Antiviral Research 85 (2010) 101–118

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Review

Strand transfer inhibitors of HIV-1 integrase: Bringing IN a new era of antiretroviral therapy

Damian J. McColl*, Xiaowu Chen

Gilead Sciences, Inc., 333 Lakeside Drive, Foster City, CA 94404, United States

ARTICLE INFO

Article history:
Received 14 October 2009
Received in revised form 5 November 2009
Accepted 10 November 2009

Keywords:
HIV-1
Integrase (IN)
Integrase strand transfer inhibitor (INSTI)
Raltegravir (RAL)
Elvitegravir (EVG)
Drug resistance

ABSTRACT

HIV-1 integrase (IN) is one of three essential enzymes (along with reverse transcriptase and protease) encoded by the viral pol gene. IN mediates two critical reactions during viral replication; firstly 3′-end processing (3′EP) of the double-stranded viral DNA ends and then strand transfer (STF) which joins the viral DNA to the host chromosomal DNA forming a functional integrated proviral DNA. IN is a 288 amino acid protein containing three functional domains, the N-terminal domain (NTD), catalytic core domain (CCD) and the C-terminal domain (CTD). The CCD contains three conserved catalytic residues, Asp64, Asp116 and Glu152, which coordinate divalent metal ions essential for the STF reaction. Intensive research over the last two decades has led to the discovery and development of small molecule inhibitors of the IN STF reaction (INSTIs). INSTIs are catalytic inhibitors of IN, and act to chelate the divalent metal ions in the CCD. One INSTI, raltegravir (RAL, Merck Inc.) was approved in late 2007 for the treatment of HIV-1 infection in patients with prior antiretroviral (ARV) treatment experience and was recently approved also for first line therapy. A second INSTI, elvitegravir (EVG, Gilead Sciences, Inc.) is currently undergoing phase 3 studies in ARV treatment-experienced patients and phase 2 studies in ARV naïve patients as part of a novel fixed dose combination. Several additional INSTIs are in early stage clinical development. This review will discuss the discovery and development of this novel class of antiretrovirals. This article forms part of a special issue of Antiviral Research marking the 25th anniversary of antiretroviral drug discovery and development, Vol 85, issue 1, 2010.

Contents

1. Introduction

Human immunodeficiency virus type 1 (HIV-1) and acquired immune deficiency disease (AIDS) have cost the lives of millions of people worldwide since the epidemic began. Currently

* Corresponding author. Tel.: +1 650 522 5821; fax: +1 650 522 5890.
E-mail address: damian.mccoll@gilead.com (D.J. McColl).

0166-3542/$ – see front matter © 2009 Published by Elsevier B.V.
doi:10.1016/j.antiviral.2009.11.004

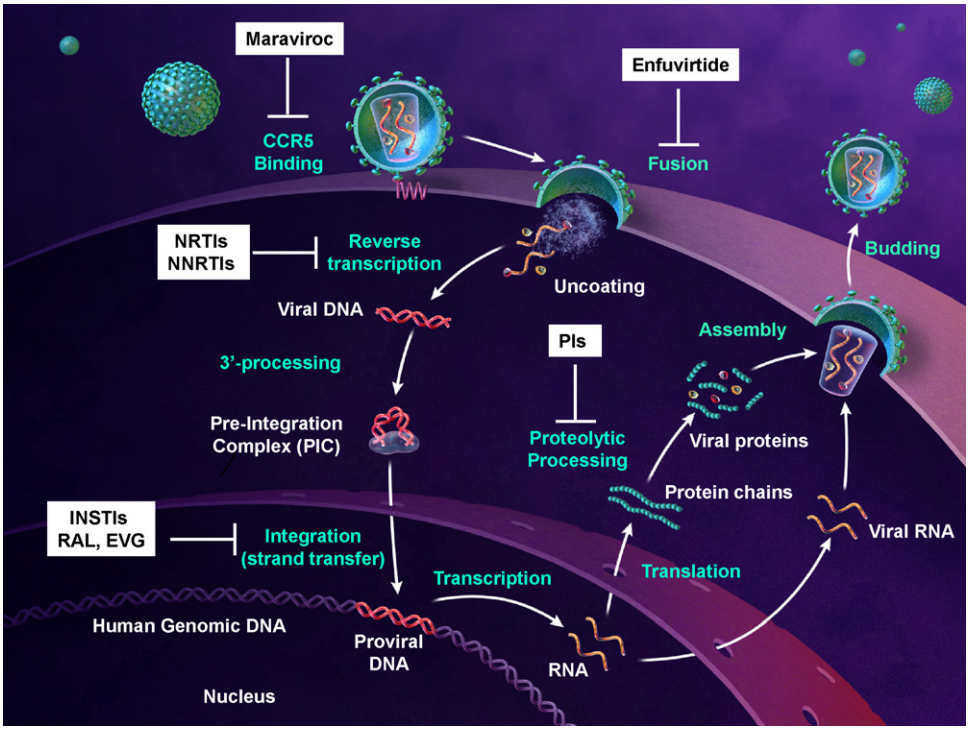

Fig. 1. HIV replication cycle.
Initial entry of HIV into a target cell can be blocked by use of the entry inhibitor maraviroc, which prevents viral interaction with the CCR5 coreceptor. Fusion of the viral membrane with the target cell membrane can be blocked by the peptidic inhibitor enfuvirtide, which prevents a conformational change in the viral Env protein needed to bring the two membranes into close proximity. Reverse transcription of the viral RNA into DNA can be blocked by nucleoside/tide reverse transcriptase inhibitors (NRTIs) which are incorporated into the viral DNA and act to chain terminate DNA synthesis. Non-nucleoside reverse transcriptase inhibitors (NNRTIs) are non-competitive inhibitors of reverse transcriptase. Integrase strand transfer inhibitors (INSTIs), such as raltegravir and elvitegravir, are active site inhibitors of the viral integrase enzyme and prevent the strand transfer reaction, the final ligation of the 3′-processed viral DNA into the host genome. Protease inhibitors (PIs) prevent the proteolytic processing of translated viral proteins by the viral protease enzyme, resulting in defective virions. Combinations of drugs from two or more of these classes when combined together form the basis of highly active antiretroviral therapy (HAART).

the World Health Organization estimates that some 33 million people are infected with HIV-1, constituting a global pandemic illness with significant social and economic impact (http://www.who.int/hiv/data/en). In response to this medical crisis, the scientific and medical communities have discovered and developed antiretroviral (ARV) drugs that directly target viral enzymes or processes essential for viral replication. These ARV drugs, most of which are small molecule inhibitors, include nucleoside/tide reverse transcriptase inhibitors (NRTIs, 8 approved drugs) (Cihlar and Ray, 2010), non-nucleoside reverse transcriptase inhibitors (NNRTIs, 4 approved drugs) (de Bethune, 2010), protease inhibitors (PIs, 9 approved drugs) (Wensing et al., 2010), and most recently the entry inhibitor (Tilton and Doms, 2010) maraviroc, and the integrase inhibitor, raltegravir. Additionally, a peptidic inhibitor, enfuvirtide, targets viral fusion. Combinations of ARV drugs used as highly active antiretroviral therapy (HAART) have become the standard of care for HIV therapy and have produced significant declines in the morbidity and mortality associated with HIV-1 infection, especially in those countries where access to ARV medications is widely available (Bartlett et al., 2007). Fixed dose combination tablets containing two or more drugs, such as Atripla® (emtricitabine/tenofovir DF/efavirenz), which represents a complete ARV therapy in one pill taken once daily (QD), have become the standard of care for ARV-naïve subjects (Arribas et al., 2008). Second generation drugs in multiple classes such as tenofovir (NRTI), etravirine (NNRTI) and darunavir (PI) have also provided additional benefit to subjects with prior ARV treatment experience and evidence of antiretroviral drug resistance (Schooley et al., 2002; Madruga et al., 2007; Lazzarin et al., 2007).

Among the three enzymes encoded by HIV-1, protease (PR) reverse transcriptase (RT), and integrase (IN), the latter was until recently an "orphan" in terms of approved ARV drugs. This changed in late 2007 with the FDA approval of raltegravir (RAL, MK-0518, Isentress®, Merck Inc.), a strand transfer inhibitor of IN (INSTI) dosed 400 mg twice-daily (BID) for the treatment of HIV-1 infection in subjects with prior ARV treatment experience. RAL was also approved by the FDA for use in first-line ARV therapy in June 2009 and is undergoing further phase 3 studies in ARV treatment-naïve subjects including investigation of once daily dosing. A second INSTI, elvitegravir (EVG, GS-9137, JTK-303, discovered by Japan Tobacco and developed by Gilead Sciences, Inc.) is undergoing phase 3 studies in ARV treatment-experienced subjects. Phase 2 studies in ARV treatment-naïve subjects are also investigating a fixed dose combination tablet containing EVG combined with emtricitabine/tenofovir DF and a novel pharmacokinetic enhancer, GS-9350, which boosts EVG systemic exposure to once-daily dosing. S/GSK-1349572 is another INSTI, which recently entered phase 2 testing, suggesting that additional drugs may become available in this new class. This review will focus on the discovery and development of HIV-1 IN strand transfer inhibitors, clinical trials of RAL and EVG and an overview of drug resistance to INSTIs, as it is currently understood.

2. Biology of HIV-1 integration

2.1. Structure and function of HIV-1 integrase

HIV-1 IN is an essential viral enzyme that binds to the double-stranded viral DNA generated by reverse transcription and

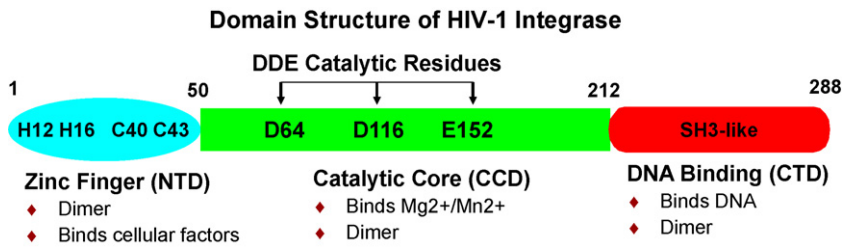

Fig. 2. Structural domains of HIV integrase.
Schematic of the domain structure of HIV integrase. Three structural and functional domains have been identified. The N-terminal domain (residues 1–50, NTD) contains a HH-CC zinc finger motif and is required for dimerization and binding of cellular factors. The catalytic core domain (residues 51–212, CCD) contains the conserved residues forming a catalytic triad (Asp64, Asp116, and Glu152) that are required to coordinate essential divalent metal ions (Mn^{2+} or Mg^{2+}). The C-terminal domain (residues 213–288, CTD) shares homology with the SH3 DNA-binding domains and binds DNA non-specifically.

mediates its integration into the cellular genomic DNA of the infected host to produce a functional provirus. Integration of the HIV-1 viral DNA into the host genomic DNA usually involves insertion into active transcription units. Once this occurs, HIV-1 transcription of new viral RNAs and production of new viral proteins can commence. Integration is the final step before irreversible and productive HIV-1 infection of a target cell is achieved (Fig. 1) (Pommier et al., 2005).

IN is encoded by the HIV-1 *pol* gene, immediately 3′ of the RT/RNAseH coding sequence. The 32 kDa IN enzyme contains 288 amino acids, divided into three structural and functional domains (Fig. 2). The functional IN enzyme is generated by proteolytic cleavage of the Gag-Pol precursor polypeptide, mediated by the viral PR enzyme, as occurs for other viral enzymes and proteins (Bushman et al., 1993, 1990; Goff, 1992; Vink and Plasterek, 1993). The three functional domains of IN include the N-terminal domain (NTD), the catalytic core domain (CCD) and the C-terminal domain (CTD) (Fig. 3) (Ellison et al., 1995; Engelman et al., 1993; Goldgur et al., 1999). Each IN monomer associates with another IN monomer to form an IN homo-dimer and these have been proposed to further associate into functional tetrameric or higher order IN complexes (Figs. 3 and 4) (Ellison et al., 1995; Engelman et al., 1993). It is estimated that approximately 50–100 copies of the IN enzyme are packaged into each newly formed virion.

Each of the three IN domains contains recognizable functional motifs (Fig. 2). The N-terminal domain (residues 1–50) contains two histidine residues (His 12 and His16) and two cysteine residues (Cys40 and Cys43), all of which are absolutely conserved and form a HH-CC zinc-finger motif that chelates one zinc atom per IN monomer (Burke et al., 1992; Cai et al., 1997). The NTD is required for higher order multimer formation, a process, which requires zinc (Zheng et al., 1996). The zinc atom acts to stabilize the fold of the NTD and is necessary for the activity of IN. The catalytic core domain (residues 51–212) contains three absolutely conserved negatively-

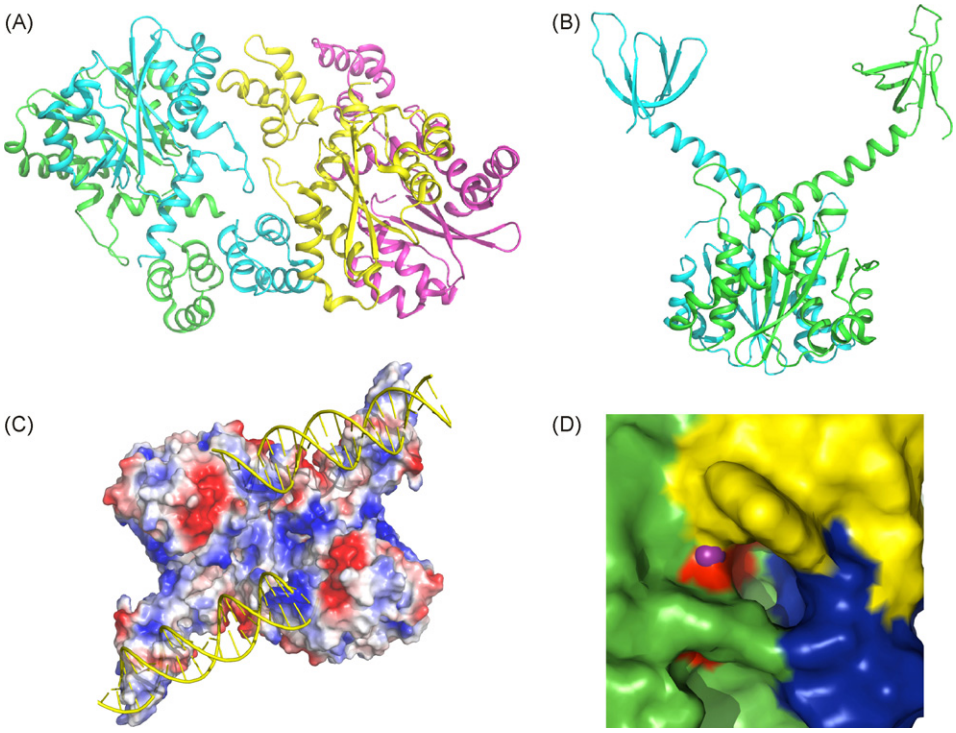

Fig. 3. Crystal structure of HIV integrase (A and B) and the DNA binding domain.
(A) Crystal structure of the NTD and CCD domains of integrase showing a model of an IN tetramer composed of a dimer of integrase dimers. Each individual integrase monomer (composed of a NTD and CCD) is shown in a distinct color. (B) Crystal structure of an integrase dimer composed of the CCD and CTD domains. (C) Hypothetical space filling model of an integrase tetramer in which each monomer contains all three domains of integrase (courtesy of X. Chen). Electrostatic surface potential is also shown (positive in blue; negative in red). A ribbon model of the viral DNA bound to the tetrameric integrase complex (in trans binding mode) is also shown. The viral DNA ends are coordinated together in close proximity. Host chromosomal DNA (not shown) most likely lies in the central groove (D) Close up view a model of an integrase active site showing how an induced hydrophobic pocket is formed upon viral DNA binding (taken from the work of Chen et al., 2008).

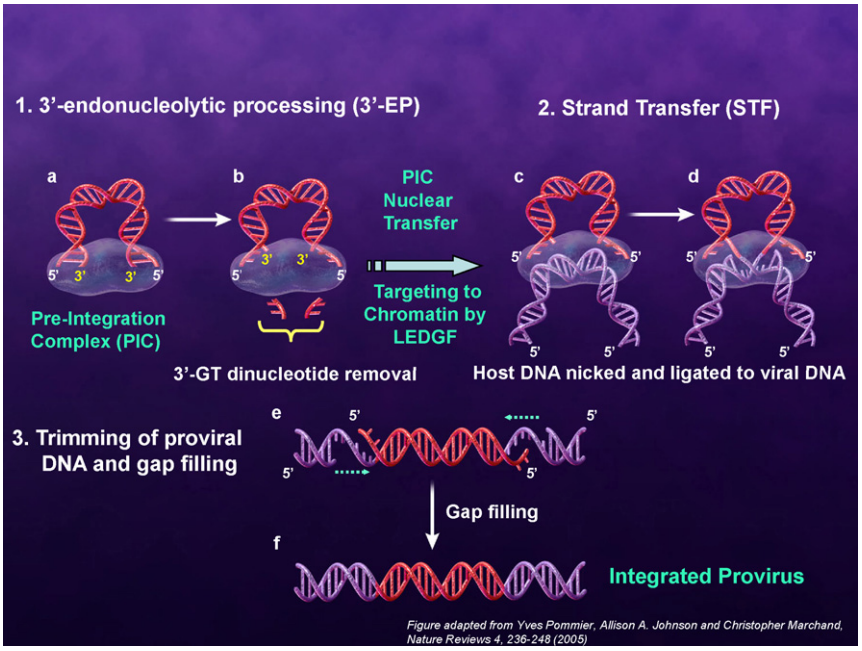

Fig. 4. Mechanism of proviral DNA integration.
The integration step of the HIV lifecycle requires two steps mediated by the integrase enzyme, 3′-end processing (3′-EP) and strand transfer reaction. The 3′-EP reaction occurs in the cytoplasm following completion of viral DNA synthesis by reverse transcriptase. The 3′-EP reaction is an endonucleolytic cleavage of the viral DNA and occurs immediately 3′ of a conserved CA dinucleotide motif. This produces a reactive 3′ hydroxyl at each end of the viral DNA. Integrase remains bound to the ends of the viral DNA which remain in close proximity to one another. This complex of viral DNA, integrase multimers and associated cellular factors form the preintegration complex (PIC). The PIC is transported across the nuclear membrane and is then targeted to chromatinized host genomic DNA via LEDGF. The second reaction catalyzed by integrase, the STF reaction then takes place. STF is 3′-end joining. Each of the 3′hydroxyl ends of the viral DNA are coordinated to attack the phosphodiester bond on the host chromosomal DNA and then ligated to the ends of the nicked chromosomal DNA. The 3′ ends of the viral DNA are positioned such that they attack the host chromosomal DNA across a span of 5 base pair along the major groove. The STF reaction results in a 5 base pair, single stranded gap at the join between the viral and chromosomal DNA and a 2 base pair "flap" at the end of the 5′end of the viral DNA. Cellular repair enzymes then fill the gap, resulting in production of the mature integrated provirus from which viral transcription can be initiated.

charged amino acids (residues Asp64, Asp116 and Glu152, also known as the DDE motif) which coordinate divalent metal ions (Mg^{2+} or Mn^{2+}). These divalent metal ions are essential for the catalysis of integration (Engelman and Craigie, 1992). Substitution of any of the residues in the DDE motif dramatically inhibits the activity of IN (Drelich et al., 1992; Kulkosky et al., 1992; van Gent et al., 1993). The CCD, and in particular the DDE motif, is conserved in all retroviral IN enzymes. The crystal structure of the CCD shows that it consists of 5 β-sheets and six α-helices that are linked by flexible loops (Chen et al., 2000; Goldgur et al., 1999; Lubkowski et al., 1998). The structural fold of the CCD is homologous to the fold of RNAseH, and the catalytic domain of eukaryotic recombinases and transposases, including the Holiday junction resolvase RuvC and the MuA tranposase (Davies et al., 2000; Dyda et al., 1994). All of these enzymes belong to the DNA processing polynucleotide transferase superfamily, enzymes that cleave and join DNA by trans-esterification (Bushman et al., 1993; Katayanagi et al., 1993; Rice et al., 1996; Rice and Mizuuchi, 1995). The CCD is essential for two key steps of the integration reaction, 3′ end processing (3′-EP) of the viral DNA and the strand transfer (STF) reaction, in which it cleaves the phosphodiester bond of the host genomic DNA and subsequently joins the 3′-processed viral DNA ends to the host genomic DNA. The CCD forms a dimer in solution and is a dimer in the functional IN enzyme (Fig. 3) (Chen et al., 2000; Goldgur et al., 1999; Lubkowski et al., 1998). As an isolated domain, the CCD can also perform the disintegration reaction (the opposite of integration) although the physiological significance, if any, of this "reverse reaction" is not understood (Bushman et al., 1993). The C-terminal domain (residues 213–288) has some structural homology with SH3 DNA binding domains and binds DNA non-specifically (Vink and Plasterek, 1993). Overall, the CTD is the least conserved of the three IN domains (Engelman et al., 1993). Coordinated action between the three domains is, however, necessary for the 3′-EP and STF reactions to be catalyzed efficiently.

2.2. Integration of HIV-1 into the human genome

The role of IN during HIV-1 replication begins in the cytoplasm after completion of reverse transcription. Ultimately, integration of the viral DNA into the host genome establishes persistent HIV-1 infection, resulting in both active and latent viral reservoirs. IN binds the newly synthesized double-stranded viral DNA and first catalyzes 3′-end processing (3′EP) (Fig. 4). IN binds to a specific, but imperfect inverted DNA sequence in the long terminal repeats (LTRs) of the viral DNA (Colicelli and Goff, 1988; Pommier et al., 2005). IN cleaves GT dinucleotides by nucleophilic attack immediately 3′ to a conserved CA dinucleotide at both 3′ ends of the viral DNA, i.e. in the U3 and U5 LTR ends (LaFemina et al., 1991; Sherman and Fyfe, 1990). The nucleophilic attack occurs on the phosphodiester bond between the deoxyguanosine and deoxyadenosine.

The 3′-EP reaction produces reactive 3′-OH ends and IN remains bound to the both of the LTR ends, forming the preintegration complex (PIC) (Miller et al., 1997). The functional PICs also contain a number of other viral proteins, including matrix, Vpr, p7/nucleocapsid and RT (Bukrinsky et al., 1993). PICs also contain a number of host cellular proteins which have been implicated in the process of integration, including barrier to autointegration factor-1 (BAF), interactor-1, heat shock protein 60 (HSP60), high mobility group protein A1 (HMG-A1) and lens epithelium derived growth factor (LEDGF, also know as p75) (Bushman, 1999; Lin and Engelman, 2003; Kalpana et al., 1994; Farnet and Bushman, 1997; Gao et al., 2003; Li et al., 2000; Maertens et al., 2003). LEDGF has been the most extensively characterized of the host proteins associated with the PIC; it appears to play a role in targeting IN and the

PIC to chromatinized DNA. LEDGF's interaction with IN, its role in integration and the potential for this interaction to be a novel drug target are described in more detail in another article in this series. Following formation of the PIC, it is actively transported across the nuclear membrane (Fig. 4).

The strand transfer reaction occurs inside the nucleus once the PIC is targeted to the host genomic DNA. IN with the viral (donor) DNA bound, binds to host genomic (acceptor) DNA and utilizes the 3′-OH ends of the viral DNA generated during the 3′-EP reaction to perform nucleophilic attack reactions on the phosphodiester bonds of the host genomic DNA. Both ends of the viral DNA are coordinated such that they remain in close proximity to one another and the 3′-OH ends of the viral DNA are ligated to the resulting 5′-phosphate ends of the host genomic DNA, with a 5-base pair stagger separating the ligation points in the genomic DNA (Fig. 4) (Pommier et al., 2005; Leavitt et al., 1992). Indeed, the 5-base pair stagger indicates that each viral DNA end attacks the host genomic DNA along the major groove. The two nucleotides at the 5′end of the viral DNA form a "flap" and are then trimmed; gap filling from the 3′-end of the host genomic DNA, probably by cellular DNA repair enzymes, completes integration (Yoder and Bushman, 2000). The specific identity of the cellular enzymes involved in gap filling is unknown. The completion of the STF reaction produces a functional integrated proviral DNA which forms the template for transcription of new viral RNAs needed for translation of viral proteins and enzymes and transcription of new full length viral RNAs needed for packaging into new virions. Each integrated provirus allows for the production of thousands of new virions, amplifying the initial infection but with the ultimate cost being the death of the infected $CD4^+$ T-cell. Without successful integration, viral replication would stall leading to a rapid decline in the viral load due to the lack of amplification of the infection. Exploration of IN as an ARV drug target was of long standing interest once the function of IN was understood. The development of STF inhibitors of IN (INSTIs) nevertheless required over a decade of basic research before representatives of this drug class began to emerge in the clinic.

3. Strand transfer inhibitors of integrase

IN and the integration reaction were initially postulated to be difficult targets for antiviral drug discovery and development. As previously noted, 50–100 copies of IN are brought into the cell by each incoming virion and only two successful integration events are required to produce the functional provirus. Unlike reverse transcription, where several thousand enzymatic turnovers by RT are needed to complete synthesis of the viral DNA, providing numerous "shots at goal" for NRTIs and NNRTIs to block reverse transcription, the relatively small number of events needed to complete integration potentially may have made it difficult to inhibit. The main impediment to discovery of INSTIs was in fact the relative complexity of the integration reaction itself. Once *in vitro* assays that mimicked functional integration became available, discovery in this field advanced relatively rapidly. Early INSTIs were discontinued in clinical development, primarily due to concerns about toxicity however persistent efforts in basic research and clinical development of this novel class have now borne fruit. INSTIs have turned out to be highly potent and effective ARV drugs which have been clinically validated in multiple clinical trials (Zolopa et al., 2007a; Cooper et al., 2008; Markowitz et al., 2007; Lennox et al., 2009a; Steigbigel et al., 2008).

Several criteria define an INSTI or any other inhibitor of integrase (Pommier et al., 2005). The candidate INSTI must be active at the appropriate point in the viral lifecycle, after reverse transcription and before maturation, as defined by time-of addition experiments. The window of activity of an INSTI is therefore about 4–16 h post infection. Secondly, treatment of infected cells with a candidate INSTI should lead to accumulation of 2-long terminal repeat circles (2-LTR circles). The 2-LTR circles occur due to the accumulation of the viral DNA and its subsequent circularization by cellular enzymes. Successful integration and production of the proviral DNA should concomitantly be decreased, a process that can be measured by Alu-PCR. Finally, treatment of HIV-1 with a putative INSTI should lead to the selection of mutations in the integrase gene in the selected viruses and these viruses should show reduced susceptibility to the selecting compound. Transfer of these mutations to recombinant integrase should also show reduced susceptibility of the resulting mutant enzyme to the inhibitor in STF assays, *in vitro*.

INSTIs are active site inhibitors in that they bind tightly and specifically to IN and chelate the divalent metal ions coordinated by the catalytic triad, i.e. the DDE motif, in the CCD of IN (Fig. 5 (Grobler et al., 2002; Marchand et al., 2003, 2002). Structure activity studies have identified that INSTIs bind to integrase following a DNA-induced conformational change, indeed, viral DNA may well form part of the inhibitor binding site (Alian et al., 2009; Chen et al., 2008). Among the identified integrase inhibitors, there are those that specifically target the STF reaction alone, and others, which block STF but also show some activity against the 3′-EP reaction. INSTIs may bind selectively to the target DNA binding site whereas bifunctional inhibitors may be able to bind to both the donor and target DNA binding sites. INSTIs bind to a conformation of integrase that is present only after processing of the 3′ processed viral DNA ends, in effect a form of allosteric inhibition as it implies blockage of a specific integrase-viral DNA complex. INSTIs may therefore be more specific in the selectivity of their mode of action with possible important implications for reduced toxicity of these compounds versus bifunctional integrase inhibitors.

INSTIs have been observed to be structurally diverse (Fig. 6) and encompass a variety of pharmacophores, however, all appear to have features in common, reflecting a likely common mode of action involving binding to divalent metal ions. Pharmacophore analysis suggests two functional domains: the metal-binding moiety, or catalytic moiety, and an enzyme-binding moiety (Marchand et al., 2002). The enzyme-binding moiety of INSTIs contains one or more aromatic hydrophobic groups and can accommodate hydrophobic groups of diverse size,; these hydrophobic groups are postulated to anchor the INSTI to a hydrophobic pocket that forms upon binding of viral DNA (Chen et al., 2008) (Fig. 3d). The enzyme binding moiety of INSTIs allows presentation of the catalytic moiety to the catalytic triad. The enzyme-binding moiety, being hydrophobic, may also enable transport of the INSTI across the cellular membrane. Unlike the enzyme-binding moiety, the catalytic moiety of the INSTIs tends to be structurally conserved. All INSTIs contain divalent metal ion binding motifs, such as catechol, 1,2-diols, β-dicarbonyls, α-hydroxy acids or quinolinols, which tend to be arranged in a planar configuration with respect to the enzyme binding domain (Mekouar et al., 1998; Hazuda et al., 2000; Neamati et al., 1998; Nicklaus et al., 1997; Zouhiri et al., 2000; Marchand et al., 2002). Evidence suggests that the catalytic moiety sequesters the divalent metal ions (either Mg^{2+} or Mn^{2+}) in the IN active site and thereby inhibits enzymatic catalysis. It has been postulated that metal ions are coordinated between Asp64 and Asp116 and potentially also between Asp116 and Glu152, based on the observation of polynucleotidyly transferases having a two metal structure (Davies et al., 2000; Dyda et al., 1994). Confirmation of the CCD of IN being the binding site of INSTIs has come from studies of selection of viral resistance to these compounds and crystallography studies.

3.1. Discovery of INSTIs

The discovery of INSTIs, like most processes in science, was not a straight path. Early approaches to inhibit the activity of IN

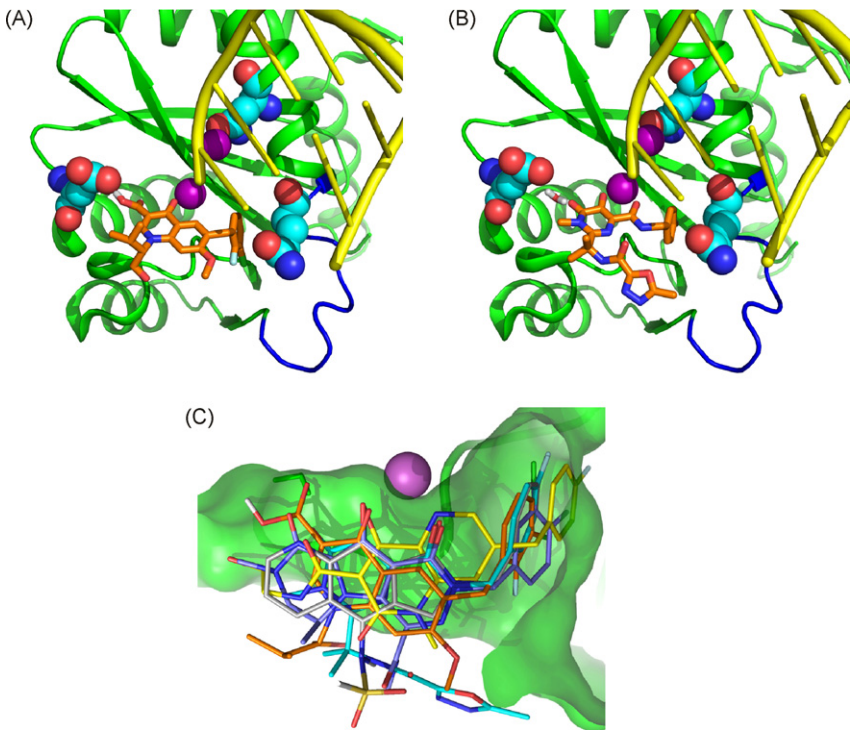

Fig. 5. Proposed model of binding modes of INSTIs (taken from Chen et al., 2008). Comparison of the proposed binding modes of RAL (panel A) and EVG (panel B) to the integrase CCD in the presence of viral DNA. The backbone fold of integrase is shown as a ribbon model (green), with the exception of the mobile loop (residues 138–151, blue), active site Mg (purple). Each inhibitor is shown as a stick model. Residues associated with primary resistance including E92, Q148 and N155 are shown as space filling models. The presence of a potential structural water is shown as a stick near residue E92. (C) Overlap of five representative INSTIs docked into the integrase active site. Despite their structural diversity, INSTIs show a common mode of metal-dependent binding to integrase. The viral DNA and the rest of integrase are omitted for clarity in this figure; the surface of the integrase active site is shown in green. INSTIs are elvitegravir (brown), raltegravir (cyan), GS-9160 (grey), GSK-364,735 (yellow) and MK-2048 (light blue). The active site Mg^{2+} is shown in purple. Note how the hydrophobic moieties on each INSTI orient themselves into the hydrophobic pocket formed upon DNA binding. Metal chelating groups on each INSTI are brought into close proximity with the active site Mg^{2+} and despite the structural diversity of these INSTIs, the metal binding groups are largely coplanar.

focused on using ribozymes and small molecules such as those with DNA-binding activity; triple helix forming oligonucleotides and aptamers were also investigated (Bouziane et al., 1996; Sioud and Drlica, 1991; Raillard and Joyce, 1996). However, these approaches, while providing *in vitro* data on the potential to inhibit IN, harbored significant risk of toxicities and did not proceed into the clinic.

The discovery of small molecule inhibitors of IN was greatly advanced by the description by Merck researchers in the early 1990's of *in vitro* assays for the analysis of IN activity including high throughput screening assays (Hazuda et al., 1994). The initial assay described in a patent by Merck in 1992 involved the use of labeled short oligonucleotide duplexes that mimicked the U3 and U5 LTR ends which acted to mimic target and donor DNAs. When combined with purified recombinant IN, this assay allowed important aspects of IN activity to be defined including the contribution of different domains to activity, the identification of the catalytic residues and early small molecule inhibitors. Early examples of IN inhibitors identified using these IN assays included quinolone antimalarials such as chloroquine and primaquine, which could be shown to inhibit IN but intercalated DNA only weakly (Fesen et al., 1993). An additional important observation was that the 3′-EP reaction could be mechanistically distinguished from the STF reaction. The dicaffeoylquinic acids were also identified as inhibitors of integrase *in vitro* during the mid-1990s (Robinson et al., 1996).

An assay for high throughput screening for IN inhibitors was published by Merck researchers in 1994 (Hazuda et al., 1994). In this assay, recombinant IN nicked the target DNA (the equivalent of the cellular DNA), which was biotinylated and formed a new phosphodiester linkage between this nicked DNA and the 3′-processed end of an immobilized LTR donor oligonucleotide. The integrated target was thus bound to the immobilized donor DNA and then detected colorimetrically by an avidin-linked alkaline phosphatase reporter. A blunt ended U5 LTR oligomer or a preprocessed U5 oligomer lacking the terminal dinucleotide could be used in this assay allowing discrimination of selective INSTIs versus those with bifunctional activity.

During this period a number of natural products, particularly from fungi, were identified as having IN inhibitory activity (Hazuda et al., 1999; Singh et al., 2003). A key example of these was the compound equisitin derived from *Fusarium* spp., which inhibited the STF reaction with an IC_{50} of 7 μM. Many of these fungal compounds contained β-hydroxy-keto groups which were later recognized as key functional groups of INSTIs. Equisitin and other related compounds were characterized using preassembled LTR-donor DNA oligonucleotide-IN complexes and in preintegration complexes isolated from HIV-1 infected cells (Hazuda et al., 1999). These compounds were not completely selective for STF as they also inhibited both 3′-EP and disintegration, however they did inhibit STF under conditions somewhat reminiscent of HIV-1 infected cells, in preassembled IN-DNA complexes. Among the pharmacophores identified in these early integrase inhibitors were the β-hydroxyketo group, diketo group or a carboxylate group. The β-diketo acid (DKA) motif, or its structural analogues was a signature pharmacophore in the INSTIs that were subsequently identified, including styrylquinolone motifs, diazide, napthylazo compounds, tricyclics, and in the case of elvitegravir, a quinolone carboxylic acid motif, which is a structural analogue of a mono-keto acid (Sato et al., 2006).

Researchers at Merck and Shionogi independently discovered the DKAs as INSTIs. Among these compounds was the compound

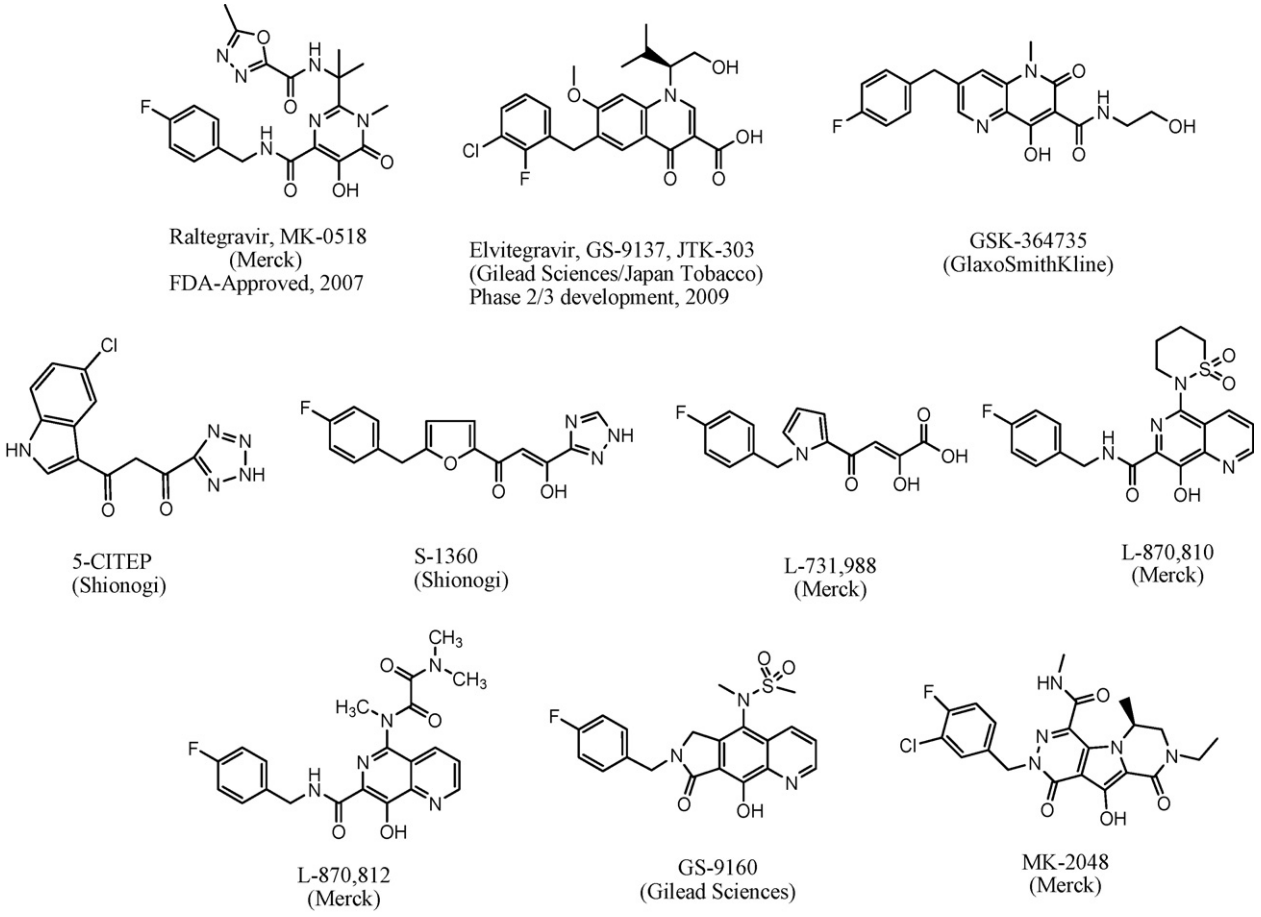

Fig. 6. Chemical structures of INSTIs.
The evolution of INSTIs from discovery to clinical trials to approval, including raltegravir (RAL, MK-0518, Isentress®, Merck Research Laboratories, approved for use in HIV infected patients in 2007); elvitegravir (in development by Gilead Sciences and discovered by Japan Tobacco, currently in phase 2/3 development); the naphthyridinone GSK-364735 (GSK/Shionogi); early diketo acid INSTIs including 5-CITEP and S-1360 (Shionogi) and L-731,988 (Merck Research Laboratories); the naphthyridines L-870,810 and L-870,812 (Merck Research Laboratories); a tricylic INSTI GS-9160 (Gilead Sciences) and a "second generation" INSTI with an enhanced resistance profile, MK-2048 (Merck Research Laboratories).

5-CITEP (Shionogi, Fig. 6) which contain an isostere of a carboxylic acid group, the tetrazole group (Goldgur et al., 1999). The selectivity of 5-CITEP for the strand transfer reaction was significantly increased with an IC_{50} of 0.65–3 μM for the strand transfer reaction, but 35 μM for the 3′-EP reaction. Furthermore, 5-CITEP was the first integrase inhibitor for which the crystal structure was solved in complex with the integrase CCD (Goldgur et al., 1999). 5-CITEP was shown to be bound in the active site, in a planar orientation and located between the three catalytic residues with the hydroxyl group located at hydrogen bonding distance to E152, one of the three catalytic residues. Further studies indicated that the binding of 5-CITEP to IN was consistent with a model, in which the DKA group interacted with the metal ion(s) coordinated by the catalytic residues of the CCD (Grobler et al., 2002). SAR studies demonstrated the capacity to identify DKA compounds that displayed specificity for the STF reaction in the presence of Mn^{2+}, but lost it in the presence of Mg^{2+}. Indoles enhanced metal dependency, which was lost in the presence of substituted phenyl groups indicating the importance of the aromatic group in binding. From these studies, a reaction in the presence of Mg^{2+} was shown to be the most stringent condition with respect to strand transfer inhibition (Marchand et al., 2003). A structurally related molecule to 5-CITEP, S-1360 was the first STF inhibitor to enter clinical trials. S-1360 had shown favorable pharmacokinetic properties in animal studies and a phase I/II study was initiated in 2001 with initial favorable results however development of this molecule did not continue (Billich, 2003).

Both Merck and Shionogi discovered DKA inhibitors of integrase in which pyrrole groups were conjugated to the phenyl groups. Among these compounds, L-731,988 and L-708,906 were highly potent and selective inhibitors of the STF reaction *in vitro* (Fig. 6) L-731,988 had an IC_{50} for strand transfer of 80 nM but had an IC_{50} for 3′-EP and disintegration that was 70- and 250-fold higher, respectively (Hazuda et al., 2000). These compounds were also used to select resistance mutations which appeared in the integrase CCD as will be further discussed. The integrase inhibitory activity of L-731,988 showed dependence from the target DNA concentration confirming that binding of the target DNA and the DKA are mutually exclusive (Espeseth et al., 2000).

The next phase in the evolution of INSTIs came in the discovery of the naphthyridine derivatives by Merck in which the carboxylate group was replaced with a suitable heterocycle, which contained a lone pair donor atom such as an 8-hydroxy-1,6-naphthyridine linked to a benzoyl substituent. These compounds were further indicative of the necessity of coplanarity of the active groups important for metal binding (Zhuang et al., 2003). L-870,812 was one of the most extensively characterized compounds of this series; in this molecule, the ketone was replaced with a benzyl amide on the naphthyridine (Fig. 6). A cyclic sulfonamide substituent on the naphthyridine further optimized oral bioavailability, pharmacokinetic parameters and cardiac safety. L-870,812 demonstrated that INSTIs could suppress retroviral replication *in vivo* (Hazuda et al., 2000). This compound had potent antiretroviral activity

against HIV-1 and SIV (IC_{95} = 250 and 350 nM, respectively) and demonstrated the capacity to suppress the viral load of rhesus macaques infected with the SHIV 89.6P recombinant strain. Among 6 macaques treated with L-870,812 starting 10 days post infection, 4 of 6 had a suppression of their viral load to undetectable levels (<25 copies/mL of viral RNA), representing a >4 $\log_{10}$ decrease compared to untreated animals. In macaques with more advanced disease, 10 days of treatment with L-870,812 produced 10–100 fold decreases in viral load; significant increases in CD4 cells were also observed. The durability of response was variable and dependent on the preexistent immune status and viral load of each subject. Two animals showed evidence of viraemia in the presence of L-870,812 and beginning at days 28 and 32, an N155H mutation was noted to have developed in the IN gene. N155H reduced susceptibility to L-870,812 by 25-fold when engineered into a site-directed mutant virus and also reduced viral infectivity by 75%, indicative of a loss of viral fitness. Residue N155 points into the active site of the CCD and forms a hydrogen bond with D116, one of the three catalytic residues. Additional work showed that another mutation at residue 155, N155S caused resistance to DKAs and cross resistance to naphthyridines (Hazuda et al., 2004). As will be further discussed, mutations at position N155, including N155H/S represent primary resistance mutations to a variety of INSTIs. Mutation of this position could disrupt interaction of these INSTIs by interfering directly with metal binding.

L-870,810 was another naphthyridine from this series (Merck, Fig. 6) that was tested in a double blind placebo controlled phase II clinical trial in HIV-1 infected subjects (Little et al., 2005). In this study, 30 ARV treatment-naïve or treatment-experienced (but otherwise off drug) subjects were dosed with either 200 mg (n = 7) or 400 mg (n = 17) L-870,810 given BID (twice daily) versus placebo (6 subjects). The highest dose group showed a median decline in viral load of 1.7 log with 6 subjects achieving <400 copies/mL and the median rise in CD4 cells was 89 cells/mL. Similar results were noted in the lower dose group. Development of L-870,810 was discontinued due to hepatotoxicity observed in long-term safety studies in dogs, however, the results of this study clearly validated INSTIs for treatment of HIV-1 infection.

3.2. Development and approval of an HIV-1 INSTI for the treatment of HIV-1

Successful development and approval of an HIV-1 INSTI for the treatment of HIV-1 infection was ultimately achieved with the discovery of raltegravir (RAL, MK-0518, Isentress, Fig. 6) by Merck Research Laboratories (Summa et al., 2008). Merck had discovered a series of DKA scaffolds active against HIV-1 IN and the HCV NS5b RNA-dependent RNA polymerase and mechanistically these compounds were expected to act similarly against these enzymes, namely via the chelation of active site metals. An interesting and key observation of this work was that a dihydroxypyrimidine caboxamide derived from DKAs in the HCV program that had no activity against HCV NS5B, acted as a potent, reversible and selective HIV INSTI with an IC_{50} of 85 nM in an enzymatic IN STF assay. Optimization of the SAR on the carboxamide led to the identification of p-flurobenzyl as the optimal amide residue and a gem-dimethyl as the optimal 2-substituent for the dihydroxy pyrimidine core. Further optimization of capping groups of the amine portion of the scaffold demonstrated that heteroaromatic groups were the most potent. Five membered ring heterocycles were also investigated and compounds bearing 2 or 3 heteroatoms were the most potent in cell based antiviral assays, particularly those with a heteroatom in the 2 position. The compound that ultimately became known as RAL was an oxadiazole with three heteroatoms and was one of the most potent compounds investigated in cell based assays with an IC_{95} of 31 nM in the presence of 50% human serum. RAL was highly selective against other enzymes working through Mg^{2+}-based mechanisms being inactive against HCV polymerase, HIV RT and RNaseH, and human α, β and γ polymerases, and showed no activity up to 10 μM concentration on a panel of 150 enzymes, channels and receptors. RAL also did not have activity against the major cytochrome P450 isoforms including 1A2, 2C9, 2D6, 3A4 and 2C9 nor did it show time dependent inhibition of 3A4. Binding affinity on cardiac HERG channels was >50 μM, suggestive of a good cardiac safety profile. Pharmacokinetic studies in animals showed good bioavailability with a multiphasic elimination, including a relatively short α-phase and a prolonged β-phase. Studies of metabolism demonstrated that RAL was primarily eliminated as a glucuronidated metabolite through bile and urine with the glucoronidation occurring on the 5-hydroxyl group of the pyrimidinone ring. Based on the need to keep the plasma concentration above the cell culture 95% inhibitory concentration (CIC_{95}) and considering a variety of key factors including the biphasic elimination, the metabolic stability, protein binding and plasma clearance, the human dosing regimen of RAL was predicted to be twice daily (BID). Metabolism of RAL occurs primarily through glucoronidation and not via Cyp3A4, therefore RAL cannot be boosted with ritonavir as is the case for EVG. Subsequent clinical development of this compound in clinical trials and its approval focused on BID dosing in both ARV experienced and naïve subjects. Raltegravir has been approved for BID dosing in both patient populations; an ongoing phase 3 trial is investigating once daily dosing (800 mg QD) of raltegravir in ARV naïve subjects.

Elvitegravir (EVG JTK-303/GS-9137, Fig. 6) is a second INSTI in phase 3 clinical development in ARV treatment-experienced subjects and is also undergoing phase 2 development in ARV naïve subjects as part of a fixed dose combination regimen. EVG was discovered by researchers at Japan Tobacco who described a new pharmacophore, specifically 4-quinolone-3-glycoxylic acid, which maintained the coplanarity observed in DKA INSTIs (Sato et al., 2006). Compounds containing the 4-quinolone-3-carboxylic acid motif, but not the 4-quinolone-3-glycoxylic acid, were inhibitors of IN, with the coplanar monoketo acid motif in 4-quinolone-3-carboxylic acids providing an alternative to the DKA motif. Elvitegravir showed potent anti-HIV activity *in vitro* against HIV-1 of multiple subtypes (EC_{50} ranging from 0.1 to 1.26 nM) as well as against HIV-2 strains (EC_{50} 1.4–2.8 nM) (Shimura et al., 2008), and both SIV and murine leukemia viruses. Elvitegravir, like raltegravir has also shown potent antiviral activity *in vivo* against HIV-1 carrying resistance mutations to multiple antiretroviral drug classes (DeJesus et al., 2006a; Zolopa et al., 2007b). Development of EVG, has focused on boosting it to QD dosing via inhibition of CyP3A4 metabolism.

More recently, Shionogi and GlaxoSmithKline described a series of two-metal binding INSTIs based on a naphthyridinone scaffold (Garvey et al., 2008). One of these, S/GSK1349572 has recently shown impressive activity in a phase 2A study, in which it was studied as a once-daily unboosted INSTI dosed at 2, 10 or 50 mg once daily (Lalezari et al., 2009). The 50 mg dose produced a −2.46 $\log_{10}$ decline in HIV RNA after a 10-day monotherapy. Resistance data suggested that this compound may have an improved resistance profile on RAL and EVG selected resistance mutations, which may allow it to be used for salvage of patients with virologic failures on the other INSTIs (Underwood et al., 2009). The structure of S/GSK1349572 has not been disclosed at this time.

3.3. Clinical trials of integrase inhibitors: raltegravir and elvitegravir

3.3.1. Raltegravir

The efficacy of RAL in HIV-1 infected individuals was first described in a proof of concept study that explored the antiviral

activity, pharmacokinetics and tolerability of the compound when dosed BID (100, 200, 400 or 600 mg BID) as 10-day monotherapy (Markowitz et al., 2006). Twenty-eight subjects, randomized 1:1:1:1, received one of the four doses of RAL and 7 subjects received placebo. Among the four dose groups tested, the mean change in HIV-1 RNA from baseline to day 10 was −1.93, −1.98, −1.66 and −2.16 $\log_{10}$ copies/mL in the 100, 200, 400 and 600 mg BID RAL dose groups, respectively, and significantly different from the placebo group (−0.17 log10 copies/mL). Similar proportions of subjects achieved <400 copies/mL (50–57%) or <50 copies/mL (13–29%) of HIV-1 RNA among the four RAL dose groups.

Protocol 004 was a double blind, randomized, controlled, 48-week dose ranging study which investigated 100, 200, 400 and 600 mg RAL BID versus efavirenz (EFV 600 mg QD), in all cases combined with tenofovir DF and lamivudine (both dosed QD), in ARV-naïve, HIV-1 infected individuals (Markowitz et al., 2007). One hundred and ninety-eight subjects were enrolled, 160 on RAL and 38 on EFV. Rapid viral load declines were observed in the RAL dose groups, with 90% of subjects on RAL achieving <400 copies/mL by Week 4. This feature of rapid early viral load declines has now been observed in clinical trials of both RAL and EVG and appears to be characteristic of INSTIs. Furthermore, HIV-1 RNA < 50 copies/mL was achieved at Weeks 2, 4 and 8 by more subjects in the RAL dose groups than on EFV. These initial differences in viral load decline between the RAL and EFV dose groups eventually diminished so the overall clinical significance of this initial rapid drop in viral load is unclear. By Week 24, both the RAL and EFV treatment groups had similar efficacy with <50 copies/mL of HIV-1 RNA achieved by 85–95% of subjects which was largely maintained through Week 48. The mean change in CD4+ T-cell count was comparable across all treatment groups at Weeks 24 and 48, with mean increases in from 144 to 221 cells/mL. The most common treatment related adverse event associated with RAL treatment was nausea; neuropyschiatric events, particularly abnormal dreams, were less common on RAL during the first eight weeks of treatment, consistent with the known side effect profile of EFV. Only one patient on RAL (600 mg dose group) discontinued due to increases in aspartate aminotransferase levels. Protocol 004 continued through 96 weeks and raltegravir demonstrated sustained and durable activity when combined with TDF and 3TC; 83% and 84% of subjects on RAL and EFV, respectively achieved <50 copies/mL.

Protocol 005 was a second phase 2 dose ranging study of RAL, a double-blind, placebo controlled study of 200, 400 and 600 mg BID doses of RAL, in combination with an optimized background regimen (OBR) in HIV-1 infected, ARV treatment-experienced subjects (Grinsztejn et al., 2007). Inclusion criteria required documented drug resistance to at least one drug in each of the NRTI, NNRTI and PI drug classes. Using the PhenoSense GT Assay to score genotypic and phenotypic resistance to NRTIs, NNRTIs and PIs, and excluding enfuvirtide, 72% of subjects ($n = 128$) had no genotypic sensitivity (GSS = 0) and 48% had no phenotypic sensitivity (PSS = 0) to the agents used in their OBRs. A median of 4 drugs were used in the OBR in all treatment arms and enfuvirtide was used in 36% of treated subjects, indicative of a highly treatment-experienced population. Overall, subjects treated with any of the three dose groups of RAL achieved significantly greater reductions in viral load compared to the placebo group. The differences in the proportion of subjects achieving <400 copies/mL on all dose groups of RAL versus placebo were 54–56% and for subjects achieving <50 copies/mL 42–53% versus placebo. Significantly greater rises in $CD4^+$ T-cell counts were also noted in the RAL groups. Similar to Protocol 004, rapid viral load declines, of up to 2.0 $\log_{10}$ copies/mL, were noted during the early phase of RAL treatment. First use of enfuvirtide was associated with improved outcomes in both the RAL and placebo groups, but the treatment benefit of RAL was still approximately 60% (with respect to achieving <400 copies/mL). The safety profile of RAL in this study was similar to placebo; only one subject in the RAL group discontinued the study due to a raised hepatic aminotransferases and which was considered to be drug related. This study demonstrated that RAL, when combined with an OBR, could provide a sustained treatment benefit in HIV-1 infected subjects with evidence of resistance to other ARVs.

The BENCHMRK-I and -II studies (Protocols 018 and 019, respectively) were two large, identically designed phase 3, multicenter, multinational, double blind studies of RAL which enrolled approximately 700 HIV-1 infected, antiretroviral treatment-experienced individuals (Cooper et al., 2008; Steigbigel et al., 2008). In each study, RAL dosed 400 mg BID versus placebo (2:1 randomization), was combined with an OBR. Subjects had documented resistance to at least one drug in each of the NRTI, NNRTI and PI classes. Overall, 232 and 230 subjects were treated with RAL in BENCHMRK-1 and II, respectively. This was a highly treatment-experienced population, with a median of 10–11 years of prior ARV experience, 95% had resistance to >1 PI, 18.6% had no active agents in their OBR as assessed by their phenotypic sensitivity score (both studies combined) and 27.8% had no active agents in their OBR as assessed by their genotypic sensitivity score (both studies combined). For each study and for both studies combined, subjects on RAL plus OBR showed significantly greater ($P < 0.001$) virologic responses than placebo; 355/458 (77.5%) of RAL subjects achieved < 400 copies/mL of HIV-1 RNA by the primary Week 16 timepoint versus 99/236 (41.9%) placebo subjects. Similar differences of 61.8% and 34.7% were noted with respect to achievement of <50 copies/mL of HIV-1 RNA by Week 16, on RAL and placebo, respectively. These antiviral responses were sustained through Week 48. Similarly, significantly greater CD4+ T-cell responses were noted in the RAL versus placebo groups through Week 48 (mean increases of 109 cells/mm^3 versus 45 cells/mm^3, respectively). When RAL was combined with first use of the protease inhibitor darunavir, the fusion inhibitor enfuvirtide or both drugs combined, 69%, 80% and 89% of subjects, respectively, achieved <50 copies/mL by Week 48, which was greater than the placebo response in all cases. RAL safety and tolerability were similar to placebo through Week 48. Treatment responses on RAL were sustained through Week 96; 62% and 57% of subjects maintained <400 copies/mL and <50 copies/mL of HIV-1 RNA, respectively, through Week 96 (Steigbigel et al., 2009).

STARTMRK is an ongoing phase 3 study of RAL in ARV treatment-naïve subjects comparing RAL to EFV, when combined with tenofovir DF and emtricitabine (Lennox et al., 2009a,b). In this study, 563 subjects were randomized 1:1 to each of the two treatment groups. Through Week 48, treatment with RAL was statistically non-inferior to treatment with EFV, with 86% and 82% of subjects treated with RAL or EFV, respectively, achieving <50 copies/mL by Week 48. Week 96 data from this trial were recently reported and showed durable and non-inferior efficacy of RAL-based therapy compared to EFV with 81% and 79% of subjects on RAL or EFV achieving <50 copies/mL through Week 96. Similar CD4 rises of 240 and 225 cells/mm^3 were observed on RAL and EFV, respectively through Week 96. On the basis of the STARTMRK data, RAL was approved for use in ARV-naïve patients in mid-2009.

Based on these studies RAL has been shown to be a potent, well tolerated drug that can be combined with existing ARVs to provide highly efficacious treatment to both ARV experienced and naïve subjects. Further clinical studies will define the strengths and weaknesses of RAL and of the INSTI class in general.

A recent example of an unexpected result was that seen in the SWITCHMRK studies, Protocols 032 and 033 (Eron et al., 2009). In these studies, HIV-1 infected subjects who were well controlled (VL < 50 copies/mL) on a stable LPV/r based regimen in combination with at least two NRTIs were randomized 1:1 to either continue their existing regimen or switch out LPV/r for RAL. Surprisingly, through Week 24, the group of subjects who switched to RAL had

a viral load response that was "not non-inferior" to remaining on LPV/r, with 87% versus 81% of subjects on lopinavir versus RAL, respectively remaining <50 copies/mL. Notably, 27/32 RAL treated subjects with virologic failure were not on their first ARV regimen at baseline and of these 18 had one prior virologic failure before commencing their LPV/r based regimen. Both nucleoside and non-nucleoside resistance mutations were noted in those with RAL failure but fewer were noted in those who remained on lopinavir and subsequently experienced virologic failure. Of note, several subjects with RAL failure in Protocol 033 had "RAL resistance only" detected at virologic failure, with no evidence of NRTI or NNRTI resistance, suggesting pure RAL failure in this subset of virologic failures. The results of the SWITCHMRK studies showed that care must be taken when considering treatment switches. Single agent switches, even those involving highly potent new agents like RAL in patients who are otherwise stably suppressed, can potentially be problematic.

3.3.2. Elvitegravir

Elvitegravir is another INSTI currently undergoing clinical development. EVG is a quinolone derivative and was originally discovered by Japan Tobacco, Inc. (where it was known as JTK-303), and was subsequently licensed by Gilead Sciences, Inc. (as GS-9137) for clinical development. Clinical development of EVG commenced in 2005 and results of phase I/II studies have been described. EVG was demonstrated to be orally bioavailable and well tolerated at once daily doses of 100, 200, 400 or 800 mg in HIV-1 negative individuals (Kawaguchi et al., 2006). EVG was also shown to be metabolized by Cyp3A4 and the pharmacokinetic profile of EVG could be boosted by ritonavir (Kearney et al., 2006). Use of ritonavir-boosting, as has been done for protease inhibitors, increased the EVG plasma half-life from three to nine hours, permitting QD dosing. A phase 1 randomized, double-blind placebo controlled proof of concept study, Study 0101, was initiated to examine the antiviral activity, tolerability, pharmacokinetics, and pharmacodynamics of EVG when given as 10-days of monotherapy (DeJesus et al., 2006b). This study investigated both unboosted and ritonavir boosted EVG in 40 HIV-1 infected treatment-naïve or treatment-experienced (but off treatment) individuals. Unboosted EVG (200, 400 or 800 mg BID, or 800 mg QD) or ritonavir boosted EVG (50 mg, boosted with 100 mg ritonavir) were compared to placebo. Each EVG dose resulted in significant reductions in viral loads, which were dependent on EVG trough levels. The most significant reductions in plasma viral load (mean reductions of approximately 2.0 $\log_{10}$ copies/mL) occurred in the groups dosed with 400 or 800 mg BID EVG in the unboosted state or in the group dosed once daily with 50 mg EVG boosted with 100 mg ritonavir. All EVG dose regimens were well tolerated and there were no discontinuations or serious adverse events. Pharmacokinetic/pharmacodynamic studies indicated that doses of 20, 50 and 125 mg EVG boosted with 100 mg ritonavir could be further investigated.

Study GS-US-183-0105 (Study 0105) was a phase 2B dose ranging study of ritonavir-boosted EVG (EVG/r) which was conducted in HIV-1 infected, ARV treatment-experienced individuals (Zolopa et al., 2007a). Study 0105 was a randomized, partially blinded (to dose of EVG) active controlled study designed to assess whether EVG (dosed at 20, 50 or 125 mg once daily, all boosted with 100 mg ritonavir) was non-inferior to an active comparator regimen composed of a ritonavir-boosted protease inhibitor (CPI/r). Subjects entering the study had to have evidence of at least one PI resistance mutation. Two hundred and seventy-eight ARV treatment-experienced subjects enrolled in this study, randomized 1:1:1:1 to receive either one of the three doses of boosted EVG (EVG/r) or a comparator boosted protease inhibitor (CPI/r), in each case combined with an OBR composed of NRTIs with or without enfuviritide. Due to a lack of drug–drug interaction data, subjects on EVG/r were initially not able to use boosted PIs in their OBR. The subsequent availability of this data allowed for a protocol change at Week 8 in which subjects on EVG/r could add either boosted darunavir or tipranavir, each of which demonstrated a lack of drug–drug interactions with EVG. Subjects enrolled in the study were highly treatment experienced with a median of 10–11 PI resistance mutations at baseline and approximately 50% had a genotypic sensitivity score of 0 for the NRTI components of their OBR. The primary treatment endpoint in this study was the time weighted average change from baseline in $\log_{10}$ HIV-1 RNA through Week 24 ($DAVG_{24}$); however due to the protocol change allowing use of protease inhibitors, an analysis of $DAVG_{16}$ was also performed; only 4 subjects receiving EVG/r added a protease inhibitor prior to Week 16. Like RAL, EVG treatment produced a rapid initial viral load decline, >2 $\log_{10}$ copies/mL by Week 2 in many cases. Among the three EVG/r arms, the EVG/r 125 mg arm was statistically superior compared to the CPI/r arm when both $DAVG_{16}$ and $DAVG_{24}$ were analyzed. Furthermore, 69% and 76% of subjects on either EVG/r 50 mg or 125 mg, respectively, achieved a $\geq$2 $\log_{10}$ copies/mL reduction in HIV-1 RNA by Week 24, which for the EVG/r 125 mg treatment group was significantly different versus the CPI/r arm. When considering patients using another fully active agent such as first use of enfuvirtide, use of EVG/r 125 mg produced significantly greater treatment responses versus the CPI/r arm, with $DAVG_{16}$ being a mean -2.5 $\log_{10}$ copies/mL versus 1.5 $\log_{10}$ copies/mL for the CPI/r arm. A significantly greater proportion of subjects on EVG/r 125 mg plus first use of enfuvirtide also achieved <50 copies/mL by Week 16 versus those on the CPI arm. Gradations in treatment response were also noted when subjects on EVG/r 125 mg were stratified on whether they had 1 or 2 active NRTIs without first use of enfuvirtide. Thus, as for other ARV drug classes, the use of other active drugs in combination therapy with INSTIs is necessary to obtain the maximum treatment benefit.

Development of EVG/r is now continuing and is currently undergoing a phase 3 program in which it is being directly compared to RAL. An ongoing phase Study GS-US-183-0145 (Study 0145) will enroll 700 HIV-1 infected ARV treatment-experienced individuals and directly compare EVG/r (dosed QD) versus RAL 400 mg (dosed BID). Subjects in this ongoing study are using either INSTI in combination with an active boosted protease inhibitor (as defined by phenotypic resistance testing) and are also allowed to use one other additional agent which can be fully or partially active, as defined by resistance testing. Due to the emergence of new ARV drugs like RAL, etravirine, and maraviroc, with the consequent availability of multiple new active agents, placebo-controlled phase 3 studies, like the BENCHMRK studies of RAL, can no longer be conducted as the standard-of-care for ARV treatment-experienced subjects has shifted. Active comparator trial designs will now have to be conducted to ensure subjects enrolling in clinical trials receive standard-of-care. EVG is also being studied in HIV-1 infected antiretroviral naïve individuals in an ongoing phase 2 study (Study 0104) in which the drug has been combined in a single tablet, once-daily, fixed dose combination regimen including a novel pharmacokinetic enhancer, GS-9350, and the NRTIs emtricitabine and tenofovir DF, and compared to the current standard of care for ARV naïve individuals, emtricitabine/tenofovir DF/efavirenz (Atripla™).

4. Resistance to integrase inhibitors

Drug resistance in HIV-1 evolves due to the lack of proof reading activity of HIV reverse transcriptase. Consequently, nearly every viral genome contains at least one mutation, some of which can impart drug resistance. During the development of INSTIs, a significant amount of information has accumulated on the IN mutations leading to drug resistance. A summary of these *in vitro* resistance selections is provided in Table 1. As currently understood, resistance to INSTIs can be mediated by a number of IN mutations that

Table 1
HIV-1 integrase mutations selected by INSTIs *in vitro*. The results of *in vitro* dose escalation and high concentration breakthrough experiments conducted by various groups using preclinical INSTIs, raltegravir and elvitegravir, are shown. Mutations selected in integrase are listed for each set of experiments.

Compound	Group	Mutations selected in HIV-1 integrase
L-731,988	Merck	M154I
L-708,906	Merck	T66I, S153Y, M154I
L-708-906	Rega	T66I, L74M, S230R
L-Chicoric Acid	UC Irvine	G140S
S-1360	Shionogi	T66I, Q146R, Q148K, N155S
S-1360	Rega	T66I, L74M, A128T, E138K, Q146K, S153A, K160D, V165I, V201I
L-870,812	Merck	N155H
L-870-810	Merck	V72I, F121Y, T125K, V151I
L-870,810	K. U. Leuven	L74M, E92Q, S230N
GS-9160	Gilead	L74M, E92V
GS-9224	Gilead	S147N, D270N
GS-9224	Gilead	L74M, G140S, Q148K
GSK-364735	GSK	F121Y, Q146R, Q148R
RAL	Merck	E138A, G140A, Q148K
RAL	Merck	T66A, Q95K, Y143C or T66A, Y143R or N155H
RAL	Tibotec	E138K, G140A, Q148R
EVG	Kyoto University	H51Y, E92Q, S147G, E157Q or T66I, Q95K, E138K Q146P, S147G
EVG	Gilead	T66I, F121Y or T66I, S153Y or T66I, R263K
EVG	GSK	T66I/A/K, V72A, E92Q/V, P145S, Q146S/L, Q148K/R
EVG	Merck	T66A, V72I, N155S or Q148R
EVG	Tibotec	T66I alone or R20K, T66I, L74M, S230R or T66I, A128T, E138K, Q148R, S230R or E92Q alone or T66A, E92Q or E92Q, E138K, Q148R

can be considered "primary" resistance mutations which are all located in the CCD of IN, close to the INSTI binding site. Within this set of mutations, which occur at codons T66, E92, Y143, Q148 and N155, varying effects on susceptibility to EVG and RAL are observed. Numerous additional IN mutations, some of which are natural polymorphisms and most of which are also found in the CCD, comprise secondary resistance mutations which increase phenotypic resistance when added to primary mutations. Many of these secondary mutations have only limited effects on susceptibility to EVG and RAL when present on their own. Some secondary resistance mutations have been noted in the CTD of IN including mutations at codons S230 and R263; however, no resistance mutations have been described in the NTD of IN. In many respects, resistance to INSTIs has features in common with that observed for other ARV drug classes, notably the accumulation of mutations leads to higher levels of resistance.

4.1. Resistance to preclinical integrase inhibitors

In vitro selection experiments with the early series of DKA INSTIs discovered by Merck Research Laboratories identified IN mutations leading to drug resistance and provided initial evidence that accumulation of these IN mutations increased the level of phenotypic resistance and reduced viral replication capacity. In early studies *in vitro* passage experiments with the DKA INSTIs L-708,906 or L-731,988 selected T66I + S153Y or T66I, L74 M and S230R IN or M154I mutations (Fikkert et al., 2003; Hazuda et al., 2000). The T66I mutant virus had 3-fold and >8-fold reduced susceptibility to L-708,906 and L-731,988, respectively; the other mutations further reduced susceptibility. Recombinant IN enzyme carrying these mutations also had reduced susceptibility to L-708,906 in a STF assay. Cross-resistance to S-1360 was also observed in both the antiviral and STF assays. The T66I IN mutation was also selected by S-1360 *in vitro* along with Q146R, Q148K and N155S IN mutations or L74M, A128T, E138K, Q146K, S153A, K160D, V165I and V201I IN mutations (Fikkert et al., 2004). HIV-1 carrying the T66I mutation had 4-fold reduced susceptibility to S-1360; the E138K and Q146K mutations further reduced susceptibility to S-1360. Another early INSTI based on a different chemical scaffold, the dicaffeoyltartaric acid INI, L-chicoric acid (L-CA) selected a G140S IN mutation *in vitro* resulting in >100-fold reduced susceptibility to L-CA and cross-resistance to L-731,988 (King et al., 2003; King and Robinson, 1998).

L-870,810 selected F121Y/T125K or V72I/F121Y/T125K or V72I/F121Y/T125K/V151I IN mutations *in vitro*. These mutations reduced susceptibility to L-870,810 but not to a structurally unrelated DKA INSTI suggesting the possibility of discordant resistance profiles between INSTIs (Hazuda et al., 2004). Independent experiments with L-870,810 also selected L74M, E92Q and S230N IN mutations, resulting in 22–110-fold reduced susceptibility to L-870,810 as the mutations accumulated (Hombrouck et al., 2008). The E92Q mutation alone conferred 18-, 17- and 3-fold reduced susceptibility to L-870,810, EVG and RAL, respectively. As previously noted, L-870,812, selected an N155H IN mutation in SHIV 89.6P IN which caused a 25-fold reduction in L-870,812 susceptibility. The naphthyridinone INI, GSK-364735 (GlaxoSmithKline) selected Q148R, Q146R and F121Y IN mutations in HIV-1 *in vitro* (Garvey et al., 2008). Site-directed mutant viruses carrying T66I, N155S, F121Y or Q148R/K IN mutations had 17-, 23-, 25-, 73- or 210-fold reduced susceptibility to GSK-364735, respectively. Gilead Sciences described pyrrolquinolone tri-cyclic analogs of L-870,810 and one compound in this series, GS-9160, selected E92V and L74M IN mutations in HIV-1 III_B IN. E92V mutant viruses had 5-fold reduced susceptibility to GS-9160 and reduced susceptibility to both L-870,810 and EVG (Jones et al., 2009). Thus *in vitro* resistance selection studies with preclinical or early INSTIs identified IN mutations at many residue positions including T66, E92, Q148 and N155, all of which are within the CCD and which have been subsequently associated with resistance to both RAL and EVG.

4.2. Resistance to raltegravir: in vitro and in vivo studies

During the development of RAL and EVG, drug resistance data sets have become available form *in vitro* selection studies and clinical data sets. In independent dose escalation experiments *in vitro*, RAL selected Q148K, E138A and G140A mutations or T66A, Q95K and Y143C/R mutations in HIV-1 III_B IN (Witmer et al., 2007). The combination of the Q148K, E138A and G140A mutations caused several hundred fold reduced susceptibility to RAL and EVG. The Y143R IN mutation caused >10-fold reduced susceptibility to RAL but had little effect on EVG susceptibility. Selection experiments *in vitro* using a high multiplicity of infection of HIV-1 HXB-2 and 100 nM of RAL resulted in breakthrough viruses carrying the N155H IN mutation. The N155H mutation caused >10 fold reduced susceptibility to RAL and EVG. Eight independent *in vitro* resistance selection experiments with RAL (at 3 μM) and HIV-1 III_B resulted in the emergence of the Q148R, E138K and G140A IN mutations in most cases (Goethals et al., 2008). Dose escalation studies with RAL *in vitro* produced similar results including selection of E138K, G140C/S. Q148K/R, N155H integrase mutations; E92Q was also observed in some viral quasi-species (Kobayashi et al., 2008).

In the RAL phase 2 study Protocol 005, 35/38 virologic failure (VF) subjects developed IN mutations (Hazuda et al., 2007). Most subjects developed either the Q148H/R/K ($n = 20$) or N155H ($n = 14$) IN mutations, either alone or combined with other IN mutations; one patient also developed the Y143R IN mutation. Additional IN mutations detected along with Q148H/R/K included L74M, E138A/K, and G140S. Q148H + G140S was the most common pattern of mutations that developed (13/35 subjects). Other IN mutations that developed with N155H included L74M, Y143H,

V151I, G163K, D232N, E92Q, G163R and T97A. Longitudinal analyses of VFs from Protocol 005 demonstrated genotypic switching among INSTI resistance mutations in many subjects (Miller et al., 2008). In particular, the initial development of an N155H or N155/Q148 pattern of IN resistance mutations often evolved towards Q148H/R/K genotypes. Other subjects developed N155H or Q148R/H/K IN mutations initially and maintained these patterns or added more IN mutations. A minority of subjects either developed Y143C/R/H IN mutations and switched to Q148H/R/K IN mutations, or developed the N155H IN mutation and switched to Y143C/R/H mutations. At the earliest time-points of VF, at which INSTI genotypic resistance was detected, the majority of VF subjects already had 2 or more IN mutations.

Similar patterns of IN resistance mutations were observed in the two phase 3 BENCHMRK studies of RAL (Steigbigel et al., 2008; Cooper et al., 2008). In BENCHMRK-1, 28/49 VF subjects developed Q148R/H/K and/or N155H IN mutations by Week 48; four subjects also developed Y143C/R/H. Other INI resistance mutations detected included L74M and E92Q. Seven subjects developed IN mutations of unknown significance, and nine subjects had no change in their IN genotype relative to baseline. Similar results were seen in the BENCHMRK-2 study which was of the same design; 29/45 VF subjects developed Q148R/H/K and/or N155H IN mutations, 2/45 developed other IN mutations including L74M, Y143C/R and S230R, 1/45 developed IN mutations of unknown significance and 13/45 had no change relative to baseline. Clonal analyses of baseline and virologic failure samples from the BENCHMRK studies ($n = 69$ subjects analyzed) confirmed that mutations at IN codons Q148 and N155 developed on separate viral genomes as did E92Q and Q148R/H/K mutations (Fransen et al., 2008). Q148 and N155 IN mutants, were linked with IN mutations G140S/A and E92Q, respectively. E92Q plus N155H had increased resistance to RAL but reduced viral replication capacity (RC) relative to either mutation alone. G140S had differential effects on RAL susceptibility when added to different Q148 IN mutants. IN RC was reduced in the majority of subjects with development of primary IN mutations, relative to the RC of their baseline samples. E138K and G140S/A IN mutations also had differential effects on RC, depending on the Q148 mutant background to which they were added. Several small clinical cohort studies have also described INSTI resistance data in ARV treatment-experienced subjects with virologic failure on RAL-containing regimens (Ceccherini-Silberstein et al., 2008; Da Silva et al., 2008; Hatano et al., 2008; Katlama et al., 2008). The patterns of IN resistance observed in these studies closely resembled those observed in RAL Phase 2/3 studies including Q148H + G140S/A, N155H, Y143C/R/H, E92Q, T97A, E138K, I151V and D232N mutations.

Development of INSTI resistance mutations in VFs in studies investigating RAL use in ARV naïve patients mirrored that observed in ARV treatment-experienced patients. In the Phase 2 study Protocol 004, 2/5 virologic failures on RAL developed IN mutations, including N155H, V151I, G163R/G and D232D/N (Markowitz et al., 2007). Likewise, in the phase 3 STARTMRK studies, virologic failure on RAL with >400 copies/mL at Week 48 occurred in 12 subjects of which 4 developed INSTI resistance mutations, including L74M, E92Q, T97A, G140S, Y143H and Q148R/H (Lennox et al., 2009a,b). In the SWITCHMRK studies, confirmed VF >400 copies/mL on RAL occurred in 12 subjects and was associated with development of INSTI resistance mutations in 8 subjects, including G140S, Y143C/S, Q148H/R, and N155H mutations.

Therefore, virologic failure on RAL is predominantly associated with the initial development of Q148R/H/K or N155H IN mutations, either alone or combination with other IN mutations. A third pathway of RAL resistance involving IN mutations Y143C/R/H was observed in a minority of subjects. Other IN mutations such as G140S/A or E92Q can be can be added to these Q148 or N155 patterns, respectively, and further amplify resistance to RAL. A number of mutual exclusions among primary INSTI resistance mutations have been noted in clonal analyses of RAL virologic failures, suggesting functional incompatibilities at the enzymatic level. Genotypic switching among RAL resistance patterns was observed in from raltegravir VFS with viruses initially expressing N155H and subsequently switching to the Q148 resistance pathway in many cases. IN residue Q148 makes a critical interaction with a 5′ terminal cytosine on the donor, i.e. viral DNA; that this residue is a site of major resistance mutations to INSTIs further highlights the importance of binding of viral DNA to IN in the formation of the binding pocket for the INSTI (Alian et al., 2009; Chen et al., 2008; Johnson et al., 2006).

4.3. Resistance to elvitegravir

The available resistance data on EVG have come from *in vitro* selection studies (summarized in Table 1), and from a phase 2 clinical trial, Study 0105. IN mutations observed to be selected *in vitro* by EVG have been independently confirmed to develop *in vivo*. Overall, the patterns of INSTI resistance mutations selected by EVG are identical in many respects with those selected by RAL, with some subtle differences in the relative frequencies of the particular primary INSTI resistance mutations selected. Thus, depending on the pattern of primary INSTI mutations selected by EVG and RAL, these two INSTIs are largely cross-resistant with one another.

Independent dose escalation experiments *in vitro* with EVG and HIV-1 III_B conducted by researchers from Kyoto University, in collaboration with Japan Tobacco, resulted in the emergence of two distinct patterns of IN mutations, either E92Q, S147G, H51Y, and E157Q or Q146P, T66I, S147G, Q95K and E138K (Shimura et al., 2008). Site-directed mutant HIV-1 containing the E92Q mutation alone or combined with the S147G, H51Y and E157Q mutations had 38–219-fold reduced susceptibility to EVG. The T66I mutant virus had 41-fold reduced susceptibility to EVG; addition of the Q146P mutation further reduced susceptibility. These patterns of IN mutations also caused cross-resistance to L-870,810, L-731,988 and S-1360.

The T66I IN mutation was also observed in two independent dose escalation selection experiments with EVG and HIV-1 III_B *in vitro*, conducted by Gilead Sciences (Jones et al., 2007). T66I was combined with either F121Y or S153Y or R263K IN mutations, the latter an IN polymorphism that was enriched. Site-directed mutant HIV-1 carrying the T66I mutation showed approximately 15-fold reduced susceptibility to EVG; addition of the F121Y or S153Y or R263K mutations further decreased EVG susceptibility to 34-, 37- and 94-fold reduced, relative to wild-type. The T66I/A/K mutations were also observed in dose escalation experiments *in vitro* with EVG performed by GlaxoSmithKline, along with V72A, E92Q/V, P145S, Q146L/S and Q148K/R IN mutations; T124A and A128T IN mutations, which may be natural IN polymorphisms, also developed (Kobayashi et al., 2008).

In virus breakthrough experiments using high multiplicity of infection, the Q148R IN mutation appeared in HIV-1 HXB2 selected with 25 nM EVG, whereas T66A, V72I and N155S IN mutations emerged under selection with 100 nM EVG (Witmer et al., 2007). Eight independent selection experiments *in vitro* using HIV-1 III_B and EVG at 3 μM concentration resulted in the emergence of multiple IN mutation patterns including T66I alone, E92Q alone, E92Q + T66A (in two independent experiments), T66I + R20K + L74M + S230R, T66I + Q148R + A128T + E138K + S230R, Q148R + E138K and Q148Q/R + E92E/Q + E138E/K. Viral pools containing these mutations showed evidence of reduced susceptibility to EVG and RAL (Goethals et al., 2008). Therefore, EVG can select several INSTI resistance mutations *in vitro* including H51Y, T66I/A/K, V72A,

L74M, E92Q/V, Q95K, F121Y, E138K, P145S, Q146L/P/S, S147G, Q148R/K, S153Y/F, N155S, E157Q, S230R and R263K (Table 1). Q148 and N155 represent IN codons at which primary INI resistance mutations to RAL also occur.

In the EVG phase 2 clinical trial, Study 0105, VFs on EVG/r 125 mg ($n = 28$) were analyzed for the development of genotypic and phenotypic resistance to INSTIs. The E92Q, Q148R/H/K and N155H mutations were the most common INSTI resistance mutations that emerged, each occurring in about 40% of subjects (McColl et al., 2007). Other INSTI resistance mutations that emerged in Study 0105 included H51Y, T66I/A/K, V72I, Q95K, E138K, G140C/S, S147G, E157Q and S230R. Thus, clinical primary INSTI resistance mutations for both EVG and RAL include mutations at codons Q148 and N155, indicative of genotypic cross resistance. However, development of the E92Q mutation appears to be more commonly associated with EVG compared to RAL, consistent with E92Q mediating a higher fold change to EVG compared to RAL (approximately 30-fold versus 6-fold, respectively). In contrast, mutations at position Y143 were more commonly observed in RAL virologic failures and have not been observed in to develop on EVG. These observations probably reflect subtle differences in the binding of the two INSTIs to the catalytic core domain. Indeed, modeling suggests a key role for residue E92 in binding of EVG to the CCD (Fig. 5). EVG and RAL phe-

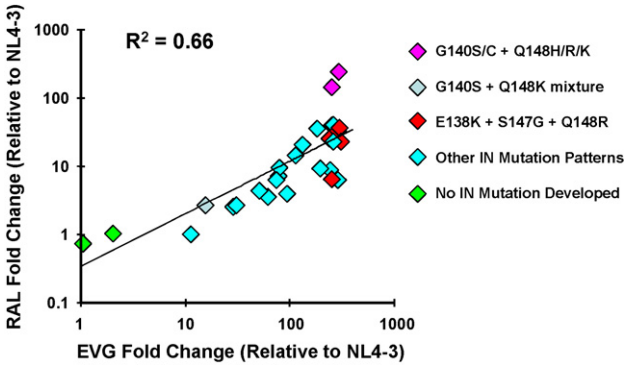

Fig. 7. Correlation of RAL versus EVG resistance.
Plot showing fold change in phenotypic susceptibility (relative to wild-type virus, NL4-3 strain) to elvitegravir versus raltegravir (PhenoSense™ Integrase Assay, Monogram Biosciences, South San Francisco) for viruses derived from virologic failures ($n = 28$) on EVG/r 125 mg (Study GS-US-183-0105). Development of reduced susceptibility to elvitegravir following virologic failure on EVG/r 125 mg was strongly correlated with reduced susceptibility to raltegravir, indicative of cross-resistance between these two drugs. Note that viruses containing mutations at integrase codon Q148 were often associated with the highest level of resistance to both INSTIs, particularly the pattern G140S/C + Q148R/H/K.

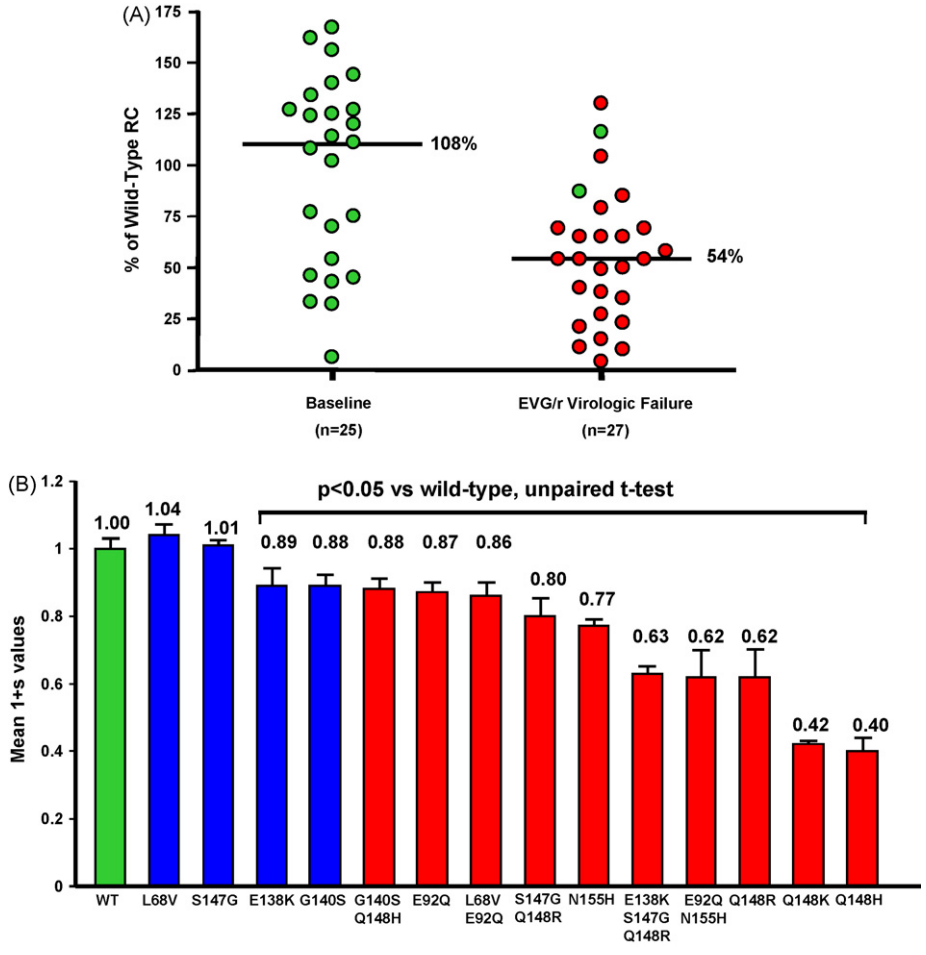

Fig. 8. Effect of integrase resistance mutations on HIV replicative capacity and relative fitness.
(A) Viral replication capacity (RC, expressed as percent RC versus wild-type, NL4-3, PhenoSense™ Integrase Assay, Monogram Biosciences, South San Francisco) of viruses derived from subjects from EVG/r study 0105. At baseline, median RC was 108%, i.e. equivalent to wild-type. At virologic failure, the median RC had fallen to 54% ($p < 0.005$ versus baseline RC) and correlated with the development of IN mutations, including E92Q, Q148R/H/K and N155H in most cases. (Data from McColl et al., 2007). (B) Competitive relative fitness scores (1 + S, versus wild-type) for site directed mutant viruses carrying integrase resistance mutations. Note that all viruses carrying one or more primary resistance mutations at IN codons E92, Q148 or N155 had significantly reduced relative fitness compared to wild-type, whereas some secondary resistance mutations, like L68V or S147G have no impact on relative fitness. Mutants at codon Q148 caused the strongest reductions in relative fitness, however addition of secondary mutations such as G140S (compare Q148H alone versus G140S + Q148H) partially compensated for the fitness defect produced by the primary resistance mutation. (Data from Goodman et al., 2008)

notypes showed a significant reduction in susceptibility to both drugs at VF with mean reductions of >151 and >28-fold, respectively. Furthermore, there was a linear relationship between EVG and RAL susceptibility in these clinical isolates, indicative of clinical cross-resistance between these two INSTIs (Fig. 7). Novel IN mutations developed in multiple subjects including L68V/I, Q95R, S119G/R and D232N/G/H mutations; the L68V/I and E92Q mutations were always associated together. Clonal analyses of Study 0105 VFs demonstrated that the L68V/I and E92Q mutations were always detected on the same viral genome and the former tended to increase resistance to both EVG and RAL (Goodman et al., 2008).

As for RAL, mutual exclusions among primary resistance mutations selected by EVG were also observed. The E92Q mutation could be found on the same viral genome as the N155H mutation but not on the same viral genome as the Q148R mutation. Viral replication capacity with respect to IN also declined significantly upon emergence of INSTI resistance mutations in both single cycle and multiple cycle competitive fitness assays (Fig. 8a and b). The E92Q, Q148R and N155H mutations also showed significantly reduced fitness relative to wild-type; viruses carrying the Q148R/H/K mutations showed the lowest fitness of the single mutants tested. Site directed mutant viruses carrying both the E92Q and Q148R mutations, or both the Q148R and N155H mutations showed even greater reductions in viral fitness compared to Q148R alone; viruses carrying these combinations of mutations may not emerge clinically due to defects in viral replication. Secondary mutations like S147G when added to Q148R caused both increased resistance to EVG and RAL and also increased viral RC, suggesting evolution to both higher levels of INSTI resistance and in some cases, higher levels of fitness (Fig. 8b). In other cases, such as E92Q + N155H, the virus has high level resistance but at the cost of reduced fitness compared to either single mutant (Goodman et al., 2008).

Longitudinal analysis showed that initial patterns of EVG resistance, like that for RAL, were relatively simple, usually involving 1–2 INSTI resistance mutations, of which the E92Q mutation was the most common occurring in 50% patients with EVG VF (Waters et al., 2009). Resistance patterns further evolved by genotypic switching among primary resistance pathways, addition of further IN mutations or remained unchanged. Genotypic switching occurred in approximately 40% of subjects and the most common genotypic switch was from an initial E92Q pattern to a Q148R/H pattern, reminiscent of RAL in which initial N155H-containing patterns switched to the Q148 pathway. Among subjects adding IN mutations, the majority started with the E92Q mutation and added a variety of other integrase mutations including H51Y, T66A, L68V, V72I, T97A, E138K, S147G and E157Q. Genotypic switching or addition of other IN mutations to an initial pattern generally resulted in higher levels of phenotypic resistance to EVG and often, cross-resistance to RAL. Subjects who developed INSTI resistance but had limited further evolution of their IN genotype had a variety of IN mutations including E92G (a less prevalent mutant at this codon), E138K, G140C/G/S, Q148R/K, N155H and P145S.

In summary, clinical resistance to EVG and RAL involves primary resistance mutations in common, notably Q148R/H/K and N155H (Fig. 9 and Table 2). Structurally, the major primary INSTI resistance mutations all occur within the CCD of IN (Fig. 9); a few secondary mutations also occur in the CCD, highlighting the probable role of the viral DNA in forming the relevant conformation of IN for binding of INSTIs. No INSTI resistance mutations have been identified in the NTD of IN with mutation H51Y being the most N-terminal INSTI resistance mutation described (Shimura et al., 2008). Mutations Q148R/H often associate with mutations E138K, G140C/S or S147G, leading to high level resistance to both EVG and RAL. E92Q and Y143C/H/R also play a role in RAL resistance but are less commonly observed in RAL failures whereas E92Q is more commonly observed

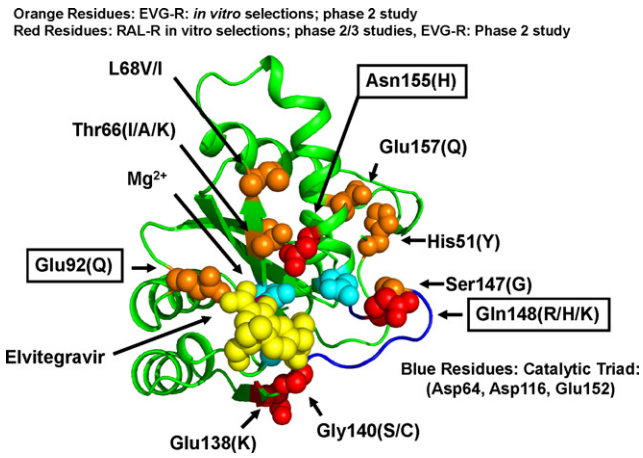

Fig. 9. Model of EVG bound to IN CCD and integrase mutations associated with resistance.
Ribbon model of the CCD of integrase (shown in green) with a space filling model of elvitegravir (shown in yellow) modeled bound to the CCD. The catalytic trial residues (Asp64, Asp116 and Glu 152) are shown in blue; the bound Mg^{2+} is shown in magenta (partially obscured in this view behind the model of elvitegravir). Residues associated with resistance are shown in orange or red and have been observed in both *in vitro* and clinical studies of elvitegravir and raltegravir. Primary resistance mutations are highlighted in boxes. Some residue associated with resistance are located close to the site of inhibitor binding and may make direct interactions with the inhibitor, such as E92, or may directly affect metal binding, such as N155. Other resistance associated residues, such as Q148, cluster on a flexible loop (shown in blue). Q148 makes a direct contact with a 5′cytosine on the viral DNA.

in EVG failures. For the N155H pathway, secondary mutations can include L74M, E92Q, T97A, V151L and G163R. For EVG, Q148R/H/K and N155H patterns are also primary resistance patterns, as is the E92Q mutation. E92Q causes 30–40 fold reduction in susceptibility to EVG compared to about 6-fold reduction to RAL (Table 2). Interestingly, the converse situation is true for the Q148H IN muta-

Table 2
Susceptibilities of IN mutant HIV-1 to INSTIs and control compounds. Fold changes in susceptibility for various single, double and triple mutants against elvitegravir, raltegravir and control ARVs (tenofovir and lopinavir). Results are taken from McColl et al. (2007) and Goodman et al. (2008).

IN mutation	Fold resistance relative to WT[a]			
	EVG	RAL	TFV	LPV
L68I	0.9	0.9	1	0.9
L68V	1.6[b]	1	1.1	0.9
E92Q	40	6	0.9	1
L68I, E92Q	43	7.6	0.8	0.8
L68V, E92Q	67[c]	14[c]	1.2	1
N155H	36	21	1.1	0.8
L68V, N155H	51	24	0.7	0.6
Q148R	109	35	0.8	0.7
L68V, Q148R	139[c]	53[c]	0.6	0.6
Q148K	50	27	0.8	0.7
G140S	4	1.6[b]	0.9	1
Q148H	5.3	18	1	0.8
G140S, Q148H	>1000	>1000	1	1.1
E138K	0.6	0.9	1.1	1.1
S147G	9.4	1	0.8	0.9
E138K, S147G, Q148R	154	34	1	0.9
E92Q, N155H	125	105	0.8	0.6
Q148R, N155H[d]	229	102	0.7	0.5

[a] Mean fold changes in susceptibility calculated from at least $n = 3$ experiments in all cases: EVG = elvitegravir, RAL = raltegravir, TFV = tenofovir, LPV = lopinavir.
[b] $p < 0.05$ (unpaired *t*-test) versus WT for mutants with <2.5-fold change to EVG or RAL.
[c] $p < 0.05$ (unpaired *t*-test) for comparison of single mutant (E92Q, N155H or Q148R) versus double mutant with L68V.
[d] This pattern of primary resistance mutations (Q148R + N155H) is unlikely to occur clinically; these mutations tend to develop on separate viral genomes as observed in both RAL and EVG clinical studies.

tion which as a single mutation causes higher levels of resistance to RAL than EVG; this may explain why the Q148H + G140C/S pattern is more prevalent in RAL versus EVG VFs (Table 2). Other mutations associated with E92Q in EVG VFs are H51Y, T66I/A/K, L68V/I, V72I, E138K, S147G and E157Q. The E138K and S147G mutations seem to be somewhat more "promiscuous" in their associations, being also commonly observed in association with Q148R in EVG virologic failures. Overall, mutations at codon Q148 also seem to confer the highest level of resistance to EVG and RAL as single mutations and when combined with secondary IN mutations, but they are also associated with the strongest reductions in viral RC (Fig. 8). Evolution of genotypic resistance to INSTIs appears to lead to higher levels of resistance and cross-resistance, and in some cases, acts to rescue viral RC.

The overlap of the INSTI resistance patterns selected by EVG and RAL and the observation that VFs can evolve multiple INSTI resistant viral quasi-species implies that sequencing from EVG to RAL (or vice versa) following VF is unlikely to be successful. Sequencing among INSTIs will probably require the development of compounds with novel modes of binding and non-overlapping resistance profiles. Merck Research Laboratories have disclosed prototypic examples of second generation INSTIs like MK-2048, which retain antiviral activity against several (but not all) key INSTI resistance mutants (Vacca et al., 2007; Wai et al., 2007); such compounds are yet to enter into clinical development. S/GSK-1349572 may have an improved resistance profile but capacity to use S/GSK1349572 in subjects with RAL virologic failure remains to be determined (Underwood et al., 2009). Molecular studies comparing the binding and dissociation of EVG, RAL and "second generation" INSTIs may help to elucidate distinct binding mechanisms of INSTIs in the CCD and their respective resistance profiles.

5. Future directions

With the emergence of INSTIs, the potential for novel first line regimens and shifts in treatment paradigms may become possible. RAL was approved in June 2009 by the FDA for use in first line ARV therapy; and EVG is in advanced clinical trials. NRTI sparing therapy may become a possibility with the emergence of INSTIs by combining them with PIs. Only large scale clinical trials can determine if such novel combinations of drugs provide an advantage in terms of long term efficacy, safety, tolerability and reduction in resistance emergence. Although MK-2048 has not entered clinical development, the implication is that like other ARV drugs classes, second generation INSTIs with orthogonal resistance profiles may emerge. Fixed dose combination products containing an INSTI, like elvitegravir/GS-9350/emtricitabine/tenofovir DF may also expand the options available for the first line therapy.

INSTIs may also have a role in pre-exposure or post-exposure prophylaxis or as antiviral microbicides for the prevention of HIV-1 transmission. Integration represents the final step before irreversible infection of a target cell and the blockage of integration leads to the production of dead-end viral DNA intermediates, the 2-LTR circles, from which viral replication cannot proceed. As such, fixed dose combinations of INSTIs with NRTIs may be highly effective combinations for prevention of HIV transmission as both act prior to integration and their combination could be expected to synergize with one another and limit the potential selection of resistance. Much remains to be learned about the long term efficacy, safety, and mode of action of INSTIs, for example the basis of the rapid viral load declines observed upon initiation of INSTI therapy and whether this has long-term clinical benefit. The molecular mechanisms of resistance mediated by the many INSTI resistance mutations described thus far also require further elucidation. The arrival of INSTIs as a new potent class of ARV signals a new era in the treatment of HIV and provides hope for continued improvement in the management of patients afflicted with this epidemic disease. Considering the experience of the previous 25 years of ARV drug development, we can be certain that the potential of INSTIs to improve ARV therapy is only beginning to be explored.

References

Alian, A., Griner, S.L., Chiang, V., Tsiang, M., Jones, G., Birkus, G., Geleziunas, R., Leavitt, A.D., Stroud, R.M., 2009. Catalytically-active complex of HIV-1 integrase with a viral DNA substrate binds anti-integrase drugs. Proceedings of the National Academy of Sciences United States of America 106, 8192–8197.

Arribas, J.R., Pozniak, A.L., Gallant, J.E., Dejesus, E., Gazzard, B., Campo, R.E., Chen, S.S., McColl, D., Holmes, C.B., Enejosa, J., Toole, J.J., Cheng, A.K., 2008. Tenofovir disoproxil fumarate, emtricitabine, and efavirenz compared with zidovudine/lamivudine and efavirenz in treatment-naive patients: 144-week analysis. Journal of Acquired Immune Deficiency Syndromes (JAIDS) 47, 74–78.

Bartlett, J.A., Chen, S.S., Quinn, J.B., 2007. Comparative efficacy of nucleoside/nucleotide reverse transcriptase inhibitors in combination with efavirenz: results of a systematic overview. HIV Clinical Trials 8, 221–226.

Billich, A., 2003. S-1360 Shionogi-GlaxoSmithKline. Current Opinion in Investigational Drugs 4, 206–209.

Bouziane, M., Cherny, D.I., Mouscadet, J.F., Auclair, C., 1996. Alternate strand DNA triple helix-mediated inhibition of HIV-1 U5 long terminal repeat integration in vitro. Journal of Biological Chemistry 271, 10359–10364.

Bukrinsky, M., Sharova, N., Stevenson, M., 1993. Human immunodeficiency virus type 1 2-LTR circles reside in a nucleoprotein complex which is different from the preintegration complex. Journal of Virology 67, 6863–6865.

Burke, C.J., Sanyal, G., Bruner, M.W., Ryan, J.A., LaFemina, R.L., Robbins, H.L., Zeft, A.S., Middaugh, C.R., Cordingley, M.G., 1992. Structural implications of spectroscopic characterization of a putative zinc finger peptide from HIV-1 integrase. Journal of Biological Chemistry 267, 9639–9644.

Bushman, F.D., 1999. Host proteins in retroviral cDNA integration. Advances in Virus Research 52, 301–317.

Bushman, F.D., Engelman, A., Palmer, I., Wingfield, P., Craigie, R., 1993. Domains of the integrase protein of human immunodeficiency virus type 1 responsible for polynucleotidyl transfer and zinc binding. Proceedings of the National Academy of Sciences United States of America 90, 3428–3432.

Bushman, F.D., Fujiwara, T., Craigie, R., 1990. Retroviral DNA integration directed by HIV integration protein in vitro (Abstract). Science 249, 1555–1558.

Cai, M., Zheng, R., Cafferey, M., Craigie, R., Clore, G.M., A.M., G., 1997. Solution structure of the N-terminal zinc binding domain of HIV-1 integrase (Abstract). Nature Structural Biology 4, 567–577.

Ceccherini-Silberstein, F., Armenia, D., D'Arrigo, R., Micheli, V., Fabeni, L., Meraviglia, P., Capetti, A., Zaccarelli, M., Trotta, M.P., Narciso, P., Antinori, A., Perno, C.F., 2008. Virological response and resistance in multi-experienced patients treated with raltegravir (poster 18). In: XVII International HIV Drug Resistance Workshop, Sitges, Spain.

Cihlar, T., Ray, A., 2010. Nucleoside and nucleotide HIV reverse transcriptase inhibitors: 25 year after zidovudine. Antiviral Research 85.

Colicelli, J., Goff, S.P., 1988. Sequence and spacing requirements of a retrovirus integration site (Abstract). Journal of Molecular Biology 199, 47–59.

Cooper, D.A., Steigbigel, R.T., Gatell, J., Rockstroh, J., Katlama, C., Yeni, P., Lazzarin, A., Clotet, B., Kumar, P., Eron, J.E., Schechter, M., Markowitz, M., Loutfy, M.R., Lennox, J.L., Zhao, J., Chen, J., Ryan, D.M., Rhodes, R.R., Killar, J.A., Gilde, L.R., Strohmaier, K.M., Meibohm, A.R., Miller, M.D., Hazuda, D.J., Nessly, M.L., DiNubile, M.J., Isaacs, R.D., Teppler, H., Nguyen, B., 2008. Subgroup and resistance analyses of raltegravir for resistance HIV-1 infection. New England Journal of Medicine 359, 355–365.

Chen, I.J., Neamati, N., Nicklaus, M.C., Orr, A., Anderson, L., Barchi Jr., J.J., Kelley, J.A., Pommier, Y., MacKerell Jr., A.D., 2000. Identification of HIV-1 integrase inhibitors via three-dimensional database searching using ASV and HIV-1 integrases as targets. Bioorganic and Medicinal Chemistry 8, 2385–2398.

Chen, X., Tsiang, M., Yu, F., Hung, M., Jones, G.S., Zeynalzadegan, A., Qi, X., Jin, H., Kim, C.U., Swaminathan, S., Chen, J.M., 2008. Modeling, analysis, and validation of a novel HIV integrase structure provide insights into the binding modes of potent integrase inhibitors. Journal of Molecular Biology 380, 504–519.

Da Silva, D., Pellegrin, I., Anies, G., Breilh, D., Wittkop, L., Morlat, P., Dupon, M., Neau, D., Pellegrin, J.L., Fleury, H., Masquelier, B., 2008. Mutational patterns in the HIV-1 integrase related to virological failures on raltegravir-containing regimens (poster 12). In: XVII International HIV Drug Resistance Workshop, Sitges, Spain.

Davies, D.R., Goryshin, I.Y., Reznikoff, W.S., Rayment, I., 2000. Three-dimensional structure of the Tn5 synaptic complex transposition intermediate. Science 289, 77–85.

de Bethune, M.P., 2010. Non-nucleoside reverse transcriptase inhibitors (NNRTIs), their discovery, development, and use in the treatment of HIV-1 infection: a review of the last 20 years (1989–2009). Antiviral Research 85, XX-XX.

DeJesus, E., Berger, D., Markowitz, M., Cohen, C., Hawkins, T., Ruane, P., Elion, R., Farthing, C., Cheng, A., Kearney, B., 2006a. The HIV integrase inhibitor GS-9137 (JTK-303) exhibits potent antiviral activity in treatment-naïve and experienced patients (oral number 36). Journal of Acquired Immune Deficiency Syndromes 43, 1–5.

DeJesus, E., Berger, D., Markowitz, M., Cohen, C., Hawkins, T., Ruane, P., Elion, R., Farthing, C., Zhong, L., Cheng, A.K., McColl, D., Kearney, B.P., 2006b. Antiviral activity,

pharmacokinetics, and dose response of the HIV-1 integrase inhibitor GS-9137 (JTK-303) in treatment-naive and treatment-experienced patients. Journal of Acquired Immune Deficiency Syndromes (JAIDS) 43, 1–5.
Drelich, M., Wilhelm, R., Mous, J., 1992. Identification of amino acid residues critical for endonuclease and integration activities of HIV-1 IN protein *in Vitro* (Abstract). Virology 188, 459–468.
Dyda, F., Hickman, A.B., Jenkins, T.M., Engelman, A., Craigie, R., Davies, D.R., 1994. Crystal structure of the catalytic domain of HIV-1 integrase: similarity to other polynucleotidyl transferases (Abstract). Science 266, 1981–1986.
Ellison, V., Gerton, J., Vincent, K.A., Brown, P.O., 1995. An essential interaction between distinct domains of HIV-1 integrase mediates assembly of the active multimer. Journal of Biological Chemistry 270, 3320–3326.
Engelman, A., Bushman, F.D., Craigie, R., 1993. Identification of discrete functional domains of HIV-1 integrase and their organization within an active multimeric complex. EMBO Journal 12, 3269–3275.
Engelman, A., Craigie, R., 1992. Identification of conserved amino acid residues critical for human immunodeficiency virus type 1 integrase function in vitro. Journal of Virology 66, 6361–6369.
Eron, J., Andrade, J., Zajdenverg, R., Workman, C., Cooper, D., Young, B., Xu, X., Nguyen, B.Y., Leavitt, R., Sklar, P., 2009. Switching from stable lopinavir/ritonavir-based to raltegravir-based combination ART resulted in a superior lipid profile at week 12 but did not demonstrate non-inferior virologic efficacy at week 24 (abstract 70aLB). In: 16th Conference on Retroviruses and Opportunistic Infections (CROI), Montréal, Canada.
Espeseth, A.S., Felock, P., Wolfe, A., Witmer, M., Grobler, J., Anthony, N., Egbertson, M., Melamed, J.Y., Young, S., Hamill, T., Cole, J.L., Hazuda, D.J., 2000. HIV-1 integrase inhibitors that compete with the target DNA substrate define a unique strand transfer conformation for integrase. Proceeding of the National Academy of Sciences United States of America 97, 11244–11249.
Farnet, C.M., Bushman, F.D., 1997. HIV-1 cDNA integration: requirement of HMG I(Y) protein for function of preintegration complexes in vitro. Cell 88, 483–492.
Fesen, M.R., Kohn, K.W., Leteurtre, F., Pommier, Y., 1993. Inhibitors of human immunodeficiency virus integrase. Proceedings of the National Academy Sciences United States of America 90, 2399–2403.
Fikkert, V., Hombrouck, A., Van Remoortel, B., De Maeyer, M., Pannecouque, C., De Clercq, E., Debyser, Z., Witvrouw, M., 2004. Multiple mutations in human immunodeficiency virus-1 integrase confer resistance to the clinical trial drug S-1360. AIDS 18, 2019–2028.
Fikkert, V., Van Maele, B., Vercammen, J., Hantson, A., Van Remoortel, B., Michiels, M., Gurnari, C., Pannecouque, C., De Maeyer, M., Engelborghs, Y., De Clercq, E., Debyser, Z., Witvrouw, M., 2003. Development of resistance against diketo derivatives of human immunodeficiency virus type 1 by progressive accumulation of integrase mutations. Journal of Virology 77, 11459–11470.
Fransen, S., Gupta, S., Danovich, R., Hazuda, D., Miller, M., Witmer, M., Petropoulos, C.J., Parkin, N.T., Huang, W., 2008. Loss of raltegravir susceptibility in treated patients is conferred by multiple non-overlapping genetic pathways (poster 7). In: XVII International HIV Drug Resistance Workshop, Sitges, Spain.
Gao, K., Gorelick, R.J., Johnson, D.G., Bushman, F., 2003. Cofactors for human immunodeficiency virus type 1 cDNA integration in vitro. Journal of Virology 77, 1598–1603.
Garvey, E.P., Johns, B.A., Gartland, M.J., Foster, S.A., Miller, W.H., Ferris, R.G., Hazen, R.J., Underwood, M.R., Boros, E.E., Thompson, J.B., Weatherhead, J.G., Koble, C.S., Allen, S.H., Schaller, L.T., Sherrill, R.G., Yoshinaga, T., Kobayashi, M., Wakasa-Morimoto, C., Miki, S., Nakahara, K., Noshi, T., Sato, A., Fujiwara, T., 2008. The naphthyridinone GSK-364735 is a novel, potent human immunodeficiency virus type 1 integrase inhibitor and antiretroviral. Antimicrobial Agents and Chemotherapy 52, 901–908.
Goethals, O., Clayton, R., Wagemans, E., Van Ginderen, M., Cummings, M., Geluykens, P., Dockx, K., Smits, V., Meersseman, G., Jochmans, D., Hallenberger, S., Hertogs, K., 2008. Resistance mutations in HIV-1 integrase selected with raltegravir or elvitegravir confer reduced susceptibility to a diverse panel of integrase inhibitors (poster 9). In: XVII International HIV Drug Resistance Workshop (IHDRW), Sitges, Spain.
Goff, S.P., 1992. Genetics of retroviral integration (Abstract). Annual Review of Genetics 26, 527–544.
Goldgur, Y., Craigie, R., Cohen, G.H., Fujiwara, T., Yoshinaga, T., Fujishita, T., Sugimoto, H., Endo, T., Murai, H., Davies, D.R., 1999. Structure of the HIV-1 integrase catalytic domain complexed with an inhibitor: a platform for antiviral drug design. Proceedings of National Academy of Sciences United States of America 96, 13040–13043.
Goodman, D.D., Hluhanich, R., Waters, J.M., Margot, N.A., Fransen, S., Gupta, S., Huang, W., Parkin, N., Borroto-Esoda, K., Svarovskaia, E.S., Miller, M.D., McColl, D.J., 2008. Integrase inhibitor resistance involves complex interactions among primary and secondary resistance mutations: a novel mutation L68V/I associates with E92Q and increases resistance (poster 13). In: XVII International HIV Drug Resistance Workshop, Stiges, Spain.
Grinsztejn, B., Nguyen, B., Katlama, C., Gatell, J., Lazzarain, A., Vittecoq, D., Gonzalex, C., Chen, J., Isaacs, R., 2007. 48 week efficacy and safety of MK-0518, a novel HIV-1 integrase inhibitor, in patients with triple-class resistant virus (abstract H-173). In: 47th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC), Chicago, IL, USA.
Grobler, J.A., Stillmock, K., Hu, B., Witmer, M., Felock, P., Espeseth, A.S., Wolfe, A., Egbertson, M., Bourgeois, M., Melamed, J., Wai, J.S., Young, S., Vacca, J., Hazuda, D.J., 2002. Diketo acid inhibitor mechanism and HIV-1 integrase: implications for metal binding in the active site of phosphotransferase enzymes. Proceedings of the National Academy of Sciences United States of America 99, 6661–6666.
Hatano, H., Lampiris, H., Huang, W., Hoh, R., Gupta, S., Fransen, S., Martin, J.N., Petropoulous, C., Deeks, S.G., 2008. Virologic and immunologic outcomes in a cohort of subjects failing integrase inhibitors (poster 2725). In: XVII International HIV Drug Resistance Workshop, Sitges, Spain.
Hazuda, D., Blau, C.U., Felock, P., Hastings, J., Pramanik, B., Wolfe, A., Bushman, F., Famet, C., Goetz, M., Williams, M., Silverman, K., Lingham, R., Singh, S., 1999. Isolation and characterization of novel human immunodeficiency virus integrase inhibitors from fungal metabolites (Abstract). Antiviral Chemistry and Chemotherapy 10, 63–70.
Hazuda, D.J., Anthony, N.J., Gomez, R.P., Jolly, S.M., Wai, J.S., Zhuang, L., Fisher, T.E., Embrey, M., Guare Jr., J.P., Egbertson, M.S., Vacca, J.P., Huff, J.R., Felock, P.J., Witmer, M.V., Stillmock, K.A., Danovich, R., Grobler, J., Miller, M.D., Espeseth, A.S., Jin, L., Chen, I.W., Lin, J.H., Kassahun, K., Ellis, J.D., Wong, B.K., Xu, W., Pearson, P.G., Schleif, W.A., Cortese, R., Emini, E., Summa, V., Holloway, M.K., Young, S.D., 2004. A naphthyridine carboxamide provides evidence for discordant resistance between mechanistically identical inhibitors of HIV-1 integrase. Proceedings of the National Academy of Sciences United States of America 101, 11233–11238.
Hazuda, D.J., Felock, P., Witmer, M., Wolfe, A., Stillmock, K., Grobler, J.A., Espeseth, A., Gabryelski, L., Schleif, W., Blau, C., Miller, M.D., 2000. Inhibitors of strand transfer that prevent integration and inhibit HIV-1 replication in cells. Science 287, 646–650.
Hazuda, D.J., Hastings, J.C., Wolfe, A.L., Emini, E.A., 1994. A novel assay for the DNA strand-transfer reaction of HIV-1 integrase. Nucleic Acids Research 22, 1121–1122.
Hazuda, D.J., Miller, M.D., Nguyen, B.Y., Zhao, J., 2007. Resistance to the HIV-integrase inhibitor raltegravir: analysis of protocol 005, a phase 2 study in patients with triple-class resistant HIV-1 infection (poster #8). In: 16th International HIV Drug Resistance Workshop, Bridgetown, Barbados.
Hombrouck, A., Voet, A., Van Remoortel, B., Desadeleer, C., De Maeyer, M., Debyser, Z., Witvrouw, M., 2008. Mutations in HIV-1 integrase confer resistance to the naphthyridine L-870,810 and cross resistance to the clinical trial drug GS-9137. Antimicrobial Agents and Chemotherapy.
Johnson, A.A., Santos, W., Pais, G.C., Marchand, C., Amin, R., Burke Jr., T.R., Verdine, G., Pommier, Y., 2006. Integration requires a specific interaction of the donor DNA terminal 5′-cytosine with glutamine 148 of the HIV-1 integrase flexible loop. Journal of Biological Chemistry 281, 461–467.
Jones, G., Ledford, R.M., Yu, F., Chen, X., Miller, M.D., Tsiang, M., McColl, D.J., 2007. In vitro resistance profile of HIV-1 mutants selected by the HIV-1 integrase inhibitor GS-9137 (JTK-303) (slides-poster #627). In: 14th Conference on Retroviruses and Opportunistic Infections, Los Angeles, CA, USA.
Jones, G.S., Yu, F., Zeynalzadegan, A., Hesselgesser, J., Chen, X., Chen, J., Jin, H., Kim, C.U., Wright, M., Geleziunas, R., Tsiang, M., 2009. Preclinical evaluation of GS-9160, a novel inhibitor of human immunodeficiency virus type 1 integrase. Antimicrobial Agents and Chemotherapy 53, 1194–1203.
Kalpana, G.V., Marmon, S., Wang, W., Crabtree, G.R., Goff, S.P., 1994. Binding and stimulation of HIV-1 integrase by a human homolog of yeast transcription factor SNF5 (Abstract). Science 266, 2002–2006.
Katayanagi, K., Okumara, M., Morikawa, K., 1993. Crystal structure of Esherichia coli RNase HI in complex with Mg2+ at 2.8 A resolution: proof for a single Mg(2+)-binding site (Abstract). Proteins 17, 337–346.
Katlama, C., Caby, F., Schneider, L., Andrade, R.M., Tubiana, A., Valantin, M.A., Ktorza, N., Malet, I., Peytavin, G., Canestri, A., Calvez, V., 2008. Virological evolution in HIV treatment-experienced patients with raltegravir-based salvage regimens (poster 11). In: XVII International HIV Drug Resistance Workshop, Sitges, Spain.
Kawaguchi, I., Ishikawa, T., Ishibashi, M., Irie, S., Kakee, A., 2006. Safety and pharmacokinetics of single oral dose of JTK-303/GS-9137, a novel HIV integrase inhibitor, in healthy volunteers (poster number 580). In: 13th Conference on Retroviruses and Opportunistic Infections, Denver, CO, USA.
Kearney, B.P., Mathias, A., Zhong, L., DeJesus, E., Markowitz, M., Hawkins, T., Cheng, A., 2006. Pharmacokinetics/pharmacodynamics of GS-9137 an HIV integrase inhibitor in treatment-naïve and experienced patients (abstract 73). In: 7th International Workshop on Clinical Pharmacology of HIV Therapy, Lisbon, Portugal.
King, P.J., Lee, D.J., Reinke, R.A., Victoria, J.G., Beale, K., Robinson Jr., W.E., 2003. Human immunodeficiency virus type-1 integrase containing a glycine to serine mutation at position 140 is attenuated for catalysis and resistant to integrase inhibitors. Virology 306, 147–161.
King, P.J., Robinson Jr., W.E., 1998. Resistance to the anti-human immunodeficiency virus type 1 compound L-chicoric acid results from a single mutation at amino acid 140 of integrase. Journal of Virology 72, 8420–8424.
Kobayashi, M., Nakahara, K., Seki, T., Miki, S., Kawauchi, S., Suyama, A., Wakasa-Morimoto, C., Kodama, M., Endoh, T., Oosugi, E., Matsushita, Y., Murai, H., Fujishita, T., Yoshinaga, T., Garvey, E., Foster, S., Underwood, M., Johns, B., Sato, A., Fujiwara, T., 2008. Selection of diverse and clinically relevant integrase inhibitor-resistant human immunodeficiency virus type 1 mutants. Antiviral Research, doi: 10.1016/j.antiviral.2008.06012.
Kulkosky, J., Jones, K.S., Katz, R.A., Mack, J.P., Skalka, A.M., 1992. Residues critical for retroviral integrative recombination in a region that is highly conserved among retroviral/retrotransposon integrases and bacterial insertion sequence transposases. Molecular and Cellular Biology 12, 2331–2338.
LaFemina, R.L., Callahan, P.L., Cordingley, M.G., 1991. Substrate specificity of recombinant human immunodeficiency virus integrase protein. Journal of Virology 65, 5624–5630.
Lalezari, J., Sloan, L., DeJesus, E., Hawkins, T., McCurdy, L., Song, I., Borland, J., Stroder, R., Chen, S., Lou, Y., Underwood, M., Fujiwara, T., Piscitelli, S., Min, S., 2009. Potent antiviral activity of S/GSK1349572, a next generation integrase inhibitor (INI),

in INI-Naïve HIV-1-infected patients: ING111521 Protocol (Abstract TUAB105). In: IAS 2009-5th Conference on HIV Pathogenesis, Treatment and Prevention, Cape Town, South Africa.

Lazzarin, A., Campbell, T., Clotet, B., Johnson, M., Katlama, C., Moll, A., Towner, W., Trottier, B., Peeters, M., Vingerhoets, J., de Smedt, G., Baeten, B., Beets, G., Sinha, R., Woodfall, B., 2007. Efficacy and safety of TMC125 (etravirine) in treatment-experienced HIV-1-infected patients in DUET-2: 24-week results from a randomised, double-blind, placebo-controlled trial. Lancet 370, 39–48.

Leavitt, A.D., Rose, R.B., Varmus, H.E., 1992. Both substrate and target oligonucleotide sequences affect in vitro integration mediated by human immunodeficiency virus type 1 integrase protein produced in *Saccharomyces cerevisiae*. Journal of Virology 66, 2359–2368.

Lennox, J., DeJesus, E., Lazzarin, A., Berger, D., Pollard, R., Madruga, J., Zhao, J., Gilbert, C., Rodgers, A., Teppler, H., Nguyen, B.Y., Leavitt, R., Sklar, P., 2009a. Raltegravir demonstrates durable efficacy through 96 weeks: results from STARTMRK, a phase III study of raltegravir-based vs. efavirenz-based therapy in treatment-naïve HIV+ patients (Poster H924b). In: 49th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC), San Francisco, CA, USA.

Lennox, J.L., DeJesus, E., Lazzarin, A., Pollard, R.B., Madruga, J.V., Berger, D.S., Zhao, J., Xu, X., Williams-Diaz, A., Rodgers, A.J., Barnard, R.J., Miller, M.D., DiNubile, M.J., Nguyen, B.Y., Leavitt, R., Sklar, P., 2009b. Safety and efficacy of raltegravir-based versus efavirenz-based combination therapy in treatment-naive patients with HIV-1 infection: a multicentre, double-blind randomised controlled trial. Lancet 374, 796–806.

Li, L., Yoder, K., Hansen, M.S., Olvera, J., Miller, M.D., Bushman, F.D., 2000. Retroviral cDNA integration: stimulation by HMG I family proteins. Journal of Virology 74, 10965–10974.

Lin, C.W., Engelman, A., 2003. The barrier-to-autointegration factor is a component of functional human immunodeficiency virus type 1 preintegration complexes. Journal of Virology 77, 5030–5036.

Little, S., Drusano, G., Schooley, R., Haas, D., Kumar, P., Hammer, S., McMahon, D., Squires, K., Asfour, R., Richman, D., Chen, J., Saah, A., Leavitt, R., Hazuda, D., Nguyen, B.Y., Protocol 004 Study Team, 2005. Antiretroviral effect of L-000870810, a novel HIV-1 integrase inhibitor, in HIV-1 infected patients (abstract 161). In: 12th Conference on Retroviruses and Opportunistic Infections, Boston, MA, USA.

Lubkowski, J., Yang, F., Alexandratos, J., Wlodawer, A., Zhao, H., Burke Jr., T.R., Neamati, N., Pommier, Y., Merkel, G., Skalka, A.M., 1998. Structure of the catalytic domain of avian sarcoma virus integrase with a bound HIV-1 integrase-targeted inhibitor. Proceedings of the National Academy of Sciences United States of America 95, 4831–4836.

Madruga, J.V., Cahn, P., Grinsztejn, B., Haubrich, R., Lalezari, J., Mills, A., Pialoux, G., Wilkin, T., Peeters, M., Vingerhoets, J., de Smedt, G., Leopold, L., Trefiglio, R., Woodfall, B., 2007. Efficacy and safety of TMC125 (etravirine) in treatment-experienced HIV-1-infected patients in DUET-1: 24-week results from a randomised, double-blind, placebo-controlled trial. Lancet 370, 29–38.

Maertens, G., Cherepanov, P., Pluymers, W., Busschots, K., De Clercq, E., Debyser, Z., Engelborghs, Y., 2003. LEDGF/p75 is essential for nuclear and chromosomal targeting of HIV-1 integrase in human cells. Journal of Biological Chemistry 278, 33528–33539.

Marchand, C., Johnson, A.A., Karki, R.G., Pais, G.C., Zhang, X., Cowansage, K., Patel, T.A., Nicklaus, M.C., Burke, T.R., Pommier, Y., 2003. Metal-dependent inhibition of HIV-1 integrase by beta-diketo acids and resistance of the soluble double-mutant (F185K/C280S) 64, 600–609.

Marchand, C., Zhang, X., Pais, G.C., Cowansage, K., Neamati, N., Burke Jr., T.R., Pommier, Y., 2002. Structural determinants for HIV-1 integrase inhibition by beta-diketo acids. Journal of Biological Chemistry 277, 12596–12603.

Markowitz, M., Morales-Ramirez, J.O., Nguyen, B.Y., Kovacs, C.M., Steigbigel, R.T., Cooper, D.A., Liporace, R., Schwartz, R., Isaacs, R., Gilde, L.R., Wenning, L., Zhao, J., Teppler, H., 2006. Antiretroviral activity, pharmacokinetics, and tolerability of MK-0518, a novel inhibitor of HIV-1 integrase, dosed as monotherapy for 10 days in treatment-naive HIV-1-infected individuals. Journal of Acquired Immune Deficiency Syndromes (JAIDS) 43, 509–515.

Markowitz, M., Nguyen, B.Y., Gotuzzo, E., Mendo, F., Ratanasuwan, W., Kovacs, C., Prada, G., Morales-Ramirez, J.O., Crumpacker, C.S., Isaacs, R.D., Gilde, L.R., Wan, H., Miller, M.D., Wenning, L.A., Teppler, H., 2007. Rapid and durable antiretroviral effect of the HIV-1 Integrase inhibitor raltegravir as part of combination therapy in treatment-naive patients with HIV-1 infection: results of a 48-week controlled study. Journal of Acquired Immune Deficiency Syndromes (JAIDS) 46, 125–133.

McColl, D.J., Fransen, S., Gupta, S., Parkin, N., Margot, N., Ledford, R., Chen, J., Chuck, S., Cheng, A.K., Miller, M.D., 2007. Resistance and cross-resistance to first generation integrase inhibitors: Insights from a phase 2 study of Elvitegravir (GS-9137) (abstract number 9). In: 16th International HIV Drug Resistance Workshop, Bridgetown, Barbados.

Mekouar, K., Mouscadet, J.F., Desmaele, D., Subra, F., Leh, H., Savoure, D., Auclair, C., d'Angelo, J., 1998. Styrylquinoline derivatives: a new class of potent HIV-1 integrase inhibitors that block HIV-1 replication in CEM cells. Journal of Medicinal Chemistry 41, 2846–2857.

Miller, M.D., Danovich, R.M., Ke, Y., Witmer, M., Zhao, J., Harvey, C., Nguyen, B.-Y., Hazuda, D., 2008. Longitudinal analysis of resistance to the HIV-1 integrase inhibitor raltegravir: results from P005, a phase 2 study in treatment experienced patients (poster). In: XVII International HIV Drug Resistance Workshop, Sitges, Spain.

Miller, M.D., Farnet, C.M., Bushman, F.D., 1997. Human immunodeficiency virus type 1 preintegration complexes: studies of organization and composition. Journal of Virology 71, 5382–5390.

Neamati, N., Hong, H., Owen, J.M., Sunder, S., Winslow, H.E., Christensen, J.L., Zhao, H., Burke Jr., T.R., Milne, G.W., Pommier, Y., 1998. Salicylhydrazine-containing inhibitors of HIV-1 integrase: implication for a selective chelation in the integrase active site. Journal of Medicinal Chemistry 41, 3202–3209.

Nicklaus, M.C., Neamati, N., Hong, H., Mazumder, A., Sunder, S., Chen, J., Milne, G.W., Pommier, Y., 1997. HIV-1 integrase pharmacophore: discovery of inhibitors through three-dimensional database searching. Journal of Medicinal Chemistry 40, 920–929.

Pommier, Y., Johnson, A.A., Marchand, C., 2005. Integrase inhibitors to treat HIV/AIDS. Nature Reviews Drug Discovery 4, 236–248.

Raillard, S.A., Joyce, G.F., 1996. Targeting sites within HIV-1 cDNA with a DNA-cleaving ribozyme. Biochemistry 35, 11693–11701.

Rice, P., Craigie, R., Davies, D.R., 1996. Retroviral integrases and their cousins. Current Opinion in Structural Biology 6, 76–83.

Rice, P., Mizuuchi, K., 1995. Structure of the bacteriophage Mu transposase core: a common structural motif for DNA transposition and retroviral integration. Cell 82, 209–220.

Robinson Jr., W.E., Cordeiro, M., Abdel-Malek, S., Jia, Q., Chow, S.A., Reinecke, M.G., Mitchell, W.M., 1996. Dicaffeoylquinic acid inhibitors of human immunodeficiency virus integrase: inhibition of the core catalytic domain of human immunodeficiency virus integrase. Molecular Pharmacology 50, 846–855.

Sato, M., Motomura, T., Aramaki, H., Matsuda, T., Yamashita, M., Ito, Y., Kawakami, H., Matsuzaki, Y., Watanabe, W., Yamataka, K., Ikeda, S., Kodama, E., Matsuoka, M., Shinkai, H., 2006. Novel HIV-1 integrase inhibitors derived from quinolone antibiotics. Journal of Medicinal Chemistry 49, 1506–1508.

Schooley, R.T., Ruane, P., Myers, R.A., Beall, G., Lampiris, H., Berger, D., Chen, S.-S., Miller, M.D., Isaacson, E., Cheng, A.K., 2002. Tenofovir DF in antiretroviral-experienced patients: results from a 48-week, randomized, double-blind study. AIDS 16, 1257–1263.

Sherman, P.A., Fyfe, J.A., 1990. Human immunodeficiency virus integration protein expressed in *Escherichia coli* possesses selective DNA cleaving activity. Proceedings of the National Academy of Sciences United States of America 87, 5119–5123.

Shimura, K., Kodama, E., Sakagami, Y., Matsuzaki, Y., Watanabe, W., Yamataka, K., Watanabe, Y., Ohata, Y., Doi, S., Sato, M., Kano, M., Ikeda, S., Matsuoka, M., 2008. Broad antiretroviral activity and resistance profile of the novel human immunodeficiency virus integrase inhibitor elvitegravir (JTK-303/GS-9137). Journal of Virology 82, 764–774.

Singh, S.B., Zink, D.L., Bills, G.F., Teran, A., Silverman, K.C., Lingham, R.B., Felock, P., Hazuda, D.J., 2003. Four novel bis-(naphtho-gamma-pyrones) isolated from Fusarium species as inhibitors of HIV-1 integrase. Bioorganic & Medicinal Chemistry Letters 13, 713–717.

Sioud, M., Drlica, K., 1991. Prevention of human immunodeficiency virus type 1 integrase expression in *Escherichia coli* by a ribozyme. Proceedings of the National Academy of Sciences United States of America 88, 7303–7307.

Steigbigel, R.T., Cooper, D.A., Eron, J.E., Gatell, J.M., Kumar, P.N., Rockstroh, J.K., Wan, H., Sklar, P., Teppler, H., Nguyen, B.Y., 2009. 96-Week results from BENCHMRK 1&2, phase III studies of raltegravir (RAL) in patients (pts) failing antiretroviral therapy (ART) with triple-class resistant HIV (Poster 571b). In: 16th Conference on Retroviruses and Opportunistic Infections (CROI), Montréal, Canada.

Steigbigel, R.T., Cooper, D.A., Kumar, P.N., Eron, J.E., Schechter, M., Markowitz, M., Loutfy, M.R., Lennox, J.L., Gatell, J.M., Rockstroh, J.K., Katlama, C., Yeni, P., Lazzarin, A., Clotet, B., Zhao, J., Chen, J., Ryan, D.M., Rhodes, R.R., Killar, J.A., Gilde, L.R., Strohmaier, K.M., Neibohm, A.R., Miller, M.D., Hazuda, D.J., Nessly, M.L., DiNubile, M.J., Isaacs, R.D., Nguyen, B.Y., Teppler, H., 2008. Raltegravir with optimized background therapy for resistant HIV-1 infection. New England Journal of Medicine 359, 339–353.

Summa, V., Petrocchi, A., Bonelli, F., Crescenzi, B., Donghi, M., Ferrara, M., Fiore, F., Gardelli, C., Gonzalez Paz, O., Hazuda, D.J., Jones, P., Kinzel, O., Laufer, R., Monteagudo, E., Muraglia, E., Nizi, E., Orvieto, F., Pace, P., Pescatore, G., Scarpelli, R., Stillmock, K., Witmer, M.V., Rowley, M., 2008. Discovery of raltegravir, a potent, selective orally bioavailable HIV-integrase inhibitor for the treatment of HIV-AIDS infection. Journal of Medicinal Chemistry 51, 5843–5855.

Tilton, J.C., Doms, R.W., 2010. Entry inhibitors in the treatment of HIV-1 infection. Antiviral Research 85.

Underwood, M., Johns, B., Sato, A., Fujiwara, T., Spreen, W., 2009. S/GSK1349572: a next generation integrase inhibitor with activity against integrase inhibitor-resistant clinical isolates from patients experiencing virologic failure while on raltegravir therapy (Poster WEPEA098). In: IAS 2009-5th Conference on HIV Pathogenesis, Treatment and Prevention, Cape Town, South Africa.

Vacca, J., Wai, J., Fisher, T., Embrey, M., Hazuda, D., Miller, M., Felock, P., Witmer, M., Gabryelski, L., Lyle, T., 2007. Discovery of MK-2048—subtle changes confer unique resistance properties to a series of tricyclic hydroxypyrrole integrase strand transfer inhibitors (Abstract). In: 4th IAS Conference on HIV Pathogenesis, Treatment and Prevention incorporating the 19th ASHM Conference, Sydney, Australia.

van Gent, D.C., Oude Groeneger, A.A., Plasterk, R.H., 1993. Identification of amino acids in HIV-2 integrase involved in site-specific hydrolysis and alcoholysis of viral DNA termini. Nucleic Acids Research 21, 3373–3377.

Vink, C., Plasterek, R.H., 1993. The human immunodeficiency virus integrase protein (Abstract). Trends Genetics 9, 443–448.

Wai, J., Fisher, T., Embrey, M., Egbertson, M., Vacca, J., Hazuda, D., Miller, M., Witmer, M., Gabryelski, L., Lyle, T., 2007. Next generation of inhibitors of HIV-1 integrase strand transfer inhibitor: structural diversity and resistance profiles (Abstract 87). In: 14th Conference on Retroviruses and Opportunistic Infections, Los Angeles, CA, USA.

Waters, J.M., Margot, N., Hluhanich, R., Svarovskaia, J., Harris, J., Borroto-Esoda, K., Miller, M.D., McColl, D.J., 2009. Evolution of resistance to the HIV integrase inhibitor (INI) elvitegravir can involve genotypic switching among primary INI resistance patterns (Poster P116). In: XVIII International HIV Drug Resistance Workshop, Fort Myers, Florida.

Wensing, A.M.J., van Maarseveen, N.M., Nijhuys, M., 2010. HIV protease inhibitors. Antiviral Research 85.

Witmer, M., Danovich, R., Ke, Y., Day, A., Gabryelski, L., Ecto, L., Lu, M., Lai, M.-T., McKenna, P., DiStefano, D., Miller, M., Hazuda, D., 2007. In vitro resistance selection studies using raltegravir: a novel inhibitor of HIV-1 integrase (abstract #17). In: 8th Annual Symposium on Antiviral Drug Resistance, Richmond, VA, USA.

Yoder, K.E., Bushman, F.D., 2000. Repair of gaps in retroviral DNA integration intermediates. Journal of Virology 74, 11191–11200.

Zheng, R., Jenkins, T.M., Craigie, R., 1996. Zinc folds the N-terminal domain of HIV-1 integrase, promotes multimerization, and enhances catalytic activity. Proceedings of the National Academy of Sciences United States of America 93, 13659–13664.

Zhuang, L., Wai, J.S., Embrey, M.W., Fisher, T.E., Egbertson, M.S., Payne, L.S., Guare Jr., J.P., Vacca, J.P., Hazuda, D.J., Felock, P.J., Wolfe, A.L., Stillmock, K.A., Witmer, M.V., Moyer, G., Schleif, W.A., Gabryelski, L.J., Leonard, Y.M., Lynch Jr., J.J., Michelson, S.R., Young, S.D., 2003. Design and synthesis of 8-hydroxy-(1,6)naphthyridines as novel inhibitors of HIV-1 integrase in vitro and in infected cells. Journal of Medicinal Chemistry 46, 453–456.

Zolopa, A.R., Lampiris, H., Blick, G., Walworth, C., Zhong, L., Chuck, S.L., Enejosa, J., Kearney, B.P., Cheng, A.K., 2007a. The HIV integrase inhibitor elvitegravir (EVG/r) has potent and durable antiretroviral activity in treatment-experienced patients with active optimized background therapy (OBT) (Oral Presentation). In: 47th Annual Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC), Chicago, IL.

Zolopa, A.R., Mullen, M., Berger, D., Ruane, P., Hawkins, T., Zhong, L., Chuck, S.L., Enejosa, J., Kearney, B.P., K, C.A., 2007b. The HIV integrase inhibitor GS-9137 has potent antiretroviral activity in treatment-experienced patients (oral presentation 143LB). In: 14th Conference on Retroviruses and Opportunistic Infections, Los Angeles, CA, USA.

Zouhiri, F., Mouscadet, J.F., Mekouar, K., Desmaele, D., Savoure, D., Leh, H., Subra, F., Le Bret, M., Auclair, C., d'Angelo, J., 2000. Structure-activity relationships and binding mode of styrylquinolines as potent inhibitors of HIV-1 integrase and replication of HIV-1 in cell culture. Journal of Medicinal Chemistry 43, 1533–1540.