FIG. 2b) at the 3′-end of the radiolabelled strand generates a 19-mer oligonucleotide that can be readily separated from the 21-mer substrate by electrophoresis. The strand-transfer reaction generates a series of products longer than 21 nucleotides (b+c, lane + IN, STP). In parts b and c differential effects of 3′-processing and strand-transfer inhibitors are shown. Note the 3′-processing inhibition in parts b but not in part c. Parts d and e illustrate high-throughput assays, which are generally used for screening integrase inhibitors. The donor DNA (generally derived from the U5 LTR) is immobilized on a micro-titre well plate (d) or bound to a magnetic bead (Dynabead) (e). Integrase 3′-processing is required to activate the donor DNA, which is then able to react with biotin-labelled target DNA. Integration can be detected after isolation of the bound donor DNA. Inhibition is measured as signal extinction. Pre-integration complex (PIC) assays detect the inhibition of integrase within PICs isolated from infected cell extracts. Such assays are generally cumbersome and use biological materials from HIV-infected cells[98]. Nevertheless, a high-throughput assay has been proposed[99].



and the requirement that putative anti-integrase inhibitors meet the four criteria listed above before concluding that integrase is indeed their antiviral target.

For some drugs, the cross-studies, especially the generation of drug-resistant viruses, have not been reported. This is often because the drugs' therapeutic indexes are too low to generate drug-resistant mutants. Examples of antiviral integrase inhibitors with potential specificity and which might represent leads for further studies are presented in FIG. 5b. Many of these inhibitors are polyhydroxylated derivatives. Thiazolothiazepines have a low cytotoxicity and do not inhibit HIV RT or protease, virus attachment or nucleocapsid formation[74]. Caffeic acid phenyl ester (CAPE) is a natural product present in propolis, also called 'bee glue,' which is the resinous substance bees use to construct and maintain their hives. CAPE was one the first integrase inhibitors reported and exhibits weak antiviral activity[9,69]. CAPE was also the first illustrative example of an inhibitor selective for the strand-transfer step, because CAPE inhibits strand transfer at concentrations up to tenfold lower than those required to inhibit 3′-processing[9,69]. Thalassiolin A, an antiviral natural flavone isolated from the Caribbean sea grass *Thalassia testudinum*, inhibits both steps of integration *in vitro* at low micromolar concentration[75]. Long-term passage of cells with thalassiolin A did not lead to resistant viruses[75]. Rolitetracycline was identified by using a pharmacophore derived from the flavones and CAPE to search the National Cancer Institute's three-dimensional chemical database[67]. It has been proposed that catechols, tetracyclines and mercaptosalicylhydrazides inhibit integrase via metal CHELATION in the enzyme active site[9,76]; this type of inhibition mechanism will be discussed further in the next section on DKAs. Lexitropsins are antiviral synthetic polyamides that inhibit integrase binding to the A/T-rich sequence at the tip of the viral LTR DNA[77] (see *att* sites in BOX 1, figure part a). Natural products with a broad spectrum of activity have also been reported to inhibit HIV-1 integrase — examples include granulatine[67] and hypericin[78], both of which are derived from lichen extracts; curcumin[79], one of the main components of turmeric; the ground root of *Curcuma longa* of the ginger family (used for curry powder); and, as recently demonstrated, the antimicrobial cationic peptide indolicidin[80]. It is notable that diketo or/and β-hydroxy-keto functions were identified as key functional motifs for many natural derivatives even before the discovery of the DKAs and DKA-like derivatives in which these functionalities play such a crucial role (for example, dihydroxynaphthoquinones[9], curcumin[79], granulatine[81], hypericin[78] and thalassiolin A[75]).

DKA derivatives: synthetic interfacial inhibitors

DKAs and their derivatives are all synthetic compounds discovered both by Shionogi & Co. Ltd[82] and the Merck Research Laboratories[56]. Shionogi's DKA (5CITEP) (FIG. 4a) was a breakthrough because it was co-crystallized with the CCD of integrase in close association with the catalytic DDE triad[82]. Before the discovery of 5CITEP, the only other reported co-crystal was a bisulphonate

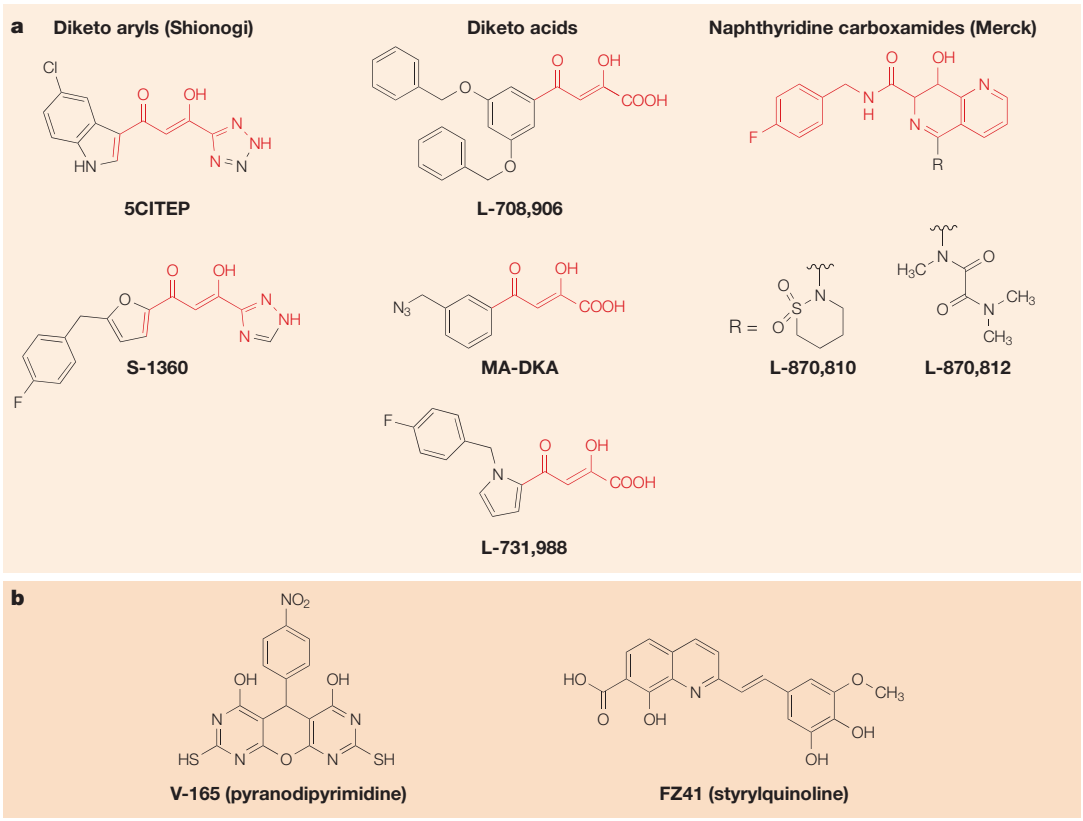


Figure 4 | **Chemical structures of antiviral integrase inhibitors. a** | Diketo aryls (DKAs) and DKA-like strand-transfer-selective inhibitors. 5CITEP is included here for discussion of the phylogeny of DKA, but is not antiviral. **b** | Potential integrase-specific inhibitors. These compounds are not selective for strand transfer. They induce drug-resistant integrase mutations in HIV infected cells, but can also inhibit other viral targets. The mutations leading to drug resistance have not been confirmed in recombinant integrase.

dimer bound to an ASV integrase dimer interface[83]. The first report of DKA derivatives from Merck[56,84] appeared at approximately the same time as the crystal structure of 5CITEP was published[82], and it was immediately clear that both the Merck and Shionogi compounds could be functionally classified together as DKAs. The Merck DKAs were identified via random screening of a 250,000-compound library[56]. As with 5CITEP, they also represented a breakthrough because of their potent antiviral activity (in the nanomolar range) and well-characterized selective targeting of integrase in HIV-infected cells[56].

Further synthetic and optimization efforts by the Merck group led to the recently disclosed 8-hydroxy-(1,6)-naphthyridine-7-carboxamide derivatives (FIG. 4a), which can still be considered DKA-like derivatives because they contain the β-hydroxy-ketone structural motif (in red in FIG. 4) and because of their common mechanism of inhibiting integrase (that is, selective inhibition of strand transfer)[7,60,85]. L-870,812 is a promising lead for therapeutic development because of its selective anti-integrase and antiviral potency, and because of its favourable pharmacokinetic profile (oral bioavailability >60% and half-life ~5 hours in rhesus macaques). The antiviral activity of L-870,812 was recently demonstrated to suppress viraemia as well as chronic infections in rhesus macaques[7] infected with simian immunodeficiency virus. A closely related derivative, L-870,810 (FIG. 4a), has entered clinical trials, as has the Shionogi derivative S-1360 (FIG. 4a)[86].

Selective inhibition of the integrase strand-transfer step at nanomolar concentrations is a trademark of DKAs[56,84,87]; although CAPE was the first strand-transfer inhibitor to be reported[9,69]. However, the benefits of such selective inhibition in treating HIV-1 infection only emerged with the characterization of the more potent DKAs. Therefore, the DKAs were the first integrase inhibitors to provide proof of the concept that selective inhibition of strand transfer can effectively decrease HIV-1 infection[7,56].

A divalent metal cofactor is required for the binding of DKA to integrase[40], and integrase inhibition can be divalent-cation dependent[40,41]. For instance, the IC_{50} of L-708,906 for 3′-processing is 2.5 μM in Mg^{2+} and 22 μM in Mn^{2+}, whereas its IC_{50} for strand transfer is comparable with either metal (~0.06 μM)[41]. The selectivity for strand transfer can also be divalent metal-dependent. Both L-708,906 and 5CITEP have roughly tenfold higher inhibition in the presence of Mn^{2+} compared with Mg^{2+} [41]. Furthermore, 5CITEP is a much better inhibitor in Mn^{2+} than in Mg^{2+} (one to two orders of magnitude more potent)[40,41]. In spite of their strand-transfer selectivity, DKAs can also inhibit 3′-processing, albeit at 30–70-fold

CHELATION
Coordination of a metal cofactor. In the case of integrase, strand-transfer inhibitors have been proposed to chelate at least one Mg^{2+} or Mn^{2+} atom (and probably two) in the DDE motif. The metal serves normally as a 'coordination bridge' between the integrase DDE motif, the viral donor cDNA and the chromosomal acceptor DNA.

higher concentrations[41,56]. Inhibition of 3′-processing by DKAs has not been observed in treated cells.

SAR studies revealed the contributions made by the acidic and aromatic portions of DKA to the inhibition of integrase. The relatively poor activity of 5CITEP in Mg^{2+} is due to the tetrazole function; this is demonstrated by the fact that replacement of the tetrazole by a carboxylate markedly increases the inhibitory activity of the hybrid compound in Mg^{2+} [40,41]. The carboxylate, therefore, might be important for metal chelation[40]. The carboxylate portion is, however, not required for binding to the integrase complex[40]. SAR studies reveal that the aromatic portion is crucial for potency[88] and for strand-transfer selectivity[41,87]. The aromatic moiety can accommodate a

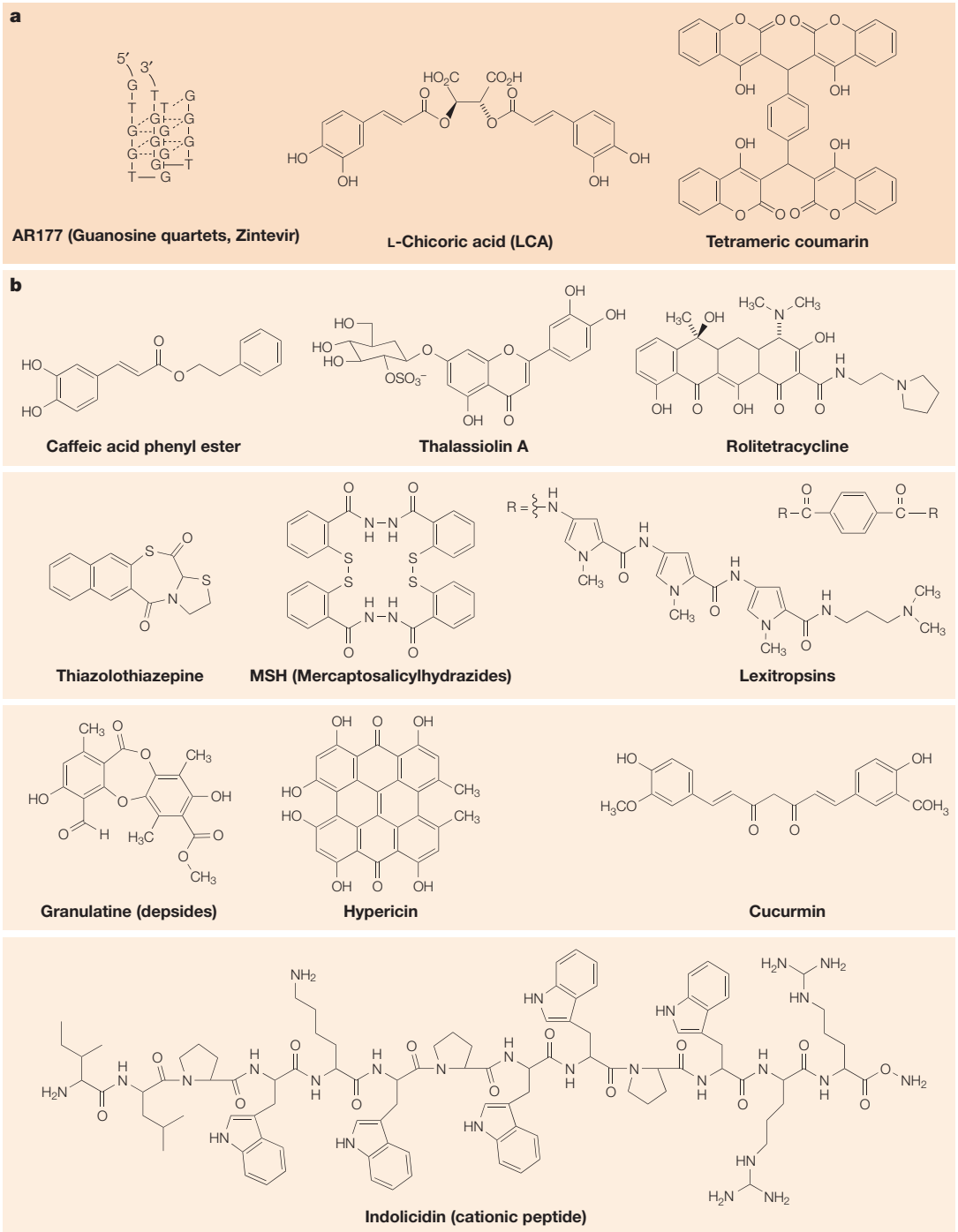


Figure 5 | **Chemical structures of antiviral integrase inhibitors. a** | Compounds inhibiting other identified viral targets. These compounds are antiviral and inhibit integrase *in vitro*, yet inhibit other viral targets in HIV infected cells. **b** | Compounds with no other identified targets. These compounds are antiviral and inhibit integrase *in vitro*, but antiviral targets have not been confirmed in HIV infected cells. The compounds in **a** and **b** do not meet all the criteria to be true integrase inhibitors.

wide range of substituents, including azido groups[89] (MA-DKA, FIG. 4a) or biphenyl ketone photo-activable groups, which were designed for drug-crosslinking studies[90]. The azido portion of the azido-containing DKAs contributes to reduced cytotoxicity[58] and could have a direct role in metal chelation[41]. The functional diketo or β-hydroxy-keto groups are known to have metal-chelating functions, and metal-dependent inhibition by DKAs and DKA-like compounds has been interpreted as indicating a direct interaction of these drugs with the divalent metal in the enzyme catalytic site[40,41]. Metal coordination could also be important for shaping the catalytic pocket of integrase and therefore the DKA-binding site[40,41,84,87].

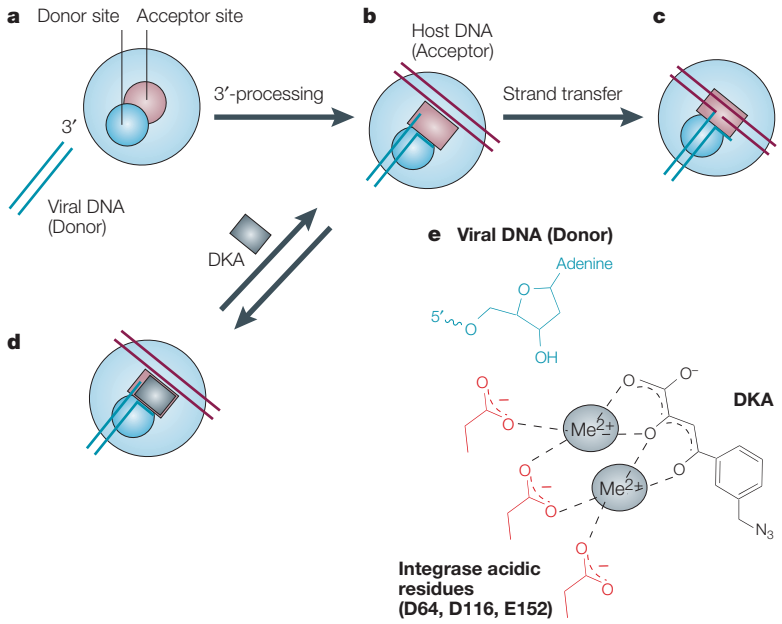
The molecular binding of DKA to integrase complexes has been a focus of research because of the importance of DKAs and DKA-like derivatives as antiviral lead compounds and their unique mechanism of action. The high selectivity of DKAs for the strand-transfer step led to a model in which the two catalytic sites are organized around the three catalytic DDE residues and the divalent metal(s) within the integrase–DNA complexes (BOX 3)[87]. In this model, the DONOR DNA site binds the donor (viral) DNA end and catalyses 3′-processing. Consequently, integrase undergoes a structural change that allows the binding of the acceptor (chromosomal) DNA in the acceptor site for strand transfer[84,87]. DKAs, it is suggested, would bind selectively to a unique conformation of the acceptor site following binding of the viral DNA and 3′-processing, which would then produce the required change in conformation in the acceptor site for accommodating the DKA ligands. According to this scheme, divalent metal coordination would be crucial for DKA binding to the acceptor site (BOX 3, figure part e). This model is supported by scintillation proximity assays with radiolabelled compounds[40,84] that demonstrated the binding of DKA to an intermediate of the integrase PIC in the presence of Mg^{2+} or Mn^{2+}. DKA binding was shown to require functional integrase, as mutant and catalytically inactive enzymes failed to support DKA binding[40]. Drug binding also requires viral DNA ends, as nonspecific DNA fails to support binding[40,84]. Finally, DKAs fail to bind in the absence of Mg^{2+} or Mn^{2+} [40] and compete with the strand-transfer target DNA[84].

The structural features of the DKA-binding site can also be inferred from mapping the amino-acid residues associated with DKA-resistance in the crystal structure of integrase (FIG. 6). Integrase mutants selected by DKA bear mutations in the CCD[56,60,62,86] (and one in the CTD[62]). The amino-acid residues within the CCD that confer resistance are clustered around the catalytic site defined by the DDE residues (FIG. 6a,c). This cluster overlaps with the cluster of residues involved in DNA binding (FIG. 6b,d). One residue (Q148) is common to both clusters and seems to be involved in the correct positioning of the viral DNA before strand transfer onto the acceptor DNA[91]. The clustering of the integrase residues involved both in drug resistance (and therefore probably in DKA binding) and in DNA contacts around the catalytic triad that coordinate the divalent metal(s) highlights the likelihood that DKAs bind at the interface of the viral (donor) DNA–integrase–metal ternary complex. DKA binding would take place specifically at the step of the catalytic cycle of integrase action that immediately precedes the complex switching from 3′-processing to

Box 3 | Interfacial inhibition: proposed mechanism of action of DKAs

One of nature's strategies for interfering with molecular interactions is to trap macromolecules undergoing transition states with their partners in dead-end complexes that are unable to complete their biological function. This type of inhibition, which we refer to as 'interfacial inhibition'[92], has been recently illustrated for two natural inhibitors, brefeldin A and camptothecin, whose modes of action have been fully elucidated by structural studies. Interfacial inhibition occurs at the protein–protein interface in the case of brefeldin A and at the protein–DNA interface in the case of camptothecin. In both systems, the drug takes advantage of transient structural and energetic conditions created by the macromolecular complex, which gives rise to 'hot spots' for drug binding. In addition to these examples, various natural compounds, such as forskolin, tubulin inhibitors and translation inhibitors, have been shown to target protein interfaces (for more details see REF. 92). In the model below, diketo aryls (DKAs) inhibit integrase at its interface with viral DNA and divalent metal.

The figure shows how DKAs block strand transfer selectively by binding at the interface of the integrase–DNA complexes. Part a shows that integrase has two proposed binding sites: the donor site for viral DNA (blue circle) and the acceptor site for host DNA (red circle). Part b shows the events following 3′-processing: the integrase–DNA complex undergoes a structural change that renders the acceptor-site competent (red rectangle) for binding host (chromosomal) DNA. In part c, and under normal conditions, binding of the host (acceptor) DNA to the acceptor site leads to strand transfer. In part d, the DKA inhibitor (grey rectangle) can only bind to the acceptor site after 3′-processing. Part e shows details of the hypothetical binding of DKAs (here MA–DKA; see FIG. 4a) at the interface of the integrase–DNA–divalent metal complex. The processed viral 3′-DNA end (in blue) is bound to integrase (the three acidic catalytic residues (DDE) are shown in red), ready to attack a host DNA phosphodiester bond (see integrase reactions in FIG. 2). It has been proposed that DKAs chelate the metal in the integrase catalytic site and stabilize the macromolecular integrase–DNA complex at the 3′-processing step of the reaction[40,41]. DKAs could therefore belong to the emerging group of interfacial inhibitors, a class of drugs with a unique new mode of action.



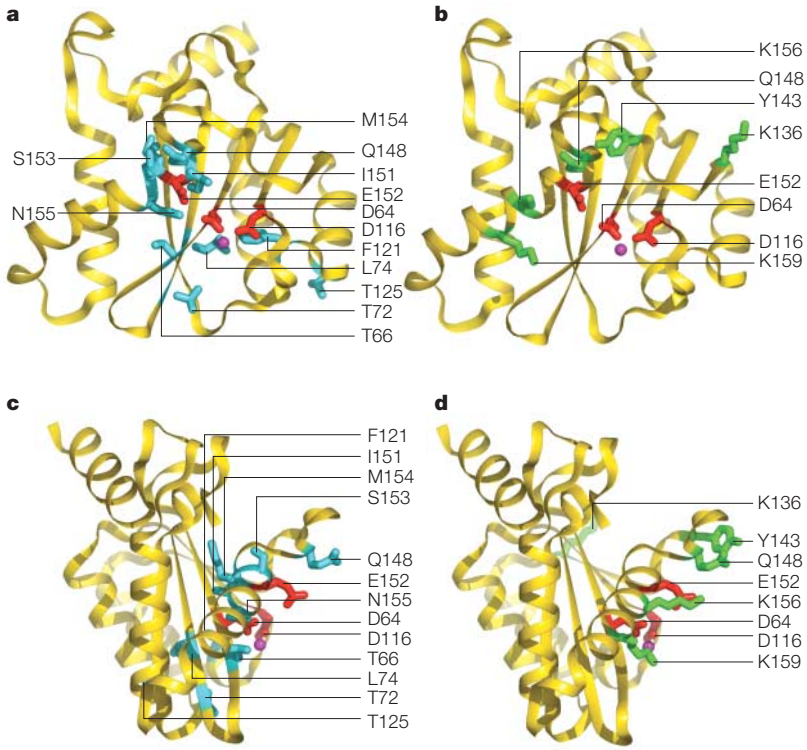


Figure 6 | **HIV-1 integrase mutations leading to resistance to DKA and naphthyridine carboxamide (DKA-like) inhibitors cluster at the interface between the integrase and DNA.** A monomer of the HIV-1 integrase catalytic core domain (CCD)[82] (PDB code: 1QS4) is shown both from the side (**a** and **b**) and from the front (**c** and **d**), with the catalytic acidic residues coloured in red (D64, D116 & E152). The orientations are similar to those in FIG. 3. The metal Mg^{2+} is shown as a magenta sphere. The amino-acid residues associated with drug resistance are coloured in blue in **a** and **c**. They cluster around the three catalytic residues (in red) and overlap with the amino-acid residues implicated in DNA binding (in green in panels **b** and **d**). DKA, diketo aryl.

strand transfer (that is, when the active PIC is assembled) (BOX 3, step b leading to steps d and e). Such binding would stabilize (trap) the 3′-processing intermediate and prevent strand transfer. We recently proposed that this mode of inhibition is a common mechanism for natural compounds that interfere with macromolecular protein–DNA complexes (such as camptothecin–topoisomerase I cleavage complex, dexrazoxane and anthracylines for the topoisomerase II–DNA complexes, or antibiotic–ribosome complexes) or protein–protein complexes (such as brefeldine A and the Arf–GEP complex or the colchicine–α/β-tubulin complex)[92] (BOX 3). We therefore propose that DKAs should be classified as INTERFACIAL INHIBITORS of macromolecular complexes.

DONOR DNA
The viral cDNA containing 3′-hydroxyl ends that act as nucleophilic donors during the strand-transfer reaction.

INTERFACIAL INHIBITOR
Interfacial inhibitors bind at the interface of two or more macromolecules (protein–protein or protein– nucleic acid). The drug takes advantage of transient structural and energetic conditions created by conformational changes in the macromolecular complex that give rise to 'hot spots' for drug binding.

Conclusions and perspectives

Remarkable progress has been made since integrase was recognized as a rational therapeutic target for the treatment of HIV infection. Recombinant integrase can be readily produced and used for high-throughput and molecular pharmacology assays. Several atomic structures of the integrase domains are available for docking studies. Two co-crystal structures of different inhibitors with integrase have been published (1A5V[83] and 1QS4[82]), and two compounds have recently been introduced in clinical trials (S-1360 and L-870,810). The mechanism of inhibition by the specific strand-transfer inhibitors (DKAs and naphthyridine carboxamide derivatives) fits the model of interfacial inhibitors of protein–nucleic acid interactions, as these drugs block a transition state of the integrase–DNA complex. Counterscreens with related enzymes, such as HIV RNase H, might be useful to assess the selectivity of integrase inhibitors, and could lead to the discovery of RNase H inhibitors that are therapeutically active against HIV and AIDS. A different therapeutic application of retroviral integrases is gene therapy. Fusing retroviral integrase to a sequence-specific DNA-binding protein is an attractive approach for delivering exogenous genes into specific chromosomal sites[93,94].

On the basis of sequence conservation of the viral cDNA *att* sites, it might be possible to design DNA-sequence-specific binding molecules that interfere selectively with integrase binding and activity at the *att* sites. Sequence-specific polyamines might represent one such approach[95]. In addition, it might be possible to develop inhibitors of integrase multimerization or inhibitors that affect the interactions between integrase and other proteins present in the PICs or that are required for integration. However, these approaches will require the development of protein-interaction assays that integrate the interfacial inhibition model[92].

The discovery of novel inhibitors and the optimization of lead compounds remains hampered by the lack of atomic structures that reveal the atomic interactions between integrase and its DNA substrates (viral and target DNA) or the atomic structure of the complete enzyme with its three domains. Interfacial inhibitors stabilizing 3′-processing might be useful for determining the atomic structure of an intermediate of integrase bound to its DNA substrate(s), and lead to the development of more potent and more selective inhibitors. Finally, the results of clinical trials with the strand-transfer inhibitors are eagerly awaited, and will affect the future development of integrase inhibitors for the prophylaxis and treatment of AIDS.

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Competing interests statement

The authors declare competing financial interests: see Web version for details.

Online links

DATABASES

The following terms in this article are linked online to:

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Vpr
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Biogs

Yves Pommier received his M.D. and Ph.D. degrees from the University of Paris, France, and has been at the National Institutes of Health (NIH) since 1981. Pommier is a member of the Molecular Target steering committee at the National Cancer Institute (NCI). He received an NIH Merit Award for his role in elucidating the function of topoisomerase enzymes as targets for anticancer drugs and Federal Technology Transfer Awards for studies on HIV-1 integrase and DNA topoisomerase inhibitors. Pommier is a programme committee member of the American Association for Cancer Research, Senior Editor of *Cancer Research* and Associate Editor of *Cancer Research*, *Molecular Pharmacology*, *Leukemia*, *The Journal of Experimental Therapeutics and Oncology*, *The International Journal of Oncology*, *Drug Resistance Updates* and *Current Medicinal Chemistry*. Pommier serves as Chair for 2004–2005 Gordon conferences on the Molecular Therapeutics of Cancer. Pommier holds several patents on inhibitors of DNA topoisomerases I and II and HIV-1 integrase inhibitors.

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Online Summary

- HIV-1 integrase is a rational target for anti-HIV therapy, and the feasibility and efficacy of integrase inhibitors in animal models has been recently demonstrated.
- Integrase catalyses the insertion of the viral cDNA ends generated by reverse transcription of the viral RNA genome into host chromosomes. The integration reaction consists of two consecutive steps: 3′-processing and strand transfer.
- Several structures of retroviral integrases have been solved. Integrase is structurally similar to other DNA-processing polynucleotide transferases, including the Tn5 and mu transposases, RuvC recombinase, RnaseH and the RNase component Argonaute. All of these contain a conserved DDE motif required for enzymatic activity. Divalent metals (almost certainly at least one, and probably two, Mg^{2+} or Mn^{2+} atoms) coordinate the integrase DDE motif, the viral cDNA and chromosomal DNA for the integration reactions.
- Integrase can be used for high-throughput screening and a variety of inhibitors from diverse chemical classes have been identified. Criteria required to demonstrate targeting of cellular integrase are reviewed.
- Diketo acids and diketo-like acids are the most promising integrase inhibitors. They are referred to as strand-transfer inhibitors because they uncouple the two integrase reactions. They can block strand transfer without affecting 3′-processing by chelating divalent cofactors in the integrase active site and by interfering with host (acceptor) DNA binding.
- Strand-transfer inhibitors probably bind at the interface of the integrase–metal cofactor–viral DNA ternary complex by chelating the divalent metal, and thereby interfering with the binding of the chromosomal target DNA. Strand-transfer inhibitors are candidate interfacial inhibitors, and represent a new mechanism of action in drug discovery.

Competing interests statement

Y.P. and C.M. are inventors on a number of pending and granted patents relating to HIV-1 integrase inhibitors.