The Studies on Novel Integrase Inhibitor of Human Immunodeficiency Virus
(ヒト免疫不全ウイルスの新規インテグラーゼ阻害剤に関する研究)

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CONTENTS

ABBREVIATIONS: HIV, human immunodeficiency virus; FIV, feline immunodeficiency virus; HTLV, human T-cell leukemia virus type 1; HAART, highly active anti-retroviral therapy; N(t)RTI, nucleoside (nucleotide) reverse transcriptase inhibitor; NNRTI, nonnucleoside reverse transcriptase inhibitor; PI, protease inhibitor; INI, integrase inhibitor; RAL, raltegravir; EVG, elvitegravir; STV, stavudine; ABC, abacavir; EFV, efavirenz; NVP, nevirapine; LPV, lopinavir; APV, amprenavir; ENF, enfuvirtide; ADV, adefovir; LTR, long terminal repeat; PA-EC_{50}, protein adjusted EC_{50}; PK, pharmacokinetic; HSA, human serum albumin; HS, human serum; AAG, α1-acid glycoprotein

PREFACE

Human immunodeficiency virus (HIV) is a RNA virus, member of Retroviridae, the lentivirus subfamily of enveloped viruses. The life cycle of HIV is as follows. 1) Binding and Fusion**:** HIV binds to a CD4 receptor and co-receptors on the surface of a CD4+ T- lymphocyte. The virus then fuses with the host cell. After fusion, the virus releases RNA into the host cell. 2) Reverse Transcription: Reverse transcriptase, an HIV enzyme, converts the single-stranded HIV RNA to double-stranded HIV DNA (provirus DNA). 3) Integration: The HIV DNA enters the host cell's nucleus, where an HIV enzyme called integrase incorporates into the chromosome of the host cell's DNA. The provirus may remain inactive for several years, producing few or no new copies of HIV. 4) Transcription: The provirus uses a host RNA polymerase to create copies of the HIV genomic material, as well as mRNA to make HIV proteins. 5) Assembly: An HIV enzyme protease processes the HIV proteins into mature proteins. As the HIV proteins come together with copies of HIV's RNA, a new virus particle is assembled. 6) Budding: The newly assembled virus pushes out ("buds") from the host cell.

To inhibit the growth of HIV, there are points of inhibition, 1) Adsorption, penetration, 2) Reverse transcription, 3) Insertion of viral genes into the chromosome, 4) Maturation of the virus by protease. The combination drug therapy (HAART; Highly Active Anti-retroviral Therapy) in clinical practice for these points have been used. Recently, novel integrase inhibitor was developed and the good tolerability and efficacy were reported. However, HIV causes a mutation in the growth every time, and lead to the emergence of resistant virus. In case of the integrase inhibitor, several pathways emerged among patients experiencing integrase inhibitor. To prevent the emergence of resistant virus, further analysis of prognostic factor associated with emergence of integrase mutations is needed.

The purpose of this study was to investigate the *in vitro* antiretroviral properties of novel

integrase inhibitors focusing on its mechanism of action and *in vitro* resistance profile. The mechanism of action was established through *in vitro* integrase enzyme assays, resistance passage experiments, activity against viral strains resistant to other classes of anti-HIV agents, and mechanistic cellular assays. This thesis consists of two chapters. In Chapter 1, selection of diverse and clinically relevant integrase inhibitor-resistant human immunodeficiency virus type 1 mutants is described. *In Vitro* Antiretroviral Properties of S/GSK1349572, a Next-Generation HIV Integrase Inhibitor is described in Chapter 2.

There are several animal viruses in the subfamily lentivirus, simian immunodeficiency virus, equine infectious anemia virus, feline immunodeficiency virus (FIV), which are important pathogen in animals. However, there is no specific antiretroviral treatment for animals. The evaluation of possibility of HIV integrase inhibitor for inhibiting FIV replication was reported. This trial may contribute to the development of a small animal treating lentiviral infection.

CHAPTER 1

Selection of diverse and clinically relevant integrase inhibitor-resistant human immunodeficiency virus type 1 mutants

INTRODUCTION

To date, twenty-three compounds are currently approved for the treatment of HIV infection. These drugs can be assigned to six classes: nucleoside (nucleotide) reverse transcriptase inhibitors [N(t)RTIs], nonnucleoside reverse transcriptase inhibitors [NNRTIs], protease inhibitors [PIs], integrase inhibitors [INIs], CCR5 antagonists and fusion inhibitors. The development of resistance to all currently marketed drugs has been observed and is a major reason for failure of therapy. Due to the high error rate of HIV-1 reverse transcriptase, drug resistance is inherent for all anti-HIV agents, and clinical data have already demonstrated resistance to INIs (22, 36). Thus, the development of new, potent antiretroviral compounds with different resistance profiles and mechanisms of action is urgently needed for patients who have multidrug-resistant HIV. In addition to these characteristics, an improved side effect profile and improved dosing convenience (once-daily dosing, fixed-dose combination pills) are desirable, because they would promote high compliance, decrease the emergence of drug-resistant variants, and thus enhance the length and quality of life. It is important to characterize the resistance profile of known INIs in order to direct research and development on new INIs.

In this chapter, I describe the development of an *in vitro* method to isolate INI resistant mutants in MT-2 cells using HIV-1 in the presence of integrase inhibitor S-1360 (4) and related compounds. I used this method to isolate mutants under the same conditions resistant to L-870,810 (14), raltegravir [RAL, MK-0518] (34, 35), elvitegravir [EVG, GS-9137] (13, 52) and the recently described S/GSK-364735 (18). Finally, the fold change (FC) of 40 INI-resistant molecular clones against various INIs was measured to directly compare resistance profiles of these INIs.

MATERIALS AND METHODS

Compounds

S/GSK-364735 and L-870,810 sodium were synthesized at GlaxoSmithKline, Research Triangle Park, NC. S-1360, compounds 1, 2, and 3, L-731,988, lamivudine, nevirapine, capravirine, efavirenz, RAL and EVG were synthesized at Shionogi Research Laboratories, Osaka, Japan.

Cells and viruses

HeLa–CD4 cells carrying a reporter β-galactosidase gene driven by HIV-1 LTR were established by transfection of HeLa cells with CD4 and β-galactosidase expression vector (24). MOLT-4 cells persistently infected with HIV-1 strain IIIB (19) and human cell lines (MT-4, MT-2, MT-1, M8166, CEM, CEMx174, Hut-102, HPB-all, HPB-Null, TL-Su, TCL-Kan, LCL-Kan, A3.01, H9, Jurkat, CESS, U937 and THP-1) were obtained from the Institute for Virus Research, Kyoto University. HeLa–CD4 cells were grown in Dulbecco's modified minimal essential medium (DMEM) containing 10% FCS and 60 μg/mL kanamycin. MOLT-4 cells and human cell lines were maintained in RPMI1640 supplemented with 10% FCS and 60 μg/mL kanamycin.

Construction of integrase gene recombinant HIV-1 molecular clones

The recombinant HIV-1 molecular clones were constructed as follows. The XbaI–EcoRI fragment from pNL-IN301 [pNL432 (1) inserted XbaI site into 5'-end of IN region] was cloned in the XbaI–EcoRI site of cloning vector pUC18. *In vitro* mutagenesis was performed with the QuikChange site-directed mutagenesis kit (Stratagene) using a pUC18 plasmid containing the IN encoding region as a template. The amplified mutated XbaI–EcoRI fragment was ligated

into pNL-IN301 to construct recombinant HIV-1 molecular clones. The plasmids were subsequently transfected into 293T cells by Lipofectamine2000 (Gibco) to generate infectious virus. Supernatants were harvested 2-3 days after transfection and were stored as cell-free culture supernatants at −80 ◦C.

Viral replication kinetics in T-cell lines

MOLT-4, Jurkat (2.5×10^4) and MT-2 cells (5×10^4) were infected with NL432 or INI-resistant viruses (T66I, Q148K and N155S) for 1h at 37 ◦C, washed and cultured in 24-well plates (1.5mL/well). Viral stocks were normalized by reverse transcriptase (RT) activity prior to infection (200,000 cpm/5×10^4 cells). The infected cells were subcultured to 5-fold dilution twice a week for MOLT-4 and Jurkat cells or once a week for MT-2 cells, and virus production in culture supernatants was titrated for RT activity.

Anti-HIV activity in MT-2 cell assay

Antiviral HIV activity of INIs was measured in the HTLV-1 transformed cell line MT-2 as previously described (17, 42) with slight modifications. Briefly, MT-2 cells were suspended in culture medium at 1×10^5 cells/mL. The cell suspension (100 μL) was added to each well of a 96-well flatbottom microtiter plate containing serial 2-fold dilutions of test compounds (50 μL/well). HIV-1 (50 μL/well) was added to each well (4-10 $TCID_{50}$/well). After 4-day of incubation at 37 ◦C, the viability of MT-2 cells was determined by the MTT method using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. The concentration achieving 50% inhibition of HIV replication (EC_{50}) was calculated by the absorbance (OD560/OD690).

Isolation of drug-resistant viruses

Virus for initiating passage work was prepared by co-culturing human T-cell lines (e.g., MT-2

cells at 1×10^5 cells/mL) with MOLT4 cells persistently infected with HIV-1 strain IIIB (1×10^5 cells/mL) for 3 days. Culture medium including suspended co-cultivated cells (0.5 mL) was used for initiating passage for selection of resistant variants. When MT-2 cells were used for the initial infection, a suspension (3×10^5 cells) was dispensed into each well of a 24-well tissue culture plate. Three wells of each culture containing 4-5 different compound concentrations (total 12 or 15 wells) were used initially. Medium containing appropriate dilutions of a test compound was distributed into the plate, and then 0.5 mL of co-cultivated MT-2 cells and MOLT-4 cells prepared as described above were added into each well. When cytopathic effect (CPE) was observed under the microscope, the culture supernatant was dispensed into a new plate, and new human T-cell suspension in medium containing a test compound was added. Every 3 or 4 days, the cells were passaged with or without addition of fresh human T-cells. If CPE was apparent, the supernatants were used to infect new human T-cells, and the concentration of compounds was held constant and/or increased 2.5- or 5-fold. When replication of viruses was ascertained by observed CPE, the infected cells were collected and used for genotypic and phenotypic analyses. To analyze mutations, DNA was extracted from infected cells using a kit (DNeasy Tissue Kit, QIAGEN) and the IN region of HIV proviral DNA was amplified by PCR using a kit (TaKaRa Taq) and specific primers. Sequencing of the products was provided by OPERON BIOTECHNOLOGIES sequencing service. The sequence of IN region derived from isolated viruses was compared to that of wild type IIIB IN region and amino acid substitutions were identified.

Phenotypic sensitivity of viral isolates and drug-resistant molecular clones

Drug sensitivity of viruses isolated during the passage study and drug-resistant molecular clones were assessed by a reporter assay with HeLa–CD4 cells. Viral isolates from the passage study were briefly expanded in fresh M8166 cells. The test compounds were diluted to

appropriate concentration with culture medium and HeLa–CD4 cell suspensions (2.5×10^4 cells/well) were dispensed into each plate. After incubation for 1 h, HIV-1 resistant viruses were added. After 3 days of incubation, the cells were lysed and supernatant of each well was used for measurement of luminescent activity using the Reporter Assay Kit-βgal (TOYOBO). The luminescent activity (RLU) was measured using a MicroBeta TRILUX instrument (Amersham Pharmacia Biotech) and EC_{50} was calculated.

RESULTS

The structures and anti-HIV activities of the compounds

The structures of the nine INIs used in this study are shown in Fig.1. The anti-HIV activity of the compounds was measured by two methods (Table 1). The EC_{50} values of the early INIs (S-1360, its related compounds, and L-731,988) were 510–2200 nM in MT-2 cells and 190–3200 nM in HeLa–CD4 cells. EC_{50} values of the newer more potent INIs (S/GSK-364735, RAL, EVG and L-870,810) were single-digit nanomolar in both assays.

Selection of T-cell line for the isolation of INI-resistant viruses

MT-2, M8166, MOLT-4, Jurkat and H9cell lines were compared for the isolation of resistant mutants against S-1360, compounds 1 and 2, and L-731,988 using HIV-1 strain IIIB. Resistant mutants emerged within shorter passage time while yielding a greater variety of mutations in MT-2 cells. Representative results are shown in Table 2. Thirteen other cell lines in addition to these five T-cell lines were compared, but nothing was equal to or better than MT-2 cells in both rapidity in the emergence and diversity of resistance mutations (data not shown). It is noted that HIV-1 IIIB replicated poorly in H9 cells even without inhibitors and needed longer passage to generate resistant mutants, and that the suppression level of HIV-1 replication by integrase inhibitors in M8166 cells was lower than in other cell lines, probably due to integrase-independent replication (39).

Next, the replication kinetics of IN-mutant viruses in T-cell lines were analyzed to establish the relationship between replication capacity and emergence of resistant viruses (Fig. 2). MT-2, Jurkat and MOLT-4 cells were infected with wild type viruses (NL432) and INI mutants, and the RT activity of supernatants was monitored. Virus with T66I substitution replicated as well as wild type in all the cell lines. However, virus with Q148K or N155S substitutions showed

either low or insignificant replication in Jurkat and MOLT-4 cells. These results were consistent with the observation that T66I was detected more often in each of the three cell types, while substitutions at either Q148 or N155 were detected less frequently and only in Jurkat or MT-2 cells (Table 2).

Optimization of the drug concentrations for passage

I selected MT-2 cells for further optimization, and examined how mutation patterns were influenced by either holding the concentration of S-1360 constant (at different concentrations) or increasing it during passage. Representative results are shown in Fig. 3. The EC_{50} value of S-1360 in MT-2 cells against wild type virus was 250 ng/mL.

Under conditions of the constant drug concentration, the greatest diversity of resistance mutations was observed when the concentration of S-1360 was 800 ng/mL (3.2-fold of EC_{50}). For example, on day 35 only T66A/I and T124A substitutions were isolated at constant 32 ng/mL, 160 ng/mL and 4000 ng/mL, while Q146R, Q148K and T66I/L74M in addition to T66A/I were isolated at constant 800 ng/mL. Based on FC data (see below), the T124A substitution alone did not increase resistance of virus and is also found as a natural polymorphism (30). Although rarely observed, the double mutant with T124A in IN-region and M184V or I in RT-region was isolated during lamivudine passage, suggesting a few viruses with T124A substitution were contained in the IIIB virus used in this study. However, there may be some unrecognized advantage for this mutation under the selective pressure of INIs.

The isolation pattern of amino acid substitutions were compared under escalating concentrations of S-1360. On day 49, substitutions of isolates were limited to the T66I and T66I/T124N mutations in the passage that started from 32 ng/mL and gradually increased up to 4000 ng/mL. In contrast, substitutions of isolates were more diverse with T66A, T66I, Q148K, N155S and T66I/L74M in the passage that started at 160 ng/mL and then increased up to 4000

ng/mL in a step-wise manner. A similar pattern of T66I, T124A, Q148K, and N155T/T124A were obtained in the passage that started at 800 ng/mL then increased to 4000 ng/mL.

Overall, regardless of whether passage was carried out under constant or escalating concentration of compounds, it appeared to be important to start the passage at a relatively high compound concentration (though less than 16-fold above EC_{50}) to isolate a greater diversity of mutant viruses.

Comparison of the time courses for the isolation of resistant viruses with INIs and NNRTIs

In the initial study described above, a longer cultivation period was needed to isolate viruses resistant to S-1360 compared to nevirapine and lamivudine (Fig. 3). Thus, the time course of isolation of resistant viruses to S-1360 was examined, Compound 3 (INIs), capravirine, efavirenz and nevirapine (NNRTIs) in parallel experiments (Table 3). Isolates from each culture were analyzed genotypically and phenotypically. It has previously been reported that 8-10 passages were required for the isolation of the highly resistant viruses for capravirine and efavirenz (FC > 20, (51); (17); (45)). In my MT-2 system, this high level of resistance was achieved more quickly compared with previously report (45). All isolates of the culture with nevirapine and some isolates of the culture with capravirine and efavirenz showed high resistance on day 14 (passage 4), and isolates of the cultures with all NNRTIs showed high resistance on day 27 (passage 8). In this experiment, a broad collection of highly resistant viruses were isolated on day 39 with the two INIs. Note that the presence of mixed populations of viruses in culture wells and/or assay may have caused the differences in FC in phenotypic evaluations of the virus with the same amino acid substitutions (e.g., T66I unbolded versus bolded in the top row of Fig. 3).

Isolation of viruses resistant to S/GSK-364735, RAL, L-870,810, or EVG

The results of resistance passage experiments with INIs that have been tested in clinical trials to date are summarized in Table 4. Genotypic and phenotypic analyses were carried out every 2 weeks. In the culture with S/GSK-364735, no amino acid substitution was found within integrase on day 13, but T124A was isolated on day 28. The following additional mutations were isolated with further passage: Q148R and F121Y on day 42, Q146R, F121Y/T124A and E10D/N17S/Q148R on day 56, G163R, E138K/Q148R, and G140S/Q148R on day 70, T66K, Q95R, V75I/T112S/Q146P on day 84. The resistant mutants against S/GSK-364735 (shown with bold letters in Table 4) were isolated when the concentration of compounds was over 6.4 ng/mL (2.7-fold of EC_{50}), and only Q148R and G140S/Q148R was isolated even at the highest concentration (160 ng/mL).

In the culture with RAL, the first amino acid substitution T124A was observed on day 14, followed by Q148K and N155H/I204T substitutions on day 28. Q148R, N155H, E92Q/M154I and Q148K/G163R substitutions were observed on day 42-56. Furthermore, a total of 13 different isolates, including Q148K/R or N155H substitution as single, double or triple mutations, were observed on day 84. Resistant mutants were isolated when RAL was over 14 ng/mL (3.6-fold of EC_{50}). The double mutations E138K/Q148K, E138(E/K)/Q148R, G140S/Q148R and V151I/N155H isolated in this study have been identified as clinical resistance mutations in patients with observed virologic failure during RAL treatment during Phase IIb (22).

In the culture with L-870,810, isolates contained the T124A substitution on day 14, and T124A and Q148R substitutions on day 28. T66K, F121Y, V151L, T124A/Q148R, E138K/Q148K, T66I/E92V/T124A, T66K/E92Q/T124A/M154I substitutions were observed at 2.9-15 ng/mL (1.8-9-fold of EC_{50}) on day 42. Finally, 13 different substitutions were isolated on day 84 in the passage with L-870,810. It is noted that the pattern of amino acid substitution in the culture with L-870,810 was different from that of RAL in the occurrences of T66K, E92

(I/Q), and F121Y substitutions. In the culture with EVG, the first amino acid substitution V151I was observed on day 13, but was absent on day 28 and later time points. Five other substitutions were observed on day 28: T66I, T124A, P145S, Q148K and T66I/T124A. All five of these latter substitutions were present during the rest of the passage with EVG. Four additional amino acid substitutions were observed on day 42; two were at T66 (T66A and T66K/T124A) and two were at Q148 (Q148R and Q148R/T124A). Finally, a total of 15 different substitutions, or combinations of substitutions, were observed on day 56 in the passage with EVG, and nine of these included T124A. Only the P145S and Q148K substitutions (FC of >350 and >1700 for EVG) were detected when 6.4 ng/mL (7.8-fold of EC_{50}) was the initial compound concentration.

Mutations which resulted in more than a 5-fold decrease in sensitivity (as measured in phenotypic assays) are shown in bold letters in Table 4. This level of resistance was first observed on day 56 in the cultures with S/GSK-364735, on day 42 with RAL and L-870,810, and on day 14 with EVG and lamivudine. In general, the phenotypic level of resistance paralleled the diversity and complexity of genotypic data, as the cultures yielding many mutations included the viruses with multiple mutations that showed the highest fold resistance in phenotypic analyses.

Sensitivity of drug-resistant molecular clones to INIs

Next, INI-resistant mutant molecular clones were constructed by site-directed mutagenesis, and determined their sensitivity to each INI (Table 5). Most of these mutations were isolated in the present passage studies, while a few were derived from the literature. Efavirenz, which was used as a control, had EC_{50} values for the mutants of up to 2.9 times that of the wild type virus. Therefore, the viruses with an EC_{50} of 3-fold or greater than that of the wild type were considered resistant in this study. It is also noted that amino acid position of 151 was different

between NL432 and IIIB, i.e., isoleucine for NL432 and valine for IIIB. V151L and V151I were isolated in the cultures with L-870,810, and with RAL and EVG, respectively in this study. To confirm the contribution for drug-resistance of the amino acid substitution at 151, I151L and V72I/F121Y/T125K/I151V mutants were constructed based on NL432.

Forty INI-resistant viruses were tested for the susceptibility to S/GSK-364735 and the other compounds. S/GSK-364735 showed a large reduction in potency against 20 mutant viruses which had greater than 10-fold increase in the EC_{50} compared to that of wild type virus (FC > 10). RAL showed greater than 10-fold increased EC_{50} against 17 viruses, EVG against 27viruses and L-870,810 against 23 viruses. Twelve mutant viruses shared a highly resistant phenotype against all five INIs (two single mutant viruses Q148K and Q148R and nine double- and one triple-mutant viruses). Thus, a high degree of cross-resistance was observed among these five different templates of two-metal binding INIs.

In contrast, differences in susceptibility to various INIs were observed for certain mutants. For example, virus with G118R was susceptible to EVG (FC of 2.6) but not to the other INIs (FCs ranging from 7.2 to 670), whereas virus with P145S had the exact opposite phenotype (FC of >350 for EVG and near wild-type level of susceptibility to all other INIs). RAL was potent against G140S/Q148K at near wild-type level (FC = 3.7) while at least a 37-fold decrease of susceptibility was observed for all other INIs. Another example of difference may be seen in that there was at least a 5-fold difference between FCs of the most and least effective INIs with 22 of the 40 mutant viruses.

Finally, it is noted that double mutants isolated at the high concentration of drug under a dose escalating protocol usually showed a higher fold resistance than that of the primary mutants. Therefore in general, the secondary mutations added a higher level of resistance to INIs.

DISCUSSION

There are several purposes of isolating drug-resistant mutants *in vitro*: [1] to confirm mechanism of action of a drug, [2] to possibly predict the clinical resistance profile, including the magnitude of genetic barrier, and [3] to ascertain the level of cross-resistance between drugs. Two concerns of passage studies are that they can take long periods of study and that *in vitro* isolated drug-resistant mutants do not necessarily reflect those isolated in clinic. It is showed that MT-2 cells were suitable for the isolation of mutant viruses resistant to a broad range of two-metal binding INIs in terms of cultivation time, variety of resistant mutants, and potential clinical relevancy.

Why did this method succeed with short cultivation times, significant diversity, and clinical relevance of mutations? One factor that may affect the variety of resistant mutants is virus copy number during the passage, especially at the beginning of cultivation with a drug. MT-2 cells and virus-producing MOLT-4 cells were co-cultivated for 3 days to generate the virus used to initiate passage in MT-2 cells. HIV production measured within 24 h of infection was moderate in MT-2 cells under cell free infection conditions even when maximum amount of virus input is used, while approximately 10-fold higher viral production was observed at 24 h in MT-2 cells after co-cultivation with HIV infected cells (data not shown). The co-cultivation infection may be providing a high titer of new viruses from MT-2 cells within 24 h, and possibly a greater diversity of spontaneous mutations at the beginning of passage. In addition, three wells were used for each concentration to increase the chance of isolating resistant mutants arising via alternate pathways. Another factor influencing the variety of resistant mutations is drug concentration. In general, as widely employed, gradual increasing of the drug concentration is an efficient technique to generate a diversity of resistant mutations quickly. However, the addition of secondary mutations to a primary mutation may improve

viral replication capacity without increasing the fold resistance to a drug. Keeping the drug concentration constant from a certain point in the passage could increase the chance of isolating such mutants. The E138K/Q148R double mutant provides an example of this, and details of this mutation will be published elsewhere. Finally, HIV replication rate was high in MT-2 cells relative to other cell lines tested, and this may have led to greater diversity of spontaneous mutations.

The first report of INI-resistant viruses used L-708,906 and L-731,988 (21). T66I, S153Y, M154I, T66I/S153Y and T66I/M154I were isolated using H9 cells infected with HIV-1 IIIB. Fifteen or twenty passages were required for the isolation of these mutants using L-706,906 or L-731,988, respectively. The isolation of L-870,810-resistant viruses using H9 cells was subsequently reported, with F121Y/T125K, isolated after 6-month of passage and V72I/F121Y/T125K, and V72I/F121Y/V151I isolated after 9-month of passage (20). In the present method described in this study, the highly resistant F121Y/T125K was isolated on day 56 in the culture with L-870,810 (16 passages), along with isolation of many other examples of complex, highly resistant mutants. In other reports using L-708,906, T66I was detected at the 35th passage, and this mutation was also isolated with S-1360 culture at the 30th passages using MT-4 cells (15, 16). In this method, various S-1360-resistant viruses were isolated at about 6-7 passages (Fig. 3 and Table 3) and L-708,906-resistant viruses containing T66I, L74M and V151I were isolated at 7 passages (data not shown). These differences in the length of passage translate into a 3-5-fold savings in time to generate mutant viruses with similar or even greater fold resistance. In addition, the variety of resistant viruses in the referenced work described above was limited, while I succeeded in isolating a variety of resistant viruses, in particular Q148K/R and N155H/S/T.

Recently disclosed mutations observed in patients failing RAL include L74M, E92Q, T97A, E138A/K, G140S, Y143H/R, Q148H/K/R, V151I, N155H, G163K/R, S230N and D232N (22).

In the present *in vitro* study, substitutions at E138K, G140S, Q148K/R, V151I, N155H, and G163R were detected in virus passaged with RAL. Furthermore, L74M, E92Q, T97A, and D232N were observed in either L-870,810- or S-1360-resistant mutants *in vitro*. In contrast, E138A, Y143H/R, Q148H, G163K, and S230N were not isolated with any INI in this study. E92Q, a signature mutant observed in clinical failure of EVG (36), was also detected in an *in vitro* isolation of resistance study using MT-2 cells and HIV-1 IIIB (47). Likewise, this mutation was isolated in my *in vitro* method. In contrast, there were several low level EVG resistance mutations which were not isolated in the previous study but that were isolated in this experiment, or vice versa. These data indicate that even when the T-cell line and HIV strain are identical, factors exist that affect which drug-resistant variants emerge at particular stages of virus passage, which in turn may result in different mutations at later passage.

Most of the amino acid substitutions of INI-resistant viruses detected in this study have been reported previously in clinical or *in vitro* studies, or as natural polymorphisms associated with INI resistance (30). However, to my knowledge, the G118S/C/N/R mutations detected during passage with Compound 3 (Table 3) were novel mutations that confer resistance of virus to other INIs (Table 5). These mutations have not been reported in clinical studies, and it remains to be determined if they will be observed in patients failing INI treatment.

In general very significant cross-resistance (i.e., high similarity of fold resistance) was observed with a panel of 40 molecular clones and the five INIs tested. But differences were also detected. For example, when comparing S/GSK-364735 and RAL, differences in fold resistance ranged from 4- to 30-fold in Q148H, N155S/T, E138K/Q148H and G140S/Q148K. G118R was highly resistant to RAL, L-870,810, and S-1360, while EVG showed wild-type sensitivity. Comparing S-1360 and EVG with other integrase inhibitors, T66I and T66I-containing double mutants were more commonly isolated. T66I with additional substitutions showed high resistance to early INIs such as L-708,906 (21). S/GSK-364735 and RAL were

effective against T66I with low FC, and viruses with T66I substitution were not detected in S/GSK-364735 and RAL cultures.

Comparing FCs indicate that the INIs tested in this study mostly have similar contact points within the two-metal binding INI site. However, the observed differences in FC must reflect at least subtle differences of how different INI scaffolds specifically fit within this pocket. Therefore, there is a possibility that future two-metal binding INIs can be developed that potently inhibit first generation INI-resistant viruses that emerged in the clinic.

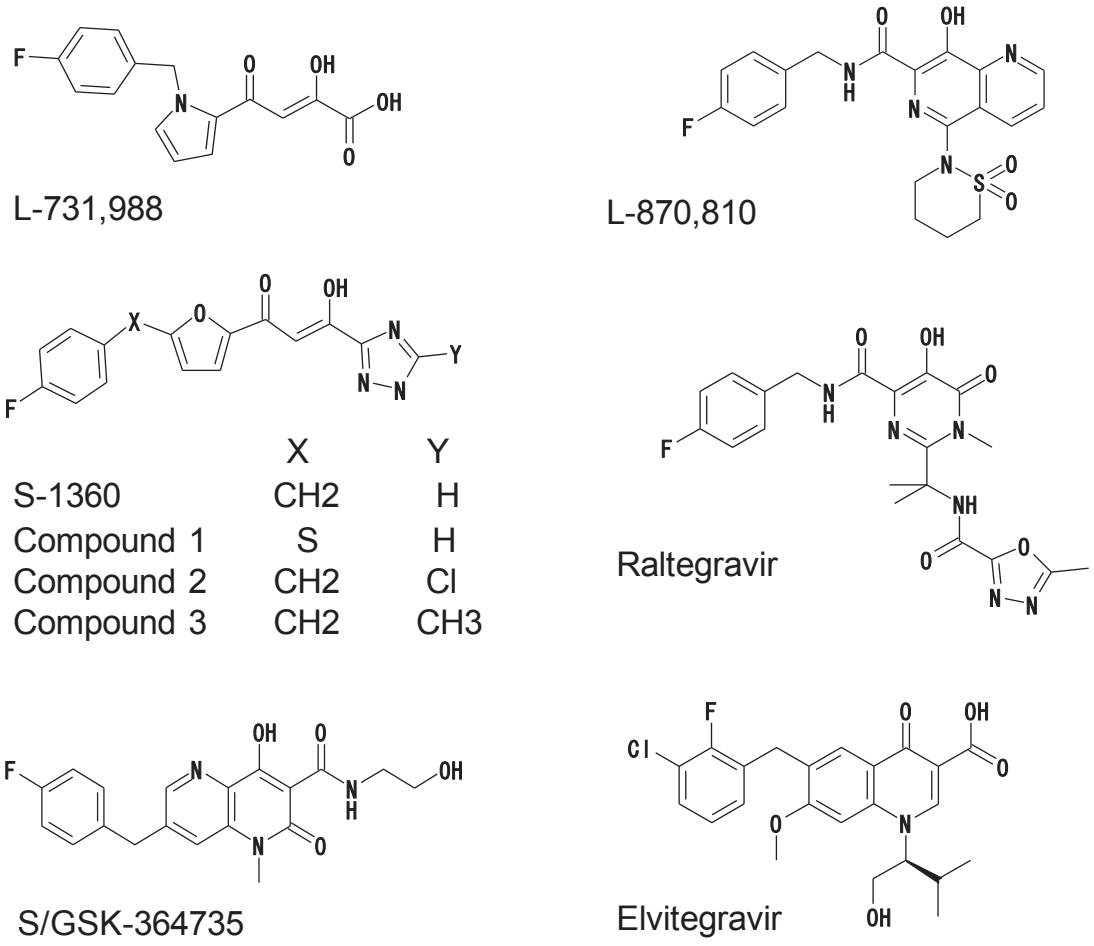


Fig. 1 Chemical structure of the HIV-1 integrase inhibitors used in this study

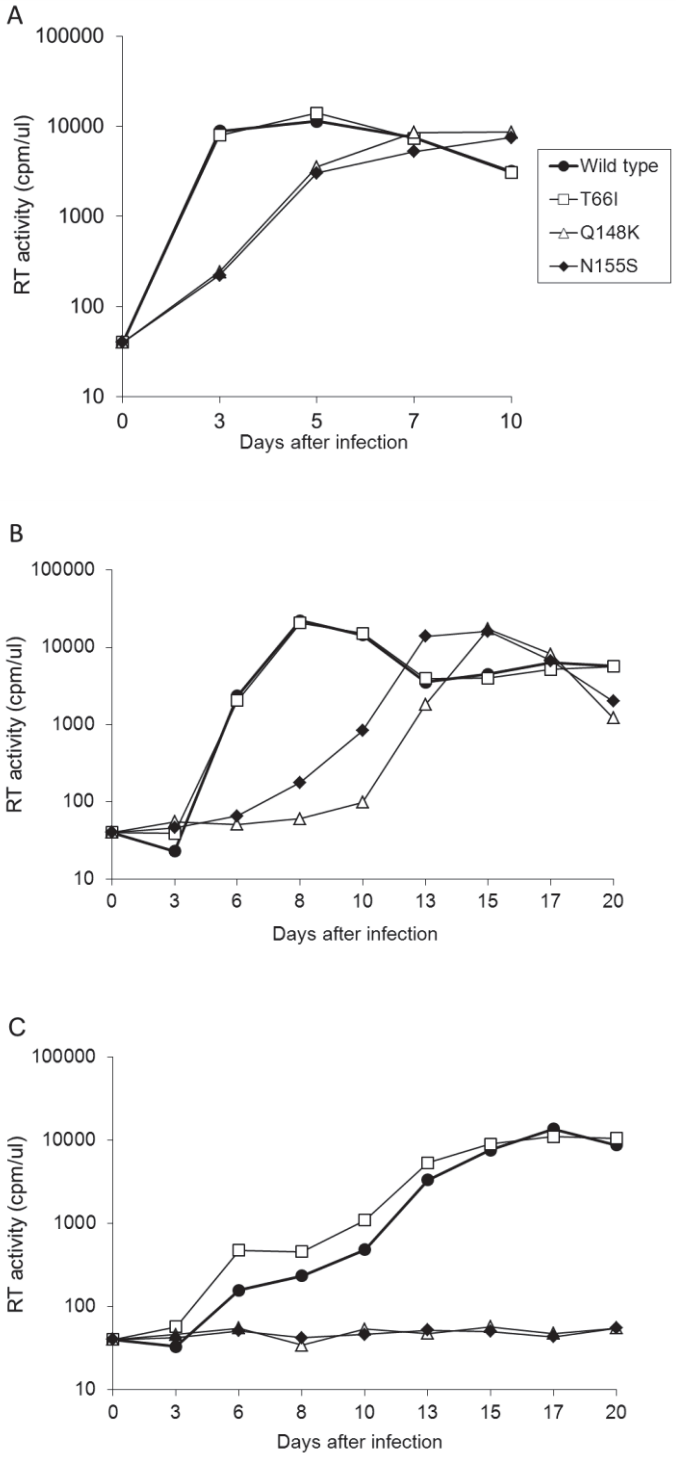
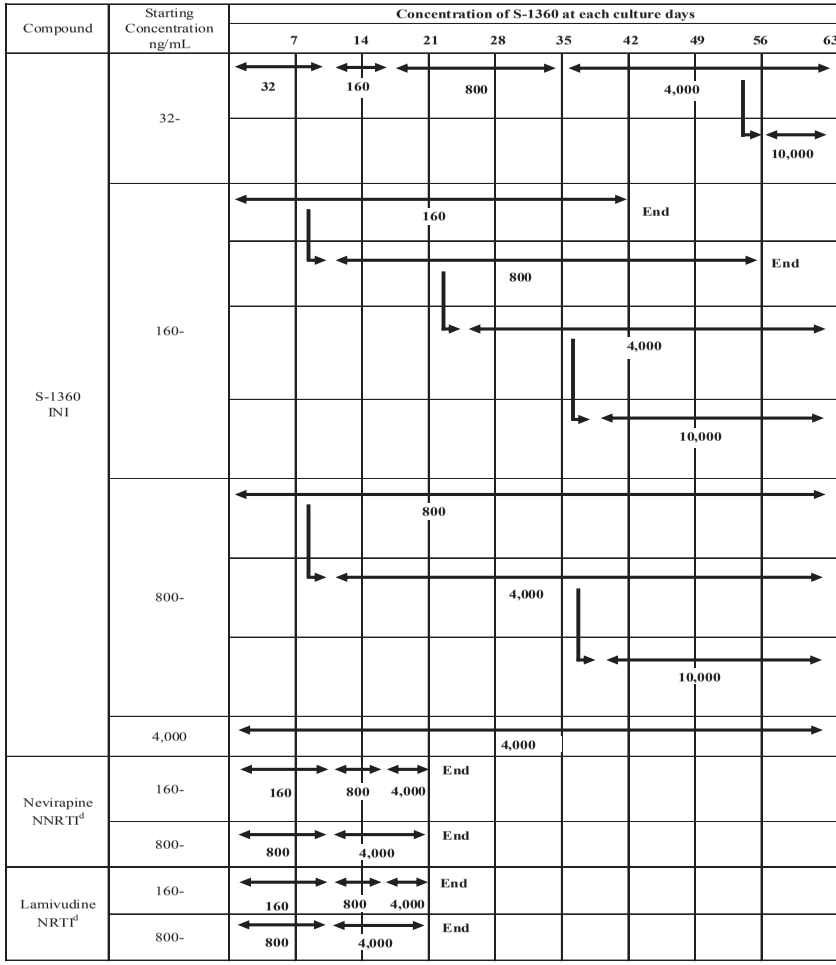


Fig. 2 Replication kinetics of mutant viruses resistant to INIs in (A) MT-2, (B) Jurkat and (C) MOLT-4 cells. Independent experiments generated the same results. Representative data are shown.



Compound	Starting Concentration ng/mL	Concentration of S-1360 at each culture days	Amino acid substitutions at each culture day for corresponding passages[a]							
			14	21[b]	28	35[b]	42	49[b]	56	63[b]
S-1360 INI	32-	32 → 160 → 800 → 4,000 → 10,000	No mutation	No mutation	T66I T124A	**T66I (3)**	T66I (2) T66I/T124N	T66I **T66I/T124N**	T66I/L74M T66I/T124N	**T66I** **T66A/T124A** T66I/L74M **T66I/T124N**
										T66I **T66A/T124A** **T66I/L74M** **T66I/T124N**
	160-	160 → End	No mutation	No mutation	T124A (3)	T124A (4)	T124A (6)			
		160 → 800 → End	ND[c]	**T66A** T124A (2)	T66A T124A (2) T66I/T124A	**T66A** **T66I (3)** **T124A (2)**	T66A T66I (2) T124A T66I/T124A(2)	T66A T66I (3) **T124A** T66I/T124A(1)	T66I (2) T66I/T124A (2) N155H/T124A T66I/L74M/T124A	
		800 → 4,000				**T66A** **T66I (2)** **Q148K**	T66I T66I/T124A Q148K N155S	**T66A** **T66I** **Q148K** **N155S** T66I/L74M	T66A T66I Q148K N155S T66I/T124A T66I/D232N	**Q148K** **N155S** **T66I/L74M** **T66I/T124A (2)** **T66I/D232N**
		4,000 → 10,000					T66I Q148K	**T66I** **N155S**	T66I Q148K N155S T66I/T124A T66I/D232N	T66I N155S **T66I/T124A** T66I/D232N **E138K/Q148K**
	800-	800 → End	No mutation	T66A T66I Q148K	T66A T66I Q148K	**T66A** **T66I** **Q146R** **Q148K** **T66I/L74M**	T66I Q146R Q148K T66I/L74M T66A/T124A	T66I Q146R **Q148K** **T66I/L74M** **T66A/T124A**	T66I Q146R Q148K T66I/L74M T66A/T124A	ND
		800 → 4,000 → End	ND	**Q148K** N155T/T124A	T124A (2) Q148K N155T/T124A	T124A (2) **Q148K** **N155T/T124A**	T66A T66I T124A Q148K N155T/T124A	**T66I (2)** T124A **Q148K** **N155T/T124A**	T66A T66I (2) T124A Q148K N155T/T124A	**T66A** T66I E92Q/T124A **E138K/Q148K** **N155T/T124A**
		4,000 → 10,000					T66A T66I T124A Q148K N155T/T124A	**T66A** **T66I** **T124A** **Q148K** **N155T/T124A**	T66A T66I E138K/Q148K N155T/T124A	**T66A** T66I **T124A** **E138K/Q148K** **N155T/T124A**
	4,000	4,000 → End	ND	No mutation	No mutation	T124A	ND	T124A	ND	**T124A** T124A/K160N
Nevirapine NNRTI[d]	160-	160 → 800 → 4,000 → End	V106A Y181C (3) V106A/Y188C V106A/F214L	**V106A** **Y181C (3)** **V106A/Y188C** **V106A/F214L**						
	800-	800 → 4,000 → End	Y181C (3)	**Y181C (3)**						
Lamivudine NRTI[d]	160-	160 → 800 → 4,000 → End	No mutation	**M184I (2)** **M184V**						
	800-	800 → 4,000 → End	M184I (3) M184V	**M184I (2)** **M184V (2)**						

Fig. 3 Isolation of S-1360-resistant viruses in various concentrations of drug. [a] Each substitution or combination shown indicates genotype from a single well. If mutants were isolated from more than one well, the number of wells was shown in parenthesis. [b] Phenotypic analysis was performed in parallel with genotypic analysis. The genotypes which showed >5-fold resistance compared with wild type were shown in bold letter. [c] ND; PCR not done [d] The genotypic analysis was performed in RT-region.

Table 1 Anti-HIV activity of INIs using MT-2 and HeLa–CD4 cells

Viruses Cells	EC_{50} Mean (SD)		
	HIV-1 IIIB MT-2 cells		HIV-1 NL432 HeLa-CD4 βgal cells
	nM	ng/mL	nM
S-1360	800 (130)	250 (30)	330 (70)
Compound 1	940 (150)	310 (50)	300 (100)
Compound 2	510 (80)	130 (20)	190 (70)
Compound 3	560 (60)	180 (20)	210 (110)
L-731,988	2,200 (300)	630 (100)	3,200 (100)
S/GSK-364735	4.4 (0.83)	2.4 (0.5)	3.6 (0.61)
L-870,810	5.2 (2.3)	1.6 (1.0)	3.0 (0.73)
Raltegravir	8.8 (1.1)	3.9 (0.5)	6.1 (0.89)
Elvitegravir	1.8 (0.28)	0.82 (0.12)	1.3 (0.31)

These data are mean values of at least 2 independent experiments performed in duplicate.

Table 2 Isolation of INI-resistant viruses using various T-cell lines

Cells	Compound	Mutation [a] (First isolation day after infection)
MT-2	S-1360	T124A (21), **N155S (28), T66I (35)**
	Compound 1	**T66I (21), Q148R (21), Q148K (21)**
	Compound 2	**G118S (14)**, T124A (21)
	L-731,988	**V72A (28), L74M (28)**, T124A (35)
M8166	S-1360	No mutation at 21 days
	Compound 1	No mutation at 21 days
	Compound 2	No mutation at 21 days
	L-731,988	No mutation at 21 days
MOLT-4	S-1360	T124A (38), **T66I/T124A (38)**
	Compound 1	T124A (38), **T66I (38), T66I/T124A (38)**
	Compound 2	T124A (38)
	L-731,988	T124A (38), **T66I/T124A (38)**
Jurkat	S-1360	T124A (38), **T66A (38)**
	Compound 1	T124A (35)
	Compound 2	T124A (38), **N155S (38)**
	L-731,988	**T66I (35)**, T124A (38)
H9	S-1360	No mutation at 31 days
	Compound 1	No mutation at 31 days
	Compound 2	No mutation at 31 days
	L-731,988	No mutation at 31 days

[a] Bold letter indicates > 5-fold resistance in phenotypic assay

Table 3 Time course of emergence of INI-resistant mutants compared to NNRTI-resistant mutants

	Days of culture (passage number)			
Compound EC50[a] (Concentration)	14[b] (4)	27[b] (8)	39[b] (11)	48 (14)
S-1360 INI EC50=250 ng/mL Initial conc. (160-800 ng/mL)	T124A (800 ng/mL)	**T66I**, E92Q, **Q148K** T124A/Q148K, T124A/Q146L T66I/V72A/T124A E10D/N17S/V72A/L74M/T124A (800-4,000 ng/mL)	**T66I, E92Q, Q148K, N155S** **T66I/T124A, T124A/Q148K, T124A/Q146L, T124A/N155S** **T66I/V72A/T124A** **E10D/N17S/V72A/L74M/T124A** (4,000 ng/mL)	T66I, E92Q, Q146L, Q148K, N155S, N155T T124A/Q146L, T124A/Q148K, T124A/N155S, T66I/V72A/T124A T66I/V72A/L74M/T124A N17S/T66I/V72A/L74M/T124A (4,000 ng/mL)
Compound 3 INI EC50=180 ng/mL Initial conc. (160-800 ng/mL)	No mutation (800 ng/mL)	**G118S**, T124A **E92Q/T124A**, G118S/T124A, T124A/Q146R (800-4,000 ng/mL)	**G118S, T124A** **E92Q/G118S, E92Q/T124A, G118S/T124A, G118C/T124A, T124A/Q146R, T124A/Q148K** (800-4,000 ng/mL)	G118S, G118C, G118N, G118R, T124A E92Q/T124A, G118S/T124A, G118C/T124A, G118N/V150I, T124A/Q146R, T124A/Q148K, T124A/N155S (800-4,000 ng/mL)
Capravirine NNRTI[c] EC50=2.0 ng/mL Initial conc. (6.4-32 ng/mL)	**L100I, Y188L, G190E, L234I** **V179D/G190E** (32 ng/mL)	**L100I, Y188L, G190E, L234I** **L100I/V106A, L100I/Y181C, L100I/Y188C, V179D/G190E, Y181C/L234I, Y188L/L234I, G190E/L234I** (160-800 ng/mL)	**Y188L, G190E, L234I** **L100I/V106A, L100I/Y181C, V106A/L234I, V106A/G190E, V106A/L234I, E138K/G190E, Y181C/L234I, Y188L/L234I** **L100I/V106A/G190E, L100I/V106A/L234F, L100I/Y181C/F227C, V106A/V179D/L234I** **V106A/Y181C/G190A/L234I** (800-4,000 ng/mL)	L100I/V106A, L100I/Y188L, L100I/G190E, L100I/L234F, E138K/G190E, Y181C/L234I L100I/V106A/V179D, L100I/V179F/Y181C, K101E/V106A/L234I, V106A/V179D/L234I, V106A/G190A/L234I, E138K/V189I/G190E, A158T/Y188L/L234I, Y181C/G190A/L234I, Y181C/M230L/L234I L100I/K103T/V106A/F227C, L100I/V179F/Y181C/F227C, K101E/V106A/G190A/L234I (800-4,000 ng/mL)
Efavirenz NNRTI[c] EC50=0.84 ng/mL Initial conc. (6.4 ng/mL)	**L100I, K103N, G190E, G190S** (32 ng/mL)	**L100I, K103N, G190E, G190S** **L100I/F227C, L100I/L234I, K103N/Y188C** (160, 800 ng/mL)	**G190E** **L100I/K103N, L100I/G190S, L100I/F227C, L100I/L234F** **L100I/K103N/L234F, L100I/Y188L** (800-4,000 ng/mL)	G190E L100I/K103N, L100I/G190S L100I/K103N/L234F, L100I/V179D/L234F, L100I/Y188L/L210V L100I/K103R/V179D/F227C (800-4,000 ng/mL)
Nevirapine NNRTI[c] EC50=33 ng/mL Initial conc. (160-800 ng/mL)	**V106A, Y181C, Y188C, G190A** **V106A/Y181C, V106A/Y188C, V106A/F214L** (800 ng/mL)	**V106A, Y181C, Y188C, Y188N, G190A** **V106A/Y181C, V106A/F214L** (4,000 ng/mL)		

[a] EC50 was determined using HIV-1 IIIB ands MT-4 cells.

[b] Phenotypic analysis was performed in parallel with genotypic analysis. The genotypes which showed >5-fold resistance compared with wild type are shown in bold letter.

[c] The genotypic analysis was performed in RT-region.

Table 4 Time course of emergence of INI-resistant mutants

	Days of culture (passage number)					
Compound EC50[a] (Concentration)	**13 or 14[b] (4)**	**28[b] (8)**	**42[b] (12)**	**56[b] (16)**	**70[b] (20)**	**84[b] (24)**
S/GSK-364735 EC50=2.4 ng/mL Initial conc. (0.26-160 ng/mL)	No mutation (0.26-160 ng/mL)	T124A (0.26-160 ng/mL)	F121Y, T124A, Q148R (1.3-160 ng/mL)	T124A, Q146R, **Q148R** F121Y/T124A **E10D/N17S/Q148R** (6.4-160 ng/mL)	Q146R, **Q148R**, G163R F121Y/T124A, **E138K/Q148R** , **G140S/Q148R** (6.4-160 ng/mL)	**T66K**, Q95R, Q146R,**Q148R** **F121Y/T124A**, **E138K/Q148R**, **G140S/Q148R** V75I/T112S/Q146P (6.4-160 ng/mL)
Raltegravir EC50=3.9 ng/mL Initial conc. (0.11-14 ng/mL)	T124A (0.11-14 ng/mL)	T124A, **Q148K** N155H/I204T (0.11-71 ng/mL)	G59E, T124A, **Q148K**, Q148R, **N155H** **N155H/I204T** (0.57-360 ng/mL)	T124A, **Q148K**, **Q148R**, **N155H** E92Q/M154I, **Q148K/G163R**, **N155H/I204T** (2.8-360 ng/mL)	T124A, **Q148K** , **Q148R** **N17S/Q148K**, **E92Q/M154I**, **G140C/Q148K** **G140S/Q148R**, **Q148K/G163R**, **V151I/N155H** **N155H/I204T** **T124A/V151I/N155H**, **G140C/Q148K/G163R** (2.8-1800 ng/mL)	T124A, **Q148K**, **Q148R** **E138K/Q148K**, **E138K/Q148R**, **G140S/Q148R** **V151I/N155H**, **N155H/I204T** **N17S/Q148K/G163R**, **T124A/V151I/N155H** **E138K/Q148K/G163R**, **G140C/Q148K/G163R** **E92Q/E138K/Q148K/M154I** (14-1800 ng/mL)
L-870,810 EC50=1.6 ng/mL Initial conc. (0.12-360 ng/mL)	T124A (0.12-360 ng/mL)	T124A, **Q148R** (0.12-360 ng/mL)	**T66K**, F121Y, T124A, Q148R, V151L **T124A/Q148R**, **E138K/Q148K** **T66I/E92V/T124A, T66K/E92Q/T124A/M154I** (0.12-360 ng/mL)	**T66K**, **E92Q**, **F121Y**, T124A, **Q148R**, V151L **T66K/T124A**, **F121Y/G163R**, **F121Y/T125K** **E138K/Q148K**, **G140S/Q148R** M22I/T97A/T124A, **T66I/E92V/T124A** **T66K/E92Q/T124A** **T66I/L74M/E92V/T124A** (0.58-360 ng/mL)	**T66K**, **F121Y**, T124A, **Q148R** **T66K/T124A**, **T66K/T125K**, **E92Q/F121Y** **E92Q/T124A**, **F121Y/T124A**, **F121Y/G163R** **T124A/Q148R**, **A128T/V151L**, **E138K/Q148K** **G140S/Q148R** M22I/T97A/T124A, **F121Y/T125K/M154I** **T66I/L74M/E92V/T124A** (2.9-360 ng/mL)	**T66K**, **F121Y**, T124A, **Q148R** **T66K/T124A**, T66K/T125K, **E92Q/F121Y** E92Q/T124A, **E92Q/G140S**, E92I/T124A, F121Y/G163R, **A128T/V151L**, **E138K/Q148K** **G140S/Q148R** M22I/T97A/T124A, **T66K/L74M/T125K**, L74M/E92Q/F121Y, **F121Y/T125K/M154I** **T124A/E138K/Q148K**, **T124A/G140S/Q148K** (2.9-360 ng/mL)
Elvitegravir EC50=0.82 ng/mL Initial conc. (0.05-32 ng/mL)	**V151I** (0.05-32 ng/mL)	**T66I**, T124A **P145S**, **Q148K** T66I/T124A (0.05-32 ng/mL)	**T66A, T66I**, T124A **P145S**, **Q148K** , **Q148R** **T66I/124A**, **T66K/T124A**, **Q148R/T124A** (0.26-32 ng/mL)	**T66I, E92Q**, T124A **P145S**, **Q148K** , **Q148R** **T66I/T124A, T66K/T124A, E92V/T124A** **P145S/T124A, Q146L/T124A, Q148R/T124A** **T66I/V72A/A128T, T66I/E92Q/T124A** **T66I/T124A/Q146L** (1.3-160 ng/mL)		
Lamivudine NRTI[c] EC50=1,400 ng/mL Initial conc. (180-920 ng/mL)	**M184V** (180-4600 ng/mL)	**M184I, M184V** (180-4600 ng/mL)	**M184I, M184V** **K82N/M184V** (180-4600 ng/mL)			

[a] EC50 was determined using HIV-1 IIIB ands MT-4 cells.

[b] Phenotypic analysis was performed in parallel with genotypic analysis. The genotypes which showed >5-fold resistance compared with wild type were shown in bold letter. Genotypes in each cell are grouped based on number of substitutions identified.

[c] The genotypic analysis was performed in RT-region.

Table 5 Fold resistance of INI-resistant molecular clones to clinically investigated INIs

	S-364735	Raltegravir	Elvitegravir	L-870,810	S-1360	Efavirenz (RT)
Wild type EC50 Mean (SD) nM	3.6 (0.61)	6.1 (0.89)	1.3 (0.31)	3.0 (0.73)	220 (50)	1.7 (0.20)
Resistant Virus[a]	Fold resistance vs. wild type					
Wild type	1	1	1	1	1	1
T66A	0.75	0.61	*4.1*	1	2.6	1.3
T66I	1.2	0.51	*8.0*	1.1	*5.3*	1.5
T66K	**17**	*9.6*	**84**	**20**	**21**	2.1
E92I	2.6	2.1	*8.0*	*4.9*	*3.8*	1.0
E92Q	*3.7*	*3.5*	**19**	*6.4*	*4.5*	1.2
E92V	2.1	1.4	*8.3*	3	*3.8*	0.93
Q95R	1.3	0.94	1.4	1.1	1.3	0.83
G118R	**>20**	*7.2*	2.6	**670**	**> 170**	0.21
G118S	*5.2*	1.2	2.1	*4.9*	**10**	0.73
F121Y	**25**	*6.1*	**36**	*8.7*	**12**	2.1
T124A	0.97	0.82	1.2	0.82	0.79	0.95
P145S	1.4	0.87	**>350**	1.1	*3.9*	2.9
Q146R	1.7	1.2	2.8	0.91	*3.4*	0.94
Q148H	*3.8*	**27**	*6.4*	**12**	**27**	2.3
Q148K	**210**	**83**	**>1700**	**22**	**63**	2.1
Q148R	**73**	**47**	**240**	**31**	**84**	1.9
I151L	*9.6*	*8.4*	**29**	**21**	**26**	2.9
S153Y	1.4	1.3	2.3	1.1	*4.2*	1.9
M154I	0.78	0.82	1.1	0.85	0.94	1.4
N155H	*7.4*	**16**	**25**	**37**	*8.3*	0.88
N155S	**23**	*6.2*	**68**	*9.4*	**36**	1.7
N155T	**22**	*5.2*	**39**	*5.7*	**65**	1.5
T66I/L74M	*4.4*	2.0	**14**	*4.1*	**16**	1.2
T66I/E92Q	*6.6*	**18**	**190**	**56**	**47**	2.0
T66K/L74M	**46**	**40**	**120**	**64**	**29**	2.0
L74M/N155H	**18**	**37**	**45**	**56**	**27**	1.3
T97A/N155H	**22**	**48**	**43**	**62**	**31**	1.7
F121Y/T124A	*8.7*	*5.5*	**18**	**11**	**24**	1.2
F121Y/T125K	**20**	**11**	**34**	**19**	**21**	1.5
E138K/Q148H	*3.9*	**34**	*7.1*	**13**	**19**	1.5
E138K/Q148K	**>177**	**330**	**371**	**> 230**	**130**	1.2
E138K/Q148R	**170**	**110**	**460**	**180**	**23**	1.0
G140C/Q148R	**>177**	**200**	**485**	**> 950**	**99**	2.8
G140S/Q148H	**>31**	**>139**	**>774**	**>327**	**48**	1.3
G140S/Q148K	**110**	*3.7*	**94**	**80**	**37**	1.3
G140S/Q148R	**>177**	**200**	**267**	**> 950**	**37**	1.5
N155H/G163R	**15**	**32**	**35**	**44**	*6.3*	1.1
V72I/F121Y/T125K	**44**	**13**	**58**	**29**	**29**	1.5
V75I/T112S/Q146P	*4.8*	1.3	**17**	1.6	*4.5*	2.2
V72I/F121Y/T125K /I151V	**16**	*7.0*	**37**	**15**	**27**	1.1

These data are mean values of at least 2 independent experiments performed in duplicate.
Fold resistance between 3 and 10 shown in italics
Fold resistance > 10 fold are shown in bold letter

[a] Molecular clone derived from pNL432

CHAPTER 2

In Vitro Antiretroviral Properties of S/GSK1349572, a Next-Generation HIV Integrase Inhibitor

INTRODUCTION

After an initial period of false starts, advances in the field of HIV integrase drug discovery since the late 1990s have been outstanding. Beginning with the discovery that molecules capable of binding two metals within the integrase active site can potently inhibit the recombinant enzyme and virus replication in cells (21), many INIs with different chemical scaffolds have proceeded from preclinical to clinical development (e.g., S-1360 (4), L-870,810 (14), S/GSK364735 (18), GS-9160 (26), raltegravir [RAL, MK-0518] (34, 35), and elvitegravir [EVG, GS-9137] (13, 52)). RAL was approved by the U.S. FDA in 2007, while EVG has progressed into phase 3 development at the time of this writing. The INI class is now recognized as among the safest and most potent anti-HIV drugs (44). However, clinical resistance to RAL and EVG has been observed, and a high degree of cross-resistance between these two agents has been demonstrated (33, 36). Furthermore, the dosing of RAL is twice daily, while once-daily administration of EVG requires a pharmacokinetic (PK) booster such as ritonavir or cobicistat (GS-9350), which raises long-term safety and/or drug-drug interaction concerns. A next-generation INI should have attributes that address these issues.

S/GSK1349572 was created by research collaboration between Shionogi and GlaxoSmithKline (GSK) and is being developed by a joint venture, Shionogi-ViiV Healthcare LLC. The aim was to provide a next-generation INI, and S/GSK1349572 was engineered to deliver a different resistance profile with once-daily unboosted dosing potential. Clinical data with healthy subjects demonstrated PKs supporting once-daily administration with a low-milligram dose, low PK variability, and excellent short-term safety/tolerability (37). The phase 2a once-daily, 10-day monotherapy study further demonstrated the potent antiretroviral activity and short-term tolerability of S/GSK1349572 (29), prompting the initiation of phase 2b (and now phase 3) clinical trials. In this chapter, I report the *in vitro* antiretroviral properties of

S/GSK1349572, focusing on its mechanism of action and *in vitro* resistance profile using panel of INI-resistant mutants and a novel method a variety of resistant viruses can be isolated.

MATERIALS AND METHODS

Compounds

S/GSK1349572 was synthesized at Shionogi Research Laboratories, Osaka, Japan. RAL and EVG were synthesized at GlaxoSmithKline, Research Triangle Park, NC. Efavirenz and lamivudine were purchased from Sequoia Research Products, Ltd., Pangbourne, United Kingdom. The structures of S/GSK1349572, RAL, and EVG are shown in Fig. 4.

Cells and viruses

MT-4 cells, a human T-cell leukemia virus type 1 (HTLV-1)-transformed human T-cell line, were maintained as described previously (12). 293T cells were maintained in Dulbecco's modified Eagle medium (DMEM)-F-12 medium containing 10% fetal bovine serum (FBS). Peripheral blood mononuclear cells (PBMCs) were derived from whole-blood samples obtained from HIV-negative donors. PBMCs were separated from whole blood by density gradient centrifugation with Ficoll-Paque Plus (GE Healthcare) according to the manufacturer's instructions and were stimulated by the addition of either 20 U/mL of interleukin-2 (IL-2) or 10% natural T-cell growth factor (ZeptoMetrix) plus 5 to 10 μg/mL of phytohemagglutinin (PHA). MOLT-4 cells persistently infected with HIV-1 IIIB and MT-2 cells (19) were obtained from S. Harada (Kumamoto University). HeLa-CD4 cells containing an HIV-1 long terminal repeat (LTR)-driven β-galactosidase reporter gene have been described previously (24). MAGI-CCR5 cells have been described previously (9). HIV-1 strain IIIB was derived from cell-free supernatants of cultures of the chronically infected cell line H93B (H9/HTLV-IIIB). HIV-1 strain Ba-L was purchased from Advanced Biotechnologies Inc. and was expanded in PHA-activated PBMCs, while HIV-1 NL432 (1) was obtained from A. Adachi (Tokushima University). Plasmid pGJ3-Luci, containing a replication-defective HIV lentiviral vector expressing luciferase (25), was licensed from Christian Jassoy (University of Leipzig) and was

used to create stocks of a vesicular stomatitis virus glycoprotein G (VSV-G)-pseudotyped self-inactivating pseudo-HIV (PHIV) lentiviral vector by cotransfection along with plasmid pVSV-G (Clontech) into CIP4 cells (a derivative of the 293T human renal epithelial cell line that expresses macrophage scavenger receptor SRA-I to improve adherence to plastic) and harvesting of the cell-free supernatant.

In vitro strand transfer assay

The inhibitory potencies of S/GSK1349572 and other INIs were measured in a strand transfer assay using recombinant HIV integrase as previously described (5). A complex of integrase and biotinylated preprocessed donor DNA-streptavidin-coated Acintillation proximity assay (SPA) beads was formed by incubating 2 μM purified recombinant integrase with 0.66μM biotinylated donor DNA-4 mg/ml streptavidin-coated SPA beads in 25 mM sodium morpholinepropanesulfonic acid (MOPS) (pH 7.2), 23 mM NaCl, and 10 mM $MgCl_2$ for 5 min at 37 °C. These beads were spun down and preincubated with diluted INIs for 60 min at 37 °C. Then a ^{3}H-labeled target DNA substrate was added to give a final concentration of 7 nM substrate, and the strand transfer reaction mixture was incubated at 37 °C for 25 to 45 min, which allowed for a linear increase in the strand transfer of donor DNA to radiolabeled target DNA. The signal was read using a Wallac MicroBeta scintillation plate reader.

Antiviral assay in MT-4 cells

MT-4 cells growing exponentially at a density of 5×10^5 or 6×10^5/ml were infected with HIV-1 strain IIIB at a viral multiplicity of infection of 0.001 or a 50% tissue culture infective dose of 4 to 10. The cells were then aliquoted to 96-well plates in the presence of varying concentrations of compounds. After incubation for 4 or 5 days, antiviral activity was determined by a cell viability assay that either measured bioluminescence with a CellTiter-Glo luminescent reagent (Promega Corporation) or measured absorbance at 560 and 690 nm using

the yellow tetrazolium MTT reagent [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide].

Pseudo-HIV assay

The antiviral activities of compounds were measured in a single-round assay using a self-inactivating PHIV lentiviral vector. CIP4 cells (2×10^4/well) infected with an amount of PHIV sufficient to produce approximately 50,000 relative light units were added to 96-well black, clear-bottom plates and were incubated for 2 days with S/GSK1349572 at varying concentrations. Infected cells were measured as a function of luciferase activity in a luminometer using the Steady-Glo reagent (Promega Corporation).

Antiviral assay in PBMCs

In one 96-well culture plate, PHA- and IL-2-stimulated PBMCs (4×10^5/well) were preincubated with a compound for 1 h, while HIV-1 strain Ba-L was mixed with the same compound in a second plate. An aliquot of the Ba-L-compound mixture was then transferred to the PBMC-compound mixture and was incubated for 7 days. After this incubation, supernatants were assayed for RT activity by incorporation of [*methyl*-^{3}H]dTTP to measure viral replication as previously described (18).

Effects of human serum and serum proteins

The effect of the presence of human serum albumin (HSA; 20 or 40 mg/mL), α_1-acid glycoprotein (AAG; 2 mg/mL), or human serum (HS; up to 30% or 50% was used, and results were extrapolated up to 100%) on the antiviral activity of S/GSK1349572 was evaluated in the PHIV and MT-4 assay systems. To estimate the effects of the fold shift in protein binding, antiviral activity was tested with the addition of various concentrations of human serum to the HIV-1 IIIB replication assay mixture in MT-4 cells as previously described (18). The protein-

adjusted half-maximal effective concentration (PA-EC_{50}) was estimated by multiplying the EC_{50} in PBMCs by the fold shift value.

Cytotoxicity assays

In vitro growth inhibition (cytotoxicity) studies were conducted with S/GSK1349572 in proliferating human leukemic and lymphomic cell lines (IM-9, U-937, MT-4, and MOLT-4) as well as in stimulated and unstimulated human PBMCs. ATP levels were quantified by using the CellTiter-Glo luciferase reagent to measure the ability of a compound to inhibit cell growth as an indicator of the compound's potential for cytotoxicity.

Mechanistic cellular studies

To determine if S/GSK1349572 was inhibiting HIV replication in cellular assays through an integrase inhibition mechanism, the effects on the synthesis of HIV NL432 DNA species in MT-4 cells were measured in a single-round infection assay using quantitative PCR methods. Quantitative PCR analysis was performed to measure the synthesis of HIV DNA species in MT-4 cells in the presence of an INI or NNRTI as described previously, with minor modifications (18). Briefly, 293T cells were transfected with the NL432 plasmid to generate infectious virus, and the supernatant was filtered through 0.45-μm-pore-size filters and was treated with DNase I. MT-4 cells were infected with HIV-1 NL432 for 1 h, incubated with dilutions of a compound, and collected after 6 or 18 h of incubation. All cells were incubated with 0.5 μM ritonavir in order to limit HIV replication to a single cycle. Total-DNA PCR to detect late RT products was performed by incubating the samples for 6 h. Nested Alu-PCR to detect integrated provirus and 2-LTR PCR to detect 2-LTR circles were performed by incubating the samples for 18 h. Reaction products were analyzed using the ABI Prism 7900HT-3 sequence detection system (Applied Biosystems).

Isolation of drug-resistant viruses

Drug-resistant viruses were isolated according to a previously described protocol (28). Briefly, the virus used for initiating passage work was prepared by coculturing MT-2 cells with MOLT-4 cells persistently infected with HIV-1 strain IIIB for 3 days. Fresh MT-2 cells were dispensed into each well of a 24-well tissue culture plate. Three wells of each culture containing several concentrations of a compound were used initially, and the virus prepared as described above was added to each well. Every 3 or 4 days, the cells were passaged with or without the addition of fresh MT-2 cells. When a cytopathic effect was observed, the supernatants were used to infect fresh MT-2 cells, and the concentration of the compound was held constant and/or increased 5-fold. Every 2 weeks, when virus replication was ascertained by observation of a cytopathic effect, the infected cells were collected and used for genotypic and phenotypic analyses. For the analysis of mutations, DNA was extracted from infected cells using the DNeasy blood and tissue kit (Qiagen), and the integrase region of HIV proviral DNA was amplified by PCR with specific primers (M-poli7, AACAAGTAGATAAATTAGTCAGT; M-poli8, TAGTGGGATGTGTACTTCTGAAC). The products were sequenced by Operon Biotechnologies' sequencing service. The sequence of the integrase region derived from isolated viruses was compared with that of wild-type IIIB, and amino acid substitutions were identified.

Construction of integrase region-recombinant HIV-1 molecular clones

The XbaI-EcoRI fragment from pNL-IN301 (the XbaI site was inserted into the 5′ end of the integrase region of pNL432 (1) and termed pNL-IN301) was cloned into the XbaI-EcoRI site of cloning vector pUC18. *In vitro* mutagenesis was performed with the QuikChange site-directed mutagenesis kit (Stratagene, a division of Agilent Technologies) using pUC18 cloned with the integrase region as a template. The mutated XbaI-EcoRI fragment was amplified and ligated into pNL-IN301 to construct a recombinant HIV-1 molecular clone. Plasmids were

subsequently transfected into 293T cells to generate infectious virus. Supernatants were harvested after 2 days of culture and were stored as cell-free culture supernatants at −80°°C.

Cross-resistance profiling of S/GSK1349572

S/GSK1349572 was evaluated against molecular clones with mutations in the integrase-, RT-, and protease-coding regions. INI-, NRTI-, and NNRTI-resistant mutants were analyzed by the reporter assay based on HeLa-CD4 cells, while PI-resistant mutants were analyzed by infectivity in MT-4 cells, with monitoring of RT activity as described previously (18). The HIV-1 wild-type infectious molecular clone pNL432 was used for site-directed mutagenesis to generate HIV clones containing mutations. Fifty INI-resistant mutants were constructed. The molecular clones with a K103N or Y188L mutation (2, 3) within the RT-coding region were used as NNRTI-resistant viruses; those with M184V (6, 48), D67N/K70R/T215Y (7), or R4 (V75I/F77L/F116Y/Q151M) (40) mutations within the RT coding region were used as NRTI-resistant viruses; and those carrying M46I/I47V/I50V (41) or L24I/M46I/L63P/A71V/G73S/V82T (27) mutations in the protease-coding region were used as PI-resistant viruses. 293T cells were subsequently transfected with the plasmids to generate infectious virus using Lipofectamine 2000 (Invitrogen Corporation). Supernatants were harvested 2 to 3 days after transfection, stored as cell-free culture supernatants at −80°C, and used for each assay.

Combination antiviral activity assay in MT-4 cells

The *in vitro* combination activity relationships of S/GSK1349572 were determined as previously described (46). Multiple concentrations of S/GSK1349572 were tested in checkerboard dilution fashion in the presence and absence of dilutions of representative approved anti-HIV drugs, adefovir, or ribavirin. The assay used HIV-1 IIIB-infected MT-4 cells, and the interaction of compound combinations was analyzed by dosewise additivity-based

calculations to quantify deviation from dosewise additivity at the 50% level. Wells containing the top concentration of compounds by themselves were compared to wells with the top concentration of compound combinations in order to show that combination effects were due to the drugs used and not simply to toxicity. Assays with the MT-4 system format were run as described previously (18). Fractional inhibitory concentration (FIC) values in the range of −0.1 to −0.2 indicate weak synergy; values that approach −0.5 indicate strong synergy; and positive values of 0.1 to 0.2 indicate weak antagonism. The effects of the anti-hepatitis B virus (anti-HBV) and anti-HCV agents adefovir and ribavirin on S/GSK1349572 IC_{50} were examined using linear regression as described previously (49). Since the HIV-1 IIIB MT-4 system is CXCR4 based, the CCR5 inhibitor maraviroc was evaluated in a checkerboard dilution format using MAGI-CCR5 cells with the Gal Screen reagent (Tropix, Bedford) for chemiluminescent endpoints, and data were analyzed as described by Prichard and Shipman (43) by using the MacSynergy II program. Synergy volumes in the range of −50 to 50 define additivity; <−50, antagonism; and >50, synergy.

RESULTS

Inhibition of recombinant HIV integrase and HIV replication by S/GSK1349572

S/GSK1349572 inhibited HIV-1 integrase-catalyzed strand transfer with IC_{50} of 2.7 nM. The EC_{50} against HIV-1 was 0.51 nM in PBMCs, 0.71 nM in MT-4 cells, and 2.2 nM in the PHIV assay, which uses a pseudotyped self-inactivating virus. Measurements of the fold shift from EC_{50} to EC_{90} ranged from 3-fold to a maximum of 4-fold; as a conservative measure, an EC_{90} 4-fold higher than the EC_{50} was used. This low-nanomolar potency was similar to the potencies of RAL and EVG (Table. 6). In the MT-4 antiviral assay, the estimated potency shift was 75-fold when the results were extrapolated to 100% human serum and 32-fold in the presence of 20 mg/ml HSA. In the PHIV assay, the potency shift was 11-fold with 40 mg/ml HSA and 2.1-fold in the presence of 2 mg/ml AAG. The extrapolated potency shift of 75-fold for S/GSK1349572 in the presence of 100% human serum was also applied to EC_{50} and EC_{90} in PBMCs, resulting in PA-EC_{50} and PA-EC_{90} values of 38 nM and 152 nM, respectively. These values were similar to those of EVG and 4.1-fold less potent than those of RAL. The 50% cytotoxic concentrations (CC_{50}) for S/GSK1349572 in proliferating IM-9, U-937, MT-4, and MOLT-4 cells were 4.8, 7.0, 14, and 15 μM, respectively. In unstimulated and stimulated PBMCs (both from the same 4 donors), the CC_{50} were 189 μM and 52 μM, respectively. Based on the EC_{50} of S/GSK1349572 against HIV-1 in PBMCs (i.e., 0.51 nM), this translates to a cell-based therapeutic index of at least 9,400.

Cellular mechanistic studies

As shown in Fig. 5, S/GSK1349572 inhibited the integration of viral DNA (Fig. 5b), with a concomitant increase in 2-LTR circles (Fig. 5c) and no effect on viral DNA production (Fig. 5a). Thus, S/GSK1349572 demonstrated the expected effects of an INI not of a RTI. Furthermore, the concentration dependency of the effects was within the range of error for the potency

observed in the inhibition of viral replication in PBMCs and MT-4 cells (11). These effects were similar to the effects observed with RAL and S/GSK364735 in a previous study (18). In contrast, NNRTI efavirenz showed decrease in total viral DNA synthesis dependent on S/GSK1349572 concentration due to inhibition of RT activity.

Cross-resistance profiling of S/GSK1349572

When tested against HIV strains resistant to marketed NNRTIs or NRTIs, S/GSK1349572 showed efficacy against five different NNRTI-resistant or NRTI-resistant viruses, with activity equivalent to that against wild-type virus (EC_{50}, 1.3 to 2.1 nM). Likewise, S/GSK1349572 showed efficacy against two different PI-resistant viruses, with activity equivalent to that against wild-type virus (EC_{50}, 0.36 and 0.37 nM).

Isolation of viruses resistant to S/GSK1349572 and other INIs

In vitro passage experiments were performed with S/GSK1349572 and RAL, with the NRTI lamivudine as a reference, starting with wild-type HIV-1 IIIB (Table 7), itself a quasispecies. In the culture with S/GSK1349572, the T124A substitution was observed first on day 14 (notably, IIIB contains ~40% T124A at baseline). The T124A/S153F substitution was observed on day 28, followed by the T124A/S153Y and L101I/T124A/S153F substitutions on day 70; these substitutions persisted throughout the remaining passages. Viruses with the S153Y substitution were isolated on days 84 and 112. Only four viruses with substitutions were observed on day 112.

Mutations that reduced susceptibility more than 5-fold, as measured by phenotypic assays, are shown in boldface in Table 7. This level of resistance was observed on day 14 with lamivudine and on day 28 with RAL. Highly resistant mutants with a high FC (>100-fold) in the EC_{50} were isolated in the presence of RAL; viruses with many of these mutations have also been isolated in the clinic from patients failing RAL-based regimens (11). In contrast, no highly resistant

mutants were isolated in the presence of S/GSK1349572 through day 112. Multiple substitutions in integrase observed during S/GSK1349572 passage conferred only a low FC (maximum, 4.1-fold). Since these viruses had relatively low FCs and these substitutions remained unchanged from day 70 to day 112, they should not significantly affect resistance to S/GSK1349572.

The dose escalation patterns of S/GSK1349572, RAL, and EVG were also compared (Fig. 6); the *in vitro* passage experiment with EVG has been described previously (28). Each passage was started with 0.26, 1.3, 6.4, and 32 nM each INI, and compound concentrations were increased in a stepwise manner. Fig. 6 shows the dose escalation patterns, starting from 1.3 nM, that permitted viral replication to continue. The concentration of RAL was increased to 160 nM on day 35, followed by 800 nM on day 77. The concentration of EVG was increased to 160 nM on day 56. In contrast, no replication was observed at S/GSK1349572 concentrations of 32 nM or greater throughout the passage experiment. Thus, the maximum concentration of S/GSK1349572 allowing replication was 6.4 nM.

Sensitivities of drug-resistant molecular clones to INIs

A panel of 50 INI-resistant site-directed mutants was constructed and tested for susceptibility to S/GSK1349572 and other INIs (Table 8). Most of these mutants were isolated during *in vitro* passage studies and/or clinical trials of RAL, while a few were derived from the literature. Efavirenz was used as a control, and the FC in its EC_{50} against the INI-resistant mutants was as high as 2.9-fold. Therefore, the viruses for which the EC_{50} was ≥3-fold that for the wild type were considered to be resistant in this study. Although substitutions involving S153 (i.e., S153F, S153Y, L101/IS153F, T124A/S153Y, and L101IT/124A/S153F) were isolated during the *in vitro* selection study with S/GSK1349572, these mutant viruses remained susceptible to S/GSK1349572. L101I and T124A are polymorphic, and all INIs showed wild-type potency against these site-directed mutants. S/GSK1349572 showed a moderate reduction in potency

(FC, <10-fold but >3-fold) against five mutant viruses (the I151L, T66K/L74M, E138K/Q148R, G140C/Q148R, and G140S/Q148R mutants), with FCs from 3.5- to 8.4-fold. Ten RAL- and seven EVG-resistant mutants had FCs within the 3- to 10-fold range. However, except for one mutant (I151L), all of these moderately RAL and EVG resistant mutants were susceptible to S/GSK1349572. Only two mutant viruses, the E138K/Q148K and Q148R/N155H mutants (the latter of which has not been demonstrated on the same genome), were shown to lead to an FC of >10-fold for S/GSK1349572; these mutants also shared a highly resistant phenotype against RAL (FCs, 330- and >140-fold, respectively) and EVG (FCs, 371- and 390-fold, respectively). In contrast, RAL and EVG showed FCs of >10-fold in their EC_{50}s against 23 and 32 mutant viruses, respectively. Notably, S/GSK1349572 is potent against all single mutants examined in my panel. Furthermore, limited cross-resistance against RAL- and/or EVG-resistant viruses was observed; S/GSK1349572 was active against most INI-resistant viruses with double or more mutations, including clinical isolates from patients with RAL treatment failure (50).

Cellular combination studies

S/GSK1349572 was tested in combination studies with 8 HIV antiretrovirals representing each class approved at the time, as well as with adefovir and ribavirin, which are likely to be coadministered to HIV patients coinfected with HBV or HCV (Table 9). No antagonism was observed with any combination, and no enhanced cytotoxicity was observed within the concentrations tested for antiviral activity. As expected, S/GSK1349572 was additive with itself. In combination with nucleoside RT inhibitors, S/GSK1349572 was synergistic with stavudine and abacavir. In combination with nonnucleoside RT inhibitors, S/GSK1349572 was synergistic with efavirenz and nevirapine. Among HIV protease inhibitors, S/GSK1349572 was synergistic with lopinavir and amprenavir. The activity of S/GSK1349572 was found to be synergistic with the effects of the fusion inhibitor enfuvirtide and was not significantly affected

by the presence of the hepatitis B drug adefovir or the hepatitis C drug ribavirin (data not shown). Finally, S/GSK1349572 was additive with the effects of maraviroc (data not shown).

DISCUSSION

It is well accepted that HIV can overcome drug therapy through the accumulation of resistance mutations. Clinical data indicate that resistant viruses against the first-generation INI drugs (RAL and EVG) have indeed arisen already (22, 36). I have engineered a new INI, S/GSK1349572, that is highly active against both wild-type HIV and many INI-resistant mutants and that has the potential for a higher genetic barrier to resistance. The EC_{50} of S/GSK1349572 against HIV-1 in PBMCs and various other cells is in the low-nanomolar to subnanomolar range (data not shown). In addition, the median EC_{50} against nine clinical isolates of HIV-2 was 0.8 nM (10). Cellular toxicity was in the micromolar range for a variety of cell types, indicating that the observed antiviral effect of S/GSK1349572 was not due to cytotoxicity.

Based on the data presented, the mechanism of inhibition is strongly suggested to be at the strand transfer step of HIV integrase, thus preventing the insertion of viral DNA into host DNA. In addition, integration reaction products obtained using blunt-end DNA as a substrate were resolved with a DNA sequencing gel, and S/GSK1349572 showed minor inhibition of 3′-end processing versus strand transfer inhibition (data not shown), indicating that S/GSK1349572 is a strand transfer inhibitor. S/GSK1349572 inhibited both the HIV integration reaction strand transfer step *in vitro* and HIV replication in cells with similar potencies. The inhibitor had no effect on total viral DNA synthesis in infected cells but blocked the integration of viral DNA into host DNA with the same potency as its antiviral effect. In addition, this INI increased the appearance of viral 2-LTR circles, a predicted by-product of integrase inhibition, with the same potency. The compound had potency against mutant viruses resistant to NRTIs, NNRTIs, and PIs similar to its potency against the wild type and consistent with action on a different antiretroviral target. The passage of virus in an S/GSK1349572-containing medium selected for viruses with mutations in the vicinity of the integrase active site, though the FCs in the EC_{50}s

against the mutants were very low. Finally, S/GSK1349572 had decreased potency against a small subset of mutant viruses resistant to known INIs.

In vitro passage with S/GSK1349572 followed a pathway significantly different from that with RAL, EVG, or lamivudine. While viruses with the ability to replicate at micromolar levels of RAL, EVG, or lamivudine were readily selected, the concentration of S/GSK1349572 that allowed for virus replication could not be raised above 32 nM. In addition, only four substitutions or combinations of substitutions (T124A, S153Y, T124A/S153Y, and L101Y/T124A/S153Y) were observed on passage with S/GSK1349572 up to day 112, and these amino acid substitutions did not cause a high FC in susceptibility to S/GSK1349572 in the phenotype assay. Although amino acid position 153 is conserved, amino acid positions 101 and 124 in HIV-1 integrase are polymorphic (8). It was also demonstrated that the selection of resistant mutations from a wild-type population was greatly delayed in S/GSK1349572-containing medium in contrast with that for first-generation INIs. Taken together, these results demonstrate that S/GSK1349572 possesses a resistance profile distinct from that of the first-generation INIs and suggest that S/GSK1349572 may possess a higher genetic barrier to the development of resistance.

The substitutions observed in the passage study with S/GSK1349572 had no effect or minimal effects on susceptibility to S/GSK1349572 or other INIs when they were introduced into a laboratory strain by site-directed mutagenesis. Moreover, the substitutions described here have not been observed so far in passage studies with other INIs, except for T124A, which is a polymorphism in the integrase region; viruses with T124A have been isolated with S-1360, S/GSK364735, RAL, L-870,810, and EVG (28). Notably, the passage with HIV-1 IIIB utilized a virus population derived from a chronically infected cell line, and at the initiation of passage, the T124A variant was present as approximately 40% of the quasispecies population. The T124A substitution alone did not contribute to virus resistance and, like L101I, is a naturally occurring polymorphism (30).

Importantly, S/GSK1349572 showed potency against all integrase-resistant single mutants in my panel at a level similar to that observed against wild-type virus or with an FC as high as 3.6-fold. This result may partially explain the observation during passage in the presence of S/GSK1349572 that no virus with high resistance to S/GSK1349572 was observed with 32 nM or higher concentrations of S/GSK1349572 in the culture medium. The hypothesis would be as follows: more than one substitution is required for very high resistance, and if single mutations conferring significant resistance did not first arise, then very high resistance would not occur during passage. In contrast, during passage with RAL and EVG (as well as with the control, lamivudine), single amino acid substitutions could cause a significant fold change; then additional secondary mutations could provide increased resistance or compensate for the decreased fitness of the virus. Overall, these data are also supportive of the potential for a higher genetic barrier of S/GSK1349572 to resistance, although clinical data will be required to fully test this hypothesis. It is noteworthy that in a 10-day phase 2a monotherapy study, the plasma steady-state geometric mean concentration at the end of a dosing interval (C_{tau}) at once-daily dosing with 50 mg was 0.83 μg/ml, 13 times the *in vitro* PA-EC_{90} (S. Min et al., submitted for publication).

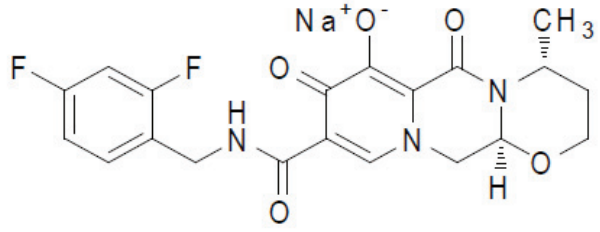
It is also striking that S/GSK1349572 had significant potency against most double or more mutants in my panel, with the exception of the E138K/Q148K and Q148R/N155H mutants (FCs, 19 and 10, respectively). While these double mutants were highly resistant to S/GSK1349572, the first mutation of these pathways (i.e., Q148K, Q148R, or N155H) was not selected in the passage study with S/GSK1349572, perhaps because the FCs for these single mutants remained similar to that for the wild type. The replication/infectivity of the E138K/Q148K mutant was higher than that with Q148K alone, but even so, its replication capacity and integration efficiency were poorer than those of the wild type (38). The Q148R and N155H mutations were not observed in the same genome in clinical isolates but were found as mixtures (32), and the replication capacity of the Q148R/N155H mutant molecular clone was poor in PBMCs

(unpublished data). It is thus noteworthy that S/GSK1349572 had limited cross-resistance to RAL- and EVG-resistant mutants, clearly distinguishing S/GSK1349572 as a next-generation INI. To better understand the distinct resistance profile of S/GSK1349572 compared to those of RAL and EVG, studies of docking into HIV-1 IN/Mg^{2+}/DNA models are in progress.

Since INIs will be used in combination regimens, it is important to evaluate the potential for synergy and antagonism. S/GSK1349572 was tested in combination assays with representatives of all approved classes of HIV therapeutics, as well as with adefovir and ribavirin, drugs likely to be coadministered to HIV patients coinfected with HBV or HCV. Ribavirin and stavudine (d4T) were used in the study as positive controls for antagonism (23). Ribavirin has no anti-HIV activity itself, and when added to d4T, ribavirin led to a decrease in its potency, thus demonstrating antagonism. No antagonism or trend toward antagonism was observed when S/GSK1349572 was tested with any class of marketed HIV drug, adefovir, or ribavirin. On the other hand, with several of the combinations, either statistically significant synergism or a trend toward synergism was seen. Similar data have been presented for other INIs (31) and raise the possibility of highly potent combinations in the clinic.

In conclusion, S/GSK1349572 has a resistance profile markedly distinct from those of RAL and EVG, and it demonstrates the potential for a higher genetic barrier to resistance. These preclinical findings formed part of the rationale for the selection of S/GSK1349572 as a candidate for clinical development, and they provide a strong foundation for its ongoing clinical investigation.

S/GSK1349572



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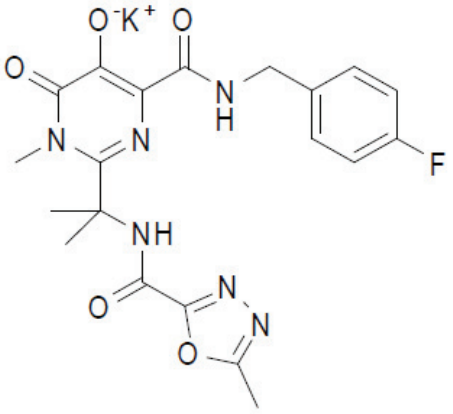

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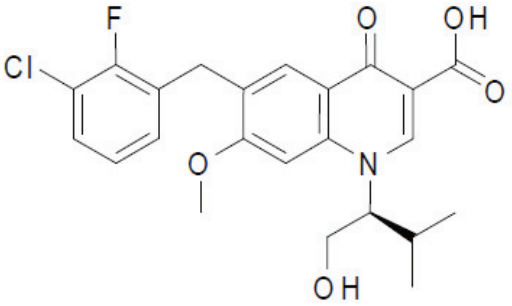

Fig. 4 Chemical structures of the HIV-1 integrase inhibitors used in this study.

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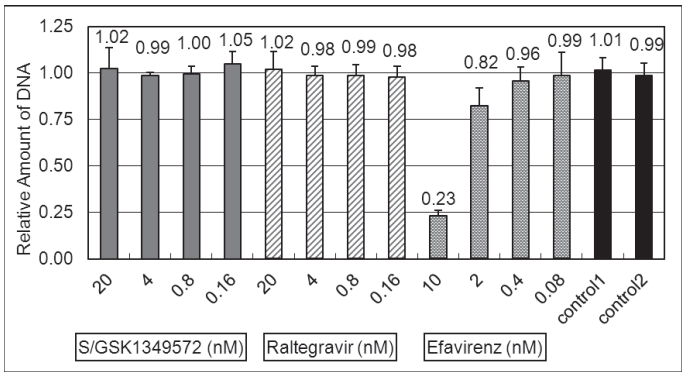

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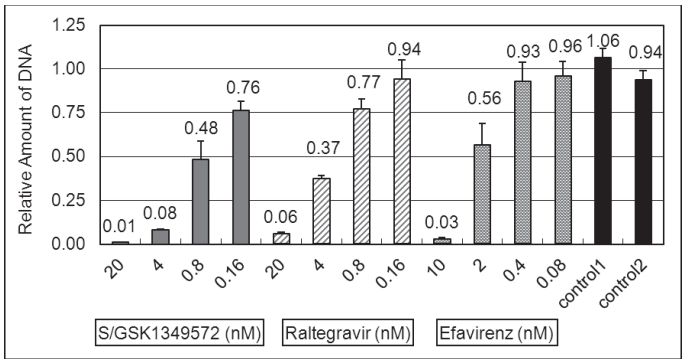

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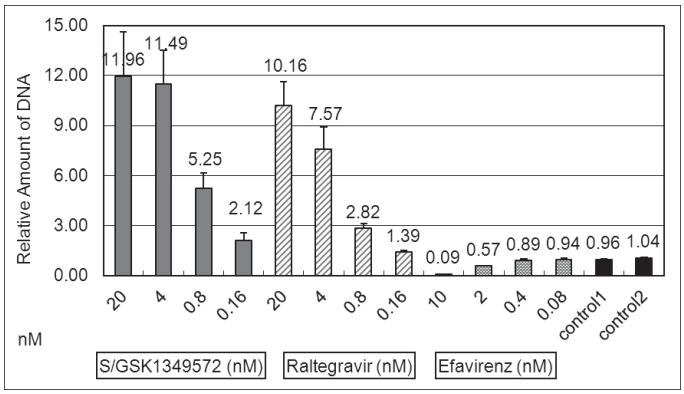

Fig. 5 Effects of inhibitors on various forms of viral DNA in MT-4 cells. The INIs S/GSK1349572 and RAL and the NNRTI efavirenz were each tested separately with MT-4 cells infected with HIV-1 NL432 to determine their effects on the amounts of total viral DNA (a), integrated viral DNA (b), and two-LTR circular viral DNA (c). The y axis of each graph represents amounts of DNA relative to the control. Each bar represents the mean value of results from three independent experiments. Error bars represent standard deviations.

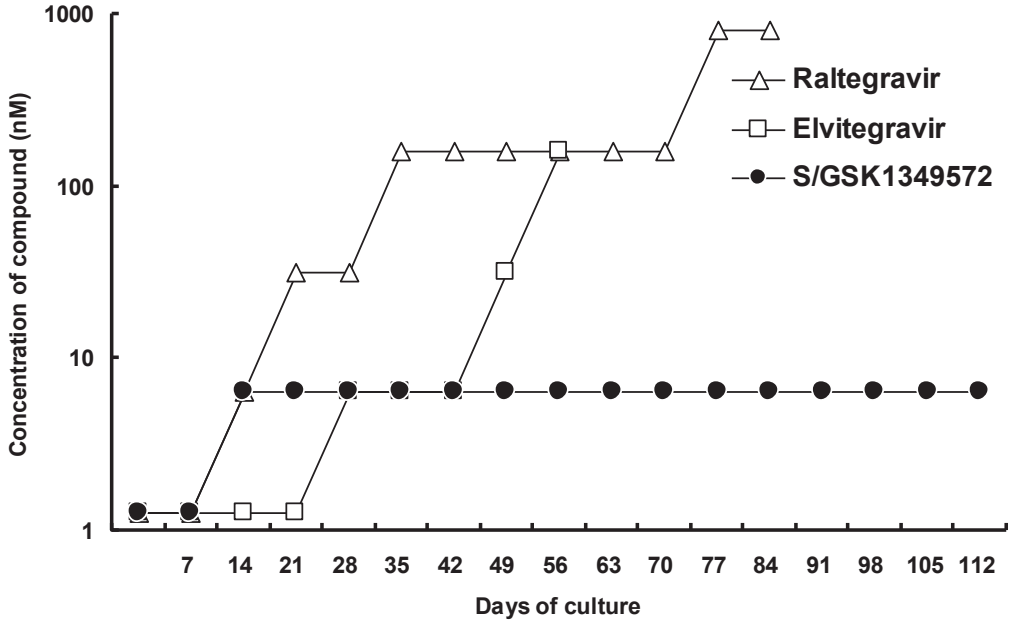

Fig. 6 Long-term culture of infected MT-2 cells with escalating concentrations of INIs. MT-2 cells were infected with strain HIV-1 IIIB and were passaged twice weekly in the presence of RAL (open triangles), EVG (open squares) or S/GSK1349572 (filled circles); the highest concentration at which the virus could replicate in cultured wells is indicated.

TABLE 6. Inhibition of recombinant HIV integrase and HIV replication by S/GSK1349572.

INI	Enzyme assay	Antiviral activity (nM)			
	IC_{50} (nM) for Strand transfer	PBMC EC_{50}	Potency Shift with 100% HS[a]	PA-EC_{50}	PA-EC_{90}
S/GSK1349572	2.7 ± 0.05 [b]	0.51 ± 0.25 [c]	75	38	152
Raltegravir[d]	3.3 ± 0.6	2 ± 1	4.7	5.6	23
Elvitegravir[d]	6 ± 1	2 ± 1	22	20	78

[a] Protein-binding fold shift was estimated from antiviral activity with various concentrations of human serum.

[b] Mean value from at least 3 experiments.

[c] HIV-1 strain Ba-L was used. Mean value from 12 experiments.

[d] Strand transfer IC_{50} and HIV replication EC_{50} for RAL and EVG were from Ref. 18.

TABLE 7. Time course of emergence of INI-resistant mutants

Inhibitor	Days of culture (passage number)							
	14[a] (4)	28[a] (8)	42[a] (12)	56[a] (16)	70[a] (20)	84[a] (24)	98[a] (28)	112[a] (32)
Initial conc. (0.26-32 nM) S/GSK1349572	(0.26-6.4 nM) T124A	(0.26-6.4 nM) T124A, T124A/S153F	(0.26-6.4 nM) T124A, T124A/S153F	(1.3-6.4 nM) T124A, T124A/S153F	(1.3-6.4 nM) T124A, T124A/S153Y, L101I/T124A/S153F	(1.3-32 nM) T124A, S153Y, T124A/S153Y, L101I/T124A/S153F	(1.3-32 nM) T124A, T124A/S153Y, L101I/T124A/S153F	(1.3-32 nM) T124A, S153Y, T124A/S153Y, L101I/T124A/S153F
Initial conc. (0.26-32 nM) Raltegravir	(0.26-32 nM) T124A	(0.26-160 nM) T124A, **Q148K**, N155H/I204T	(1.3-160 nM) G59E, T124A, **Q148K**, Q148R, **N155H**, **N155H/I204T**	(6.4-800 nM) T124A, **Q148K**, **Q148R**, **N155H**, E92Q/M154I, **Q148K/G163R**, **N155H/I204T**	(6.4-4000 nM) T124A, **Q148K**, **Q148R**, **N17S/Q148K**, **E92Q/M154I**, **G140C/Q148K**, **G140S/Q148R**, **Q148K/G163R**, **V151I/N155H**, **N155H/I204T**, **T124A/V151I/N155H**, **G140C/Q148K/G163R**	(32-4000 nM) T124A, **Q148K**, **Q148R**, **E138K/Q148K**, **E138K/Q148R**, **G140S/Q148R**, **V151I/N155H**, **N155H/I204T**, **N17S/Q148K/G163R**, **T124A/V151I/N155H**, **E138K/Q148K/G163R**, **G140C/Q148K/G163R**, **E92Q/E138K/Q148K/M154I**		
Initial conc. (0.26-32 nM) L-870,810	(0.26-32 nM) T124A	(0.26-32 nM) T124A, **Q148R**	(0.26-32 nM) **T66K**, F121Y, T124A, Q148R, V151L, **T124A/Q148R**, **E138K/Q148K**, **T66I/E92V/T124A**, **T66K/E92Q/T124A/M154I**	(1.3-160 nM) **T66K**, **E92Q**, **F121Y**, T124A, **Q148R**, V151L, **TT66K/T124A**, **F121Y/G163R**, **F121Y/T125K**, **E138K/Q148K**, **G140S/Q148R**, M22I/T97A/T124A, **T66I/E92V/T124A**, **T66K/E92Q/T124A**, **T66I/L74M/E92V/T124A**	(6.4-800 nM) **T66K**, **F121Y**, T124A, **Q148R**, **T66K/T124A**, **T66K/T125K**, **E92Q/F121Y**, **E92Q/T124A**, **F121Y/T124A**, **F121Y/G163R**, **T124A/Q148R**, **A128T/V151L**, **E138K/Q148K**, **G140S/Q148R**, M22I/T97A/T124A, **F121Y/T125K/M154I**, **T66I/L74M/E92V/T124A**	(6.4-800 nM) **T66K, F121Y,** **T124A, Q148R,** **T66K/T124A,** **T66K/T125K,** **E92Q/F121Y,** **E92Q/T124A,** **E92Q/G140S,** **E92I/T124A,** **F121Y/G163R,** **A128T/V151L,** **E138K/Q148K,** **G140S/Q148R,** **M22I/T97A/T124A,** **T66K/L74M/T125K,** **L74M/E92Q/F121Y,** **F121Y/T125K/M154I,** **T124A/E138K/Q148K,** **T124A/G140S/Q14**		
Initial conc. (0.8-4 μM) Lamivudine[c]	(0.8-4 μM) **M184V**	(0.8-20 μM) **M184I, M184V**	(0.8-20 μM) **M184I, M184V,** **K82N/M184V**					

[a] Concentration of inhibitor at each culture day were indicated in parenthesis. Phenotypic analysis was performed in parallel with genotypic analysis. The genotypes which showed >5-fold resistance compared with wild type were shown in bold letter.

[a] The genotypic analysis was performed in RT-region

TABLE 8. Fold changes in EC_{50}s against molecular clones with IN substitutions

Virus or IN Substitution(s) of resistant virus	Mean fold change in EC_{50} from that for wild-type virus[a]			
	S/GSK1349572	Raltegravir	Elvitegravir	Efavirenz
Wild type[c]	1.0	1.0	1.0	1.0
T66A	0.26 ± 0.010	0.61 ± 0.090	4.1[b]	1.3[b]
T66I	0.26 ± 0.090	0.51 ± 0.10	8.0 ± 1.7	1.5 ± 0.23
T66K	2.3 ± 0.35	9.6 ± 1.3	**84 ± 29**	2.1 ± 0.28
E92I	1.5 ± 0.19	2.1 ± 0.62	8.0[b]	1.0[b]
E92Q	1.6 ± 0.12	3.5 ± 1.4	**19 ± 8.1**	1.2 ± 0.22
E92V	1.3 ± 0.20	1.4 ± 0.18	8.3[b]	0.93[b]
L101I	1.4 ± 0.36	1.2 ± 0.12	NT	NT
G118S	1.1 ± 0.21	1.2 ± 0.30	4.9[b]	0.73[b]
F121Y	0.81 ± 0.12	6.1 ± 1.3	**36 ± 23**	2.1 ± 0.14
T124A	0.95 ± 0.19	0.82 ± 0.080	1.2 ± 0.29	0.95 ± 0.12
G140S	0.86 ± 0.30	1.1 ± 0.22	2.7 ± 0.63	1.1 ± 0.12
Y143C	0.95 ± 0.26	3.2 ± 0.57	1.5 ± 0.46	1.2 ± 0.23
Y143H	0.89 ± 0.010	1.8 ± 0.38	1.5 ± 0.047	1.3 ± 0.32
Y143R	1.4 ± 0.29	**16 ± 3.9**	1.8 ± 0.16	0.98 ± 0.041
P145S	0.49 ± 0.08	0.87 ± 0.20	**>350**	2.9 ± 0.28
Q146R	1.6 ± 0.17	1.2 ± 0.26	2.8 ± 0.72	0.94 ± 0.40
Q148H	0.97 ± 0.090	**13 ± 5.0**	7.3 ± 2.3	1.4 ± 0.83
Q148K	1.1 ± 0.19	**83 ± 6.6**	**>1700**	2.1 ± 0.26
Q148R	1.2 ± 0.21	**47 ± 9.3**	**240 ± 91**	1.9 ± 0.21
I151L	3.6 ± 3.6	8.4 ± 4.7	**29**[b]	2.9[b]
S153F	1.6 ± 0.20	1.3 ± 0.14	2.8 ± 0.93	2.0 ± 0.23
S153Y	2.5 ± 1.1	1.3 ± 0.19	2.3 ± 0.49	1.9 ± 0.18
M154I	0.93 ± 0.27	0.82 ± 0.18	1.1 ± 0.18	1.4 ± 0.14
N155H	0.99 ± 0.35	8.4 ± 1.8	**25 ± 7.8**	1.1 ± 0.044
N155S	1.4 ± 0.36	6.2 ± 1.9	**68 ± 26**	1.7 ± 0.09
N155T	1.9 ± 0.32	5.2 ± 2.0	**39**[b]	1.5[b]
T66I/L74M	0.35 ± 0.08	2.0 ± 0.81	**14**[b]	1.2[b]
T66I/E92Q	1.2 ± 0.19	**18 ± 3.6**	**190 ± 100**	2.0 ± 0.20
T66K/L74M	3.5 ± 0.94	**40 ± 13**	**120 ± 33**	2.0 ± 0.25
L74M/N155H	0.91 ± 0.17	**28 ± 12**	**42 ± 8.6**	1.1 ± 0.24
E92Q/N155H	2.5 ± 1.2	**>130**	**320 ± 39**	1.9 ± 0.71
T97A/N155H	1.1 ± 0.46	**26 ± 7.9**	**37 ± 6.9**	1.1 ± 0.14
L101I/S153F	2.0 ± 0.11	1.3 ± 0.54	2.6 ± 0.23	1.7 ± 0.12
F121Y/T125K	0.98 ± 0.35	**11 ± 0.49**	**34**[b]	1.5[b]
E138A/Q148R	2.6 ± 0.47	**110**[b]	**260 ± 12**	1.2 ± 0.090
E138K/Q148H	0.89 ± 0.19	**17 ± 5.9**	6.7 ± 1.5	1.0 ± 0.47
E138K/Q148K	**19 ± 8.0**	**330 ± 75**	**371**[b]	1.2[b]
E138K/Q148R	4.0 ± 1.1	**110 ± 37**	**460 ± 230**	1.0 ± 0.31
G140C/Q148R	4.9 ± 1.8	**200 ± 42**	**485**[b]	2.8[b]
G140S/Q148H	2.6 ± 1.4	**>130**	**>890**	1.7 ± 0.99
G140S/Q148K	1.5 ± 0.10	3.7 ± 1.3	**94 ± 53**	1.3 ± 0.39
G140S/Q148R	8.4 ± 4.0	**200 ± 5.3**	**267**[b]	1.5[b]
Y143H/N155H	1.7 ± 0.27	**38**[b]	**16 ± 4.6**	1.5 ± 0.28
Q148R/N155H	**10 ± 1.4**	**>140**	**390**[b]	1.7 ± 0.15
N155H/G163K	1.4 ± 0.40	**23 ± 7.2**	**35 ± 4.4**	1.1 ± 0.10
N155H/G163R	1.1 ± 0.18	**17 ± 5.9**	**35 ± 12**	1.1 ± 0.16
N155H/D232N	1.4 ± 0.25	**20 ± 3.9**	**36 ± 7.1**	0.92 ± 0.050
V72I/F121Y/T125K	1.3 ± 0.54	**13 ± 7.1**	**58**[b]	1.5[b]
L101I/T124A/S153F	1.9 ± 0.24	1.4 ± 0.14	2.0 ± 0.4	1.5 ± 0.24
E138A/S147G/Q148R	1.9 ± 0.89	**27 ± 3.7**	**130 ± 8.8**	0.83 ± 0.032
V72I/F121Y/T125K /I151V	1.2 ± 0.32	7.0 ± 2.8	**37**[b]	1.1[b]

[a] Each value represents the mean standard deviation for 3 to 5 independent experiments, each performed in duplicate. FCs of >10-fold are shown in boldface.

NT, not tested.

[b] Mean FC from one or two experiments.

[c] Strain NL432 was used for wild-type HIV-1.

TABLE 9. Deviation from additivity for S/GSK1349572 with marketed HIV drugs

	Deviation from Additivity[a]			
Compound	Mean	S.E..	p-value	Interaction
STV	-0.473	0.083	0.001	Synergy
ABC	-0.641	0.095	0.001	Synergy
EFV	-0.356	0.102	0.005	Synergy
NVP	-0.246	0.087	0.015	Synergy
LPV	-0.310	0.082	0.005	Synergy
APV	-0.332	0.085	0.003	Synergy
ENF	-0.245	0.110	0.034	Synergy
ADV	-0.179	0.168	0.168	Additive
S/GSK1349572	0.031	0.151	0.423	Additive

[a]Mean deviation from additivity values. Values in the range of -0.1 to -0.2 indicate weak synergy, values that approach -0.5 indicate strong synergy, and positive values of 0.1 to 0.2 indicate weak antagonism.

STV; stavudine, ABC; abacavir, EFV; efavirenz, NVP; nevirapine, LPV; lopinavir, APV; amprenavir, ENF; enfuvirtide, ADV; adefovir.

CONCLUSION

The purpose of this study was to investigate the *in vitro* antiretroviral properties of novel integrase inhibitors focusing on its mechanism of action and *in vitro* resistance profile. The mechanism of action was established through *in vitro* integrase enzyme assays, resistance passage experiments, activity against viral strains resistant to other classes of anti-HIV agents, and mechanistic cellular assays.

Resistance passage studies were conducted with five INIs that have been tested in clinical trials to date: a new naphthyridinone-type INI S/GSK-364735, raltegravir, elvitegravir, L-870,810 and S-1360. Resistance mutations against S-1360 and related diketoacid-type compounds could be isolated from infected MT-2 cell cultures. Q148R and F121Y were the two main pathways of resistance to S/GSK-364735. Q148R/K and N155H, which were found in patients failing raltegravir treatment in Phase IIb studies, were observed during passage with raltegravir with this method. The overall resistance pattern of S/GSK-364735 was similar to that of raltegravir and other INIs. However, different fold resistances of particular mutations were noted among different INIs, reflecting a potential to develop INIs with distinctly different resistant profiles.

S/GSK1349572 is a next-generation HIV INI designed to deliver potent antiviral activity with a low-milligram once-daily dose requiring no PK booster. In addition, S/GSK1349572 demonstrates activity against clinically relevant IN mutant viruses and has potential for a high genetic barrier to resistance. In a variety of cellular antiviral assays, S/GSK1349572 inhibited HIV replication with low-nanomolar or subnanomolar potency and with a selectivity index of 9,400. The PA-EC_{50} extrapolated to 100% human serum was 38 nM. When virus was passaged in the presence of S/GSK1349572, highly resistant mutants were not selected, but mutations that effected a low FC in the EC_{50} were identified in the vicinity of the integrase

active site. S/GSK1349572 demonstrated activity against site-directed molecular clones containing the raltegravir-resistant signature mutations Y143R, Q148K, N155H, and G140S/Q148H, while these mutants led to a high FC in the EC_{50} of raltegravir. Either additive or synergistic effects were observed when S/GSK1349572 was tested in combination with representative approved antiretroviral agents; no antagonistic effects were seen. These findings demonstrate that S/GSK1349572 would be classified as a next-generation drug in the INI class, with a resistance profile markedly different from that of first-generation INIs.

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