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

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ABSTRACT

Resistance passage studies were conducted with five INIs (integrase inhibitors) 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. In establishing the passage system and starting from concentrations several fold above the EC_{50} value, resistance mutations against S-1360 and related diketoacid-type compounds could be isolated from infected MT-2 cell cultures from day 14 to 28. 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 fold resistance of 40 mutant molecular clones versus wild type virus was compared with these five INIs. 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.

1. Introduction

To date, many anti-HIV agents have been developed within separate classes—NRTI, NNRTI, PI and entry inhibitors. A new class of antiretroviral, integrase inhibitors (INIs), is exemplified by the recently approved raltegravir. The goals for developing other anti-HIV agents within a new class or already existing classes are better efficacy, less toxicity, a more convenient dosing regimen and overcoming drug-resistant viruses. 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 (Hazuda et al., 2007; McColl et al., 2007). It is important to characterize the resistance profile of known INIs in order to direct research and development on new INIs. In this paper, we describe the development of an in vitro method to isolate INI-resistant mutants in MT-2 cells using HIV-1 IIIB first using the integrase inhibitor S-1360 (Billich, 2003) and related compounds. We then used this method to isolate mutants under the same conditions resistant to L-870,810 (Egbertson et al., 2006), raltegravir (Markowitz et al., 2006, 2007), elvitegravir (DeJesus et al., 2006; Zolopa et al., 2007) and the recently described S/GSK-364735 (Garvey et al., 2008). Finally, the fold resistance of 40 INI-resistant molecular clones against various INIs was measured to directly compare resistance profiles of these INIs.

2. Materials and methods

2.1. 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 (3TC), nevirapine, capravirine, efavirenz, raltegravir and elvitegravir were synthesized at Shionogi Research Laboratories, Osaka, Japan.

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2.2. 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 (Isaka et al., 1999). MOLT-4 cells persistently infected with HIV-1 strain IIIB (Harada et al., 1985) 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 RPMI 1640 supplemented with 10% FCS and 60 μg/mL kanamycin.

2.3. 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 (Adachi et al., 1986) 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.

2.4. 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 1 h at 37 °C, washed and cultured in 24-well plates (1.5 mL/well). Viral stocks were normalized by 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.

2.5. 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 (Pauwels et al., 1988; Fujiwara et al., 1998) 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 flat-bottom 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 (OD_{560}/OD_{690}).

2.6. 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 MOLT-4 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.

2.7. 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, USA). The concentration achieving 50% inhibition of HIV infection (EC_{50}) was calculated.

3. Results

3.1. 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, raltegravir, elvitegravir and L-870,810) were single-digit nanomolar in both assays.

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

Using HIV-1 IIIB as described in Section 2, we compared MT-2, M8166, MOLT-4, Jurkat and H9 cell lines for the isolation of resistant mutants against S-1360, compounds 1 and 2, and L-731,988. 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. In addition to these five T-cell lines, we compared thirteen other cell lines, but nothing was equal to or better

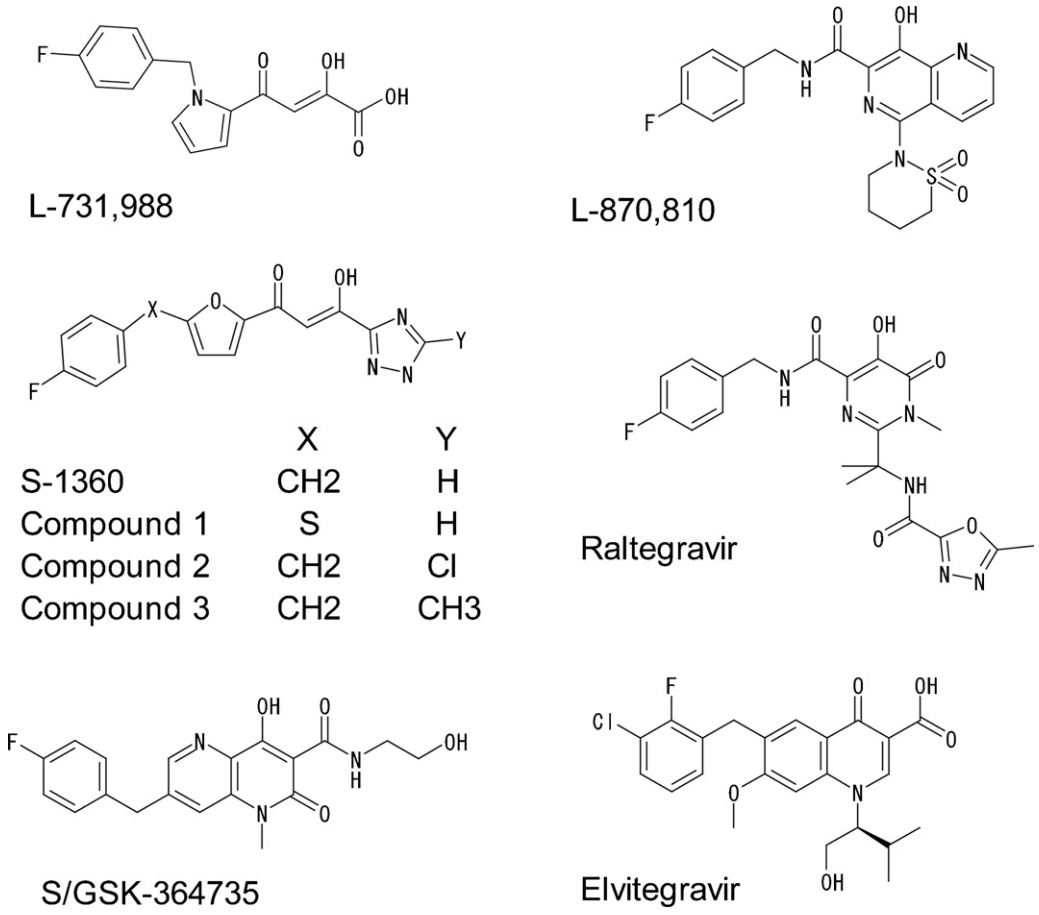


Fig. 1. Chemical structures of the HIV-1 integrase inhibitors used in this study.

than MT-2 cells in both rapidity 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 (Nakajima et al., 2001).

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 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 and in each of the three cell types, while substitutions at either Q148 or N155

Table 1
Anti-HIV activity of INIs using MT-2 cells.

	EC_{50} mean (S.D.)		
	HIV-1 IIIB virus		HIV-1 NL432 virus
	MT-2 cells		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 two independent experiments performed in duplicate.

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

Cells	Compound	Mutation (first isolation day after infection)[a]
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.

were detected less frequently and only in Jurkat or MT-2 cells (Table 2).

3.3. Optimization of the drug concentrations for passage

We 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, we observed the greatest diversity of resistance mutations when S-1360 was at 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 fold resistance (FR) data (see below), the T124A substitution alone did not increase resistance of virus and is also found as a natural polymorphism (Lataillade et al., 2007). Although rarely observed, the double mutant with T124A in IN-region and M184V or I in RT-region was isolated during 3TC 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 integrase inhibitors.

We also compared the isolation pattern of amino acid substitutions under escalating concentrations of S-1360. On day 49, 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, 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.

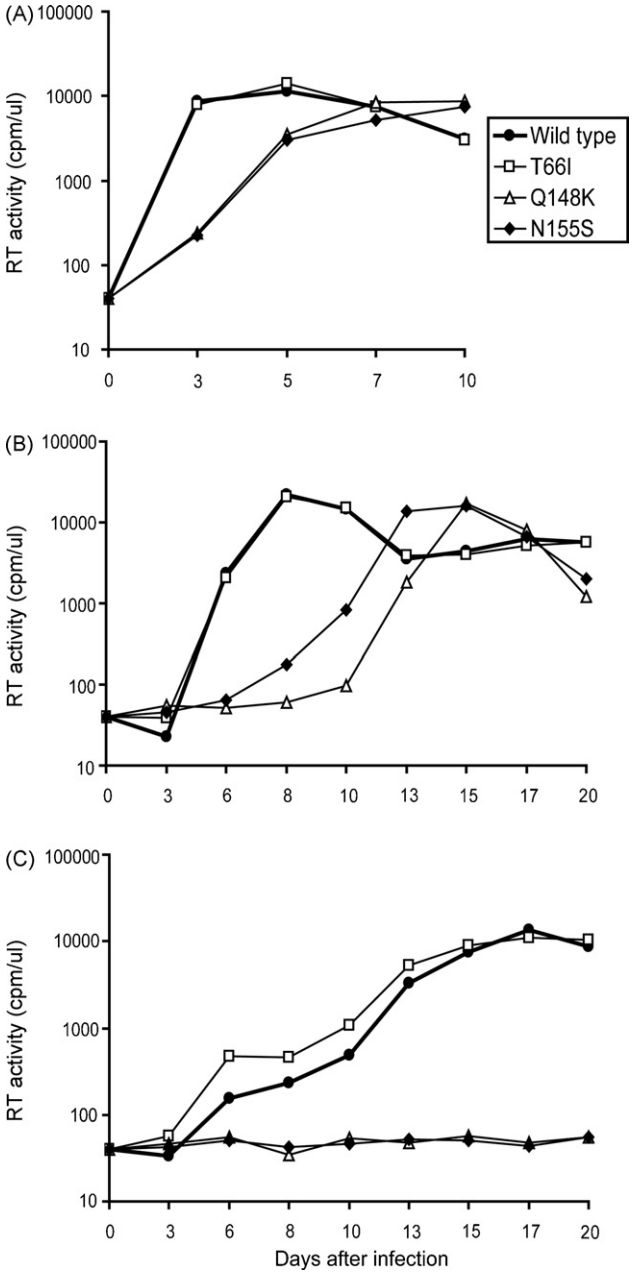
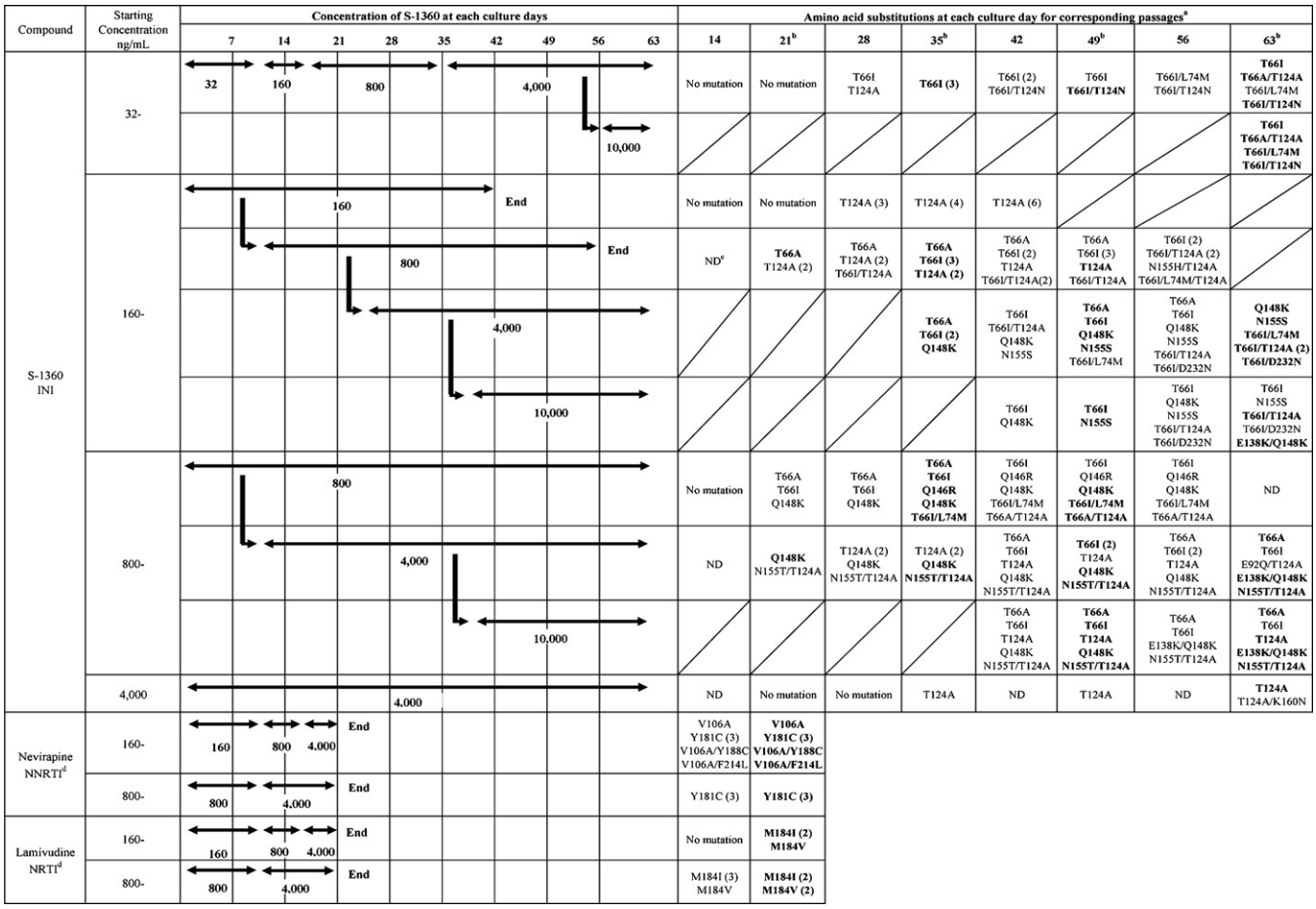


Fig. 2. Replication kinetics of mutant viruses resistant to INIs in (A) MT-2, (B) Jurkat and (C) MOLT-4 cells. Independent experiments generated the same results. Representative data are shown.

3.4. 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, we next examined the time course of isolation of resistant viruses to S-1360, 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 (FR > 20, Young et



Compound	Starting Concentration ng/mL	Concentration of S-1360 at each culture days	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)			
		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	T66I (2) T66I/T124A (2) N155H/T124A T66I/L74M/T124A	
		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**
		10,000					T66I Q148K	**T66I N155S**	T66I Q148K N155S T66I/T124A T66I/D232N	T66I N155S **T66I/T124A** T66I/D232N **E138K/Q148K**
	800-	800	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
		4,000	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**
		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	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 of substitutions indicates genotype from a single well. The number of wells were shown in parenthesis when the mutants were isolated from more than one well. [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. [c]ND; PCR not done. [d]The genotypic analysis was performed in RT-region.

al., 1995; Fujiwara et al., 1998; Sato et al., 2006). In our MT-2 system, this high level of resistance was achieved more quickly compared with our previously report (Sato et al., 2006). 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 fold resistance 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).

3.5. Isolation of viruses resistant to S/GSK-364735, raltegravir, L-870,810, or elvitegravir

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 raltegravir, 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 raltegravir 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 raltegravir treatment during Phase IIb (Hazuda et al., 2007).

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 raltegravir in the occurrences of T66K, E92(I/Q), and F121Y substitutions.

In the culture with elvitegravir, the first amino acid substitution V151I was observed on day 13, but was absent on day 28 and

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

Compound EC_{50}[a] (concentration)	Days of culture (passage number)			
	14[b] (4)	27[b] (8)	39[b] (11)	48 (14)
S-1360 INI EC_{50} = 250 ng/mL	T124A	**T66I**, E92Q, **Q148K** T124A/Q148K T124A/Q146L T66I/V72A/T124A E10D/N17S/V72A/L74M/T124A	**T66I**, **E92Q**, **Q148K**, **N155S** **T66I/T124A**, **T124A/Q148K** **T124A/Q146L T124A/N155S** **T66I/V72A/T124A** **E10D/N17S/V72A/L74M/T124A**	T66I, E92Q, Q146L, Q148K N155S, N155T T124A/Q146L T124A/Q148K T124A/N155S T66I/V72A/T124A T66I/V72A/L74M/T124A N17S/T66I/V72A/L74M/T124A
Initial conc. (160–800 ng/mL)	(800 ng/mL)	(800–4000 ng/mL)	(4000 ng/mL)	(4000 ng/mL)
Compound 3 INI EC_{50} = 180 ng/mL	No mutation	**G118S**, T124A, **E92Q/T124A** G118S/T124A T124A/Q146R	**G118S, T124A E92Q/G118S** **E92Q/T124A**, **G118S/T124A** **G118C/T124A T124A/Q146R** **T124A/Q148K**	G118S, G118C, G118N G118R, T124A E92Q/T124A, G118S/T124A G118C/T124A G118N/V150I T124A/Q146R T124A/Q148K T124A/N155S
Initial conc. (160–800 ng/Ml)	(800 ng/mL)	(800–4000 ng/mL)	(800–4000 ng/mL)	(800–4000 ng/mL)
Capravirine NNRTI[c] EC_{50} = 2.0 ng/mL	**L100I**, **Y188L**, **G190E**, **L234I** **V179D/G190E**	**L100I**, **Y188L**, **G190E**, **L234I** **L100I/V106A**, **L100I/Y181C** **L100I/Y188C V179D/G190E** **Y181C/L234I**, **Y188L/L234I** **G190E/L234I**	**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**	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
Initial conc. (6.4–32 ng/mL)	(32 ng/mL)	(160–800 ng/mL)	(800–4000 ng/mL)	(800–4000 ng/mL)
Efavirenz NNRTI[c] EC_{50} = 0.84 ng/mL	**L100I**, **K103N**, **G190E** **G190S**	**L100I**, **K103N**, **G190E** **G190S L100I/F227C** **L100I/L234I, K103N/Y188C**	**G190E**, **L100I/K103N** **L100I/Y188L**, **L100I/G190S** **L100I/F227C**, **L100I/L234F** **L100I/K103N/L234F**	G190E, L100I/K103N L100I/G190S L100I/K103N/L234F L100I/V179D/L234F L100I/Y188L/L210V L100I/K103R/V179D/F227C
Initial conc. (6.4 ng/mL)	(32 ng/mL)	(160–800 ng/mL)	(800–4000 ng/mL)	(800–4000 ng/mL)
Nevirapine NNRTI[c] EC_{50} = 33 ng/mL	**V106A**, **Y181C**, **Y188C** **G190A V106A/Y181C** **V106A/Y188C V106A/F214L**	**V106A**, **Y181C**, **Y188C** **Y188N**, **G190A** **V106A/Y181C V106A/F214L**		
Initial conc. (160–800 ng/mL)	(800 ng/mL)	(4000 ng/mL)		

[a] EC_{50} 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.

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 elvitegravir. 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 elvitegravir, and nine of these included T124A. Only the P145S and Q148K substitutions (FR of >350 and >1700 for elvitegravir) 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 3. This level of resistance was first observed on day 56 in the cultures with S/GSK-364735, on day 42 with raltegravir and L-870,810, and on day 14 with elvitegravir 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.

3.6. Sensitivity of drug-resistant molecular clones to integrase inhibitors

Next, we constructed INI-resistant mutant molecular clones 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, we considered the viruses with an EC_{50} of 3-fold or greater than that of the wild type to be 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 raltegravir and elvitegravir, respectively in our study. To confirm the contribu-

Table 4
Time course of emergence of INI-resistant mutants

Compound EC50[a] (concentration)	Days of culture (passage number)					
	13 or 14[b] (4)	28[b] (8)	42[b] (12)	56[b] (16)	70[b] (20)	84[b] (24)
S/GSK-364735 INI EC_{50} = 2.4 ng/mL	No mutation	T124A	F121Y, T124A, Q148R	T124A, Q146R, **Q148R** F121Y/T124A **E10D/N17S/Q148R**	Q146R, **Q148R**, G163R F121Y/T124A **E138K/Q148R G140S/Q148R**	**T66K**, Q95R, Q146R, **Q148R F121Y/T124A E138K/Q148R G140S/Q148R** V75I/T112S/Q146P
Initial conc. (0.26–160 ng/mL)	(0.26–160 ng/mL)	(0.26–160 ng/mL)	(1.3–160 ng/mL)	(6.4–160 ng/mL)	(6.4–160 ng/mL)	(6.4–160 ng/mL)
Raltegravir INI EC_{50} = 3.9 ng/mL	T124A	T124A, **Q148K** N155H/I204T	G59E, T124A, **Q148K** Q148R, **N155H N155H/I204T**	T124A, **Q148K**, **Q148R N155H**, E92Q/M154I **Q148K/G163R N155H/I204T**	T124A, **Q148K**, **Q148R N17S/Q148K**, **E92Q/M154I G140C/Q148K G140S/Q148R Q148K/G163R V151I/N155H N155H/I204T T124A/V151I/N155H G140C/Q148K/G163R**	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. (0.11–14 ng/mL)	(0.11–14 ng/mL)	(0.11–71 ng/mL)	(0.57–360 ng/mL)	(2.8–360 ng/mL)	(2.8–1800 ng/mL)	(14–1800 ng/mL)
L-870,810 INI EC_{50} = 1.6 ng/mL	T124A	T124A, **Q148R**	**T66K**, F121Y, T124A Q148R, V151L **T124A/Q148R E138K/Q148K T66I/E92V/T124A T66K/E92Q/T124A/M154I**	**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**	**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**	**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**
Initial conc. (0.12–360 ng/mL)	(0.12–360 ng/mL)	(0.12–360 ng/mL)	(0.12–360 ng/mL)	(0.58–360 ng/mL)	(2.9–360 ng/mL)	(2.9–360 ng/mL)
Elvitegravir INI EC_{50} = 0.82 ng/mL	**V151I**	**T66I**, T124A, **P145S Q148K**, T66I/T124A	**T66A**, **T66I**, T124A, **P145S Q148K**, **Q148R**, **T66I/124A T66K/T124A**, **Q148R/T124A**	**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**		
Initial conc. (0.05–32 ng/mL)	(0.05–32 ng/mL)	(0.05–32 ng/mL)	(0.26–32 ng/mL)	(1.3–160 ng/mL)		
Lamivudine NRTI[c] EC_{50} = 1400 ng/mL	**M184V**	**M184I, M184V**	**M184I, M184V K82N/M184V**			
Initial conc. (180–920 ng/mL)	(180 4600 ng/mL)	(180–4600 ng/mL)	(180–4600 ng/mL)			

[a] EC_{50} 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

Wild type EC_{50} nM ± (S.D.)	S/GSK-364735	Raltegravir	Elvitegravir	L-870,810	S-1360	Efavirenz (RT)
	3.6 (0.61)	6.1 (0.89)	1.3 (0.31)	3.0 (0.73)	330 (70)	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*	1.0	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 two independent experiments performed in duplicate. Fold resistance between 3 and 10 shown in italics. Fold resistance >10 folds are shown in bold letter.

[a] Molecular clone derived from pNL432.

tion for drug-resistance of the amino acid substitution at 151, we constructed I151L and V72I/F121Y/T125K/I151V mutations using 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 (FR > 10). Raltegravir showed greater than 10-fold increased EC_{50} against 17 viruses, elvitegravir against 27 viruses 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 elvitegravir (FR of 2.6) but not to the other INIs (FRs ranging from 7.2 to 670), whereas virus with P145S had the exact opposite phenotype (FR of >350 for elvitegravir and near wild-type level of susceptibility to all other INIs). Raltegravir was potent against G140S/Q148K at near wild-type level (FR = 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 FRs 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.

4. 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. Our data show that MT-2 cells were suitable for the isolation of mutant viruses resistant to a broad range of two-metal binding integrase

inhibitors 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. As described in the Section 2, we used a 3-day co-cultivation of MT-2 cells and virus-producing MOLT-4 cells 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, we used three wells 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 (Hazuda et al., 2000). 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 (Hazuda et al., 2004). 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 (Fikkert et al., 2003, 2004). In our 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 we succeeded in isolating a variety of resistant viruses, in particular Q148K/R and N155H/S/T.

Recently disclosed mutations observed in patients failing raltegravir include L74M, E92Q, T97A, E138A/K, G140S, Y143H/R, Q148H/K/R, V151I, N155H, G163K/R, S230N and D232N (Hazuda et al., 2007). In the present in vitro study, substitutions at E138K, G140S, Q148K/R, V151I, N155H, and G163R were detected in virus passaged with raltegravir. 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 elvitegravir (McColl et al., 2007), was also detected in an in vitro isolation of resistance study using MT-2 cells and HIV-1 IIIB (Shimura et al., 2008). Likewise, this mutation was isolated in our in vitro method. In contrast, there were several low level elvitegravir resistance mutations which were not isolated in the previous study but that were isolated in our 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 (Lataillade et al., 2007). However, to our 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 integrase inhibitors (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.

We observed in general very significant cross-resistance (i.e., high similarity of fold resistance) 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 raltegravir, differences in fold resistance ranged from 4- to 30-fold in Q148H, N155S/T, E138K/Q148H and G140S/Q148K. G118R was highly resistant to raltegravir, L-870,810, and S-1360, while elvitegravir showed wild-type sensitivity. Comparing S-1360 and elvitegravir with other integrase inhibitors, T66I and T66I-containing double mutants were more commonly isolated. T66I with additional substitutions showed high resistance to early integrase inhibitors such as L-708,906 (Hazuda et al., 2000). S/GSK-364735 and raltegravir were effective against T66I with low fold resistance, and viruses with T66I substitution were not detected in S/GSK-364735 and raltegravir cultures.

Comparing fold resistances 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 fold resistance 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 integrase inhibitors can be developed that potently inhibit first generation INI-resistant viruses that emerged in the clinic.

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