Selective resistance profiles emerging in patient-derived clinical isolates with cabotegravir, bictegravir, dolutegravir, and elvitegravir

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Abstract

Background: Integrase strand transfer inhibitors (INSTIs) are recommended for first-line HIV therapy based on their relatively high genetic barrier to resistance. Although raltegravir (RAL) and elvitegravir (EVG) resistance profiles are well-characterized, resistance patterns for dolutegravir (DTG), bictegravir (BIC), and cabotegravir (CAB) remain largely unknown. Here, in vitro drug selections compared the development of resistance to DTG, BIC, CAB, EVG and RAL using clinical isolates from treatment-naive primary HIV infection (PHI) cohort participants (n = 12), and pNL4.3 recombinant strains encoding patient-derived Integrase with (n = 5) and without (n = 5) the E157Q substitution.

Results: Patient-derived viral isolates were serially passaged in PHA-stimulated cord blood mononuclear cells in the presence of escalating concentrations of INSTIs over the course of 36–46 weeks. Drug resistance arose more rapidly in primary clinical isolates with EVG (12/12), followed by CAB (8/12), DTG (8/12) and BIC (6/12). For pNL4.3 recombinant strains encoding patient-derived integrase, the comparative genetic barrier to resistance was RAL > EVG > CAB > DTG and BIC. The E157Q substitution in integrase delayed the advent of resistance to INSTIs. With EVG, T66I/A, E92G/V/Q, T97A or R263K (n = 16, 3, 2 and 1, respectively) arose by weeks 8–16, followed by 1–4 accessory mutations, conferring high-level resistance (> 100-fold) by week 36. With DTG and BIC, solitary R263K (n = 27), S153F/Y (n = 7), H51Y (n = 2), Q146R/K (n = 3) or S147G (n = 1) mutations conferred low-level (< 3-fold) resistance at weeks 36–46. Similarly, most CAB selections (n = 18) resulted in R263K, S153Y, S147G, H51Y, or Q146L solitary mutations. However, three CAB selections resulted in Q148R/K followed by secondary mutations conferring high-level cross-resistance to all INSTIs. EVG-resistant viruses (T66I/R263K, T66I/E157Q/R263K, and S153A/R263K) retained residual susceptibility when switched to DTG, BIC or CAB, losing T66I by week 27. Two EVG-resistant variants developed resistance to DTG, BIC and CAB through the additional acquisition of E138A/Q148R and S230N, respectively. One EVG-resistant variant (T66I) acquired L74M/G140S/S147G, L74M/E138K/S147G and H51Y with DTG CAB and BIC, respectively.

Conclusions: Second generation INSTIs show a higher genetic barrier to resistance than EVG and RAL. The potency of CAB was lower than BIC and DTG. The development of Q148R/K with CAB can result in high-level cross-resistance to all INSTIs.

Keywords: HIV-1, Integrase inhibitors, Antiretroviral drug resistance, Cell culture selections, Primary HIV infection isolates, HIV subtypes
Background

Over the past 40 years, remarkable advances in antiretroviral therapy has enabled people living with HIV to enjoy longer life expectancy and an improved quality of life. Despite these advances, ongoing development of more robust and durable drug regimens remain critical to avoid the long-term risk of drug resistance and treatment failure [1, 2]. Presently, integrase strand transfer inhibitors (INSTIs) are the favored class of drugs in first-line combination therapy based on their high potency, improved tolerability, low toxicity and high genetic barrier to resistance [3–5].

Raltegravir (RAL) and elvitegravir (EVG) were the first INSTIs to be approved in 2007 and 2012, respectively [6, 7]. Although highly efficacious in the management of HIV, both RAL and EVG were shown to be prone to the development of resistance when used in salvage therapy without other active antiretroviral drugs [8, 9]. The resistance and cross-resistance profile for RAL and EVG have been well described [8, 10, 11]. Drug resistance is associated with the accumulation of primary resistance substitutions and relevant compensatory substitutions along several pathways including the (1) N155H and G140A/G148R/H/Q pathways conferring high level cross-resistance to RAL and EVG; (2) the T66I or E92Q/G pathways leading to resistance to RAL and EVG; (3) the Y143R/H/C RAL-specific resistance pathway [4, 12].

The emergence of viruses displaying resistance and cross-resistance to RAL and EVG spurred research into the development of “second-generation” INSTIs [4, 12]. These include dolutegravir (DTG) that was approved in 2013, bictegravir (BIC) that was approved in the US in February 2018 and cabotegravir (CAB) that is in phase III clinical development with anticipated release in 2019 [13–15]. Dolutegravir displays a higher genetic barrier to resistance than RAL and EVG, retaining efficacy against many RAL- and EVG-resistant variants [16–18]. In the SAILING clinical study, only 4 of 354 treatment-experienced, INSTI-naïve patients treated with dolutegravir acquired integrase-inhibitor resistance substitutions upon virological failure. Two of these individuals acquired the R263K mutation [19] that had previously been selected in culture by DTG to confer low-level resistance [20]. Interestingly, R263K-containing viruses do not seem able to acquire compensatory substitutions that might increase levels of resistance [21–24]. In cell culture selections with DTG, the emergence of singleton R263K, S153Y or H51Y mutations confers low-level resistance (<3-fold resistance) and contributes to a significant negative impact on viral replicative fitness [4, 20, 25, 26]. DTG has been shown to have a longer binding half-life to the HIV integrase enzyme than either RAL or EVG which may help to explain why it maintains activity against most RAL or EVG resistant variants [27, 28].

The recent phase 3 randomized GS-US-380-1489 and GS-US-380-1490 clinical trials demonstrated bictegravir BIC, co-formulated with tenofovir alafenamide and emtricitabine, to be non-inferior to DTG, when co-formulated with either abacavir and lamivudine or tenofovir alafenamide and emtricitabine at week 48 [13, 15, 29]. No resistance to any drug was observed with either BIC- or DTG-based regimens. BIC and DTG showed high barriers to in vitro resistance in MT-2 cells, with emergent M50I and R263K conferring low-fold resistance in the nM range [30]. BIC, like DTG, appears to show broad-range potency against viruses with primary INSTI mutations conferring resistance to RAL or EVG [31].

CAB, a structural analogue of DTG, displays unique physicochemical and pharmacokinetic properties that permits formulation as a single oral tablet for daily dosing and as a long-acting nanosuspension for monthly to quarterly intramuscular injection. Toxicity profiles for CAB, like that reported for DTG, BIC and RAL, include headache, nausea and diarrhea, [5, 13, 32, 33]. Long-acting CAB showed mild to moderate injection-site reactions that rarely resulted in treatment discontinuation (<1%) [14]. The ability of injectable CAB to achieve and maintain clinically relevant plasma concentrations for 16 weeks may circumvent the need for daily dosing, allowing for long-acting treatment and pre-exposure prophylaxis (PrEP) regimens [14]. The LATTE-2 clinical trial showed non-inferiority of the two-drug oral CAB plus rilpivirine (RIL) regimen to the CAB-abacavir-lamivudine drug regimen, with 2/115 experiencing virological failure. One patient acquired virus with the Q148R mutation, conferring phenotypic resistance to RAL, EVG and CAB, in association with the K103N, E138G, and K238T, conferring cross-resistance to non-nucleoside RT inhibitors (NNRTIs) [14]. Similarly, at week 48 in the Phase 2 Latte study, the injectable long-acting formulation of CAB and RIL was non-inferior to etravirenz on a dual nucleoside reverse transcriptase inhibitor (NRTI) backbone with one virological failure in injectable CAB/RIL arm harbouring viruses acquiring the INSTI-associated Q148R mutation with the E138Q NNRTI-resistant mutation [32].

To gain a better understanding of the possibility of resistance to newer INSTIs, we performed in vitro cell culture selections with DTG, BIC, CAB and EVG against primary isolates from newly infected persons harbouring subtype B (n = 7) and non-B subtype (n = 5) infections. In addition, recombinant strains of HIV-1 with integrase derived from clinical isolates assessed the impact of E157Q substitution on emergent resistance to relevant INSTIs was assessed at weeks 8, 16, 24 and 46.
We observed a higher genetic barrier to DTG and BIC as compared to CAB across the different viral subtypes.

Results
Differential selection of resistance to newer integrase inhibitors
This study was designed to compare the differential ability of a panel of patient-derived clinical isolates (n = 12) and recombinant strains (n = 10) to develop resistance to the integrase inhibitors, DTG, BIC, CAB and EVG. Baseline integrase natural polymorphisms, viral subtype and GenBank accession numbers are summarized in Tables 1 and 2.

Primary viral isolates from PHI cohort participants harbouring subtype B (n = 7) and non-B subtype (n = 5) infections were serially passaged over the course of 46 weeks in the presence of escalating concentrations of DTG, BIC, CAB and EVG (Table 1). Tissue culture selections were performed using PHA-stimulated CBMCs since newly-infected persons typically use the CCR5 receptor and do not grow in MT-2 cells.

Cell culture selections of resistance were conducted in parallel with DTG, BIC, CAB and EVG under the same conditions. DTG, BIC and CAB exhibited high subnanomolar potency against primary HIV-1 isolates grown in CBMCs with baseline inhibitory concentrations (IC50s) of 0.25 ± 0.25 nM, 0.25 ± 0.15 nM and 0.13 ± 0.02 nM, respectively (mean ± SD, n = 10 isolates). The progress of viral outgrowth in the presence of stepwise increasing concentrations of INSTIs was monitored over the course of 46 weeks. Drug-dose escalations for individual INSTIs were based on weekly RT assays, performed in the presence and absence of drug [34]. Genotyping at weeks 8, 16, 24–27, and 36–46 ascertained the differential acquisition and accumulation of drug-associated mutations.

The drug dose-escalations and viral outgrowth over time for HIV clinical isolates 5326 and 96USSN20 are illustrated in Fig. 1. The drug-dose escalations with DTG and BIC progressed at rates that were considerably slower than CAB or EVG. With DTG and BIC, the acquisitions of solitary mutations, including R263K, H51Y, or S153F, conferred low-level resistance to DTG or BIC. These resistance mutations conferred a negative impact on viral replication reflected in lower RT activity, precluding further escalations of DTG or BIC drug concentrations (Fig. 1).

In contrast, the respective first appearance of R263K or S153F mutations by 96USSN20 and 5326 viral strains with CAB at weeks 8, was followed by the serial accumulation of mutations along the Q148K/R resistance pathway leading to viral escape by week 48 (Fig. 1). The development of resistance to CAB progressed more slowly than EVG. For the 96USSN20 isolate, the acquisition of T66I, S147G, Q146R, and S147G conferred viral escape from EVG at week 25 (Fig. 1). Resistance codons Q146R and Q95R for this CRF002_AG isolate have been hitherto unreported. The Q146P is a reported mutation selected in vitro with EVG, reducing RAL and

Table 1 Baseline natural integrase polymorphisms for the HIV-1 clinical isolates used for the in vitro selections with integrase inhibitors

| Isolate ID | GenBank accession number | Subtype | Natural polymorphisms in integrase |
|------------|--------------------------|---------|-----------------------------------|
| 14514      | KT988124                 | B       | K7R, D10E, S17N, M50I, K111Q, T112V, G123S, T125V, R127K, I220L, Y227F, N232D, D256E |
| 10387      | KX7140173                | B       | D10E, V31I, L45Q, K111E, I113V, G123S, A124T, T125A, R127K, M154I, V201I, D207N, N232D, L234I |
| 10249      | KX714014                 | B       | D10E, V31I, L45Q, L101I, K111E, I113V, G123S, A124T, R127K, D207N, N232D, L234I |
| 14624      | KX714018                 | B       | D10E, S17N, A23V, L28I, S39C, V27I, L101I, G123S, R127K, N232D |
| 14637      | KT988125                 | B       | D10E, E11D, R20K, V31I, S39N, M50I, S119T, G123S, A124N, R127K, G193E, V201I, D286N |
| 14947      | KT988126                 | B       | D10E, E11D, I21T, V31I, S39N, M50I, S119T, G123S, A124N, R127K, G193E, V201I, T218S, D286N |
| 5326       | KX714021                 | B       | K7R, D10E, E11D, K14R, V31I, V72I, M50I, V72I, L101I, G123S, A124N, R127K, S195T, I203M, I220L, Y227F, N232D |
| 4742       | MG805951                 | C       | D10E, V31I, S39C, V72I, I64M, Q95P, F100Y, L112V, G123S, A124N, T125A, R127K, D167E, V201I, I203M, D278A, S283G |
| 10947      | MG805955                 | C       | D6E, D25E, V31I, L45Q, M50I, V72I, P90S, T93S, F100Y, L101I, G106A, T112V, G123S, T125A, R127K, K136Q, V201I, T218I, N232D, L234I, R269K, D278A, S283G |
| 6343       | MG805950                 | CRF01_AE| D10E, K14R, A21T, V31I, S39N, T112V, G123S, T125A, R127K, G134N, I135V, K136R, V167E, V201I, T218I, D232E, L234I, R269K, S283G |
| 14515      | MG805952                 | CRF02_AG| D10E, E11D, R20K, A21T, V31I, V72I, L101I, T112V, G123S, T125A, R127K, G134N, K136Q, D167E, V201I, N232D, L234I, L236E, V249I, S283G |
| 96USSN20   | MG805953                 | AG      | D10E, K14R, V31I, V72I, L101I, T112V, G123S, T125A, R127K, G134N, I135V, K136R, V201I, T206S, N232D, L234I, L256E, R269K, S283G |
| pNL4.3     | B                        |         | D10E, I113V, S119R, G123S, A124T, R127K, V151I, L234V |
The Q95K is a rare non-polymorphic accessory resistance mutation conferring little if any effect on drug susceptibility to INSTIs [7]. The acquisition of R263K with S153A by isolate 5326 conferred >100-fold resistance to EVG.

Overall, variations in the acquisition of resistance to antiretroviral drugs are multifactorial, dependent on
drug efficacy and viral heterogeneity [1]. The genetic barrier to resistance is defined by the number of mutations required to confer resistance, the level of resistance conferred by the acquired specific mutation(s) (ranging from 2 to >1000 fold) and the resultant costs of resistance substitutions on viral replicative fitness [1]. The patterns of viral outgrowth at the final passage (week 46) for the panel of primary HIV-1 clinical isolates (n=12) under selective pressure with DTG, BIC, CAB and EVG are shown in Table 3. Collectively, resistance selections proceeded at a considerably slower rate for DTG and BIC than CAB or EVG. By 46 weeks, resistance substitutions were observed in 12/12, 8/12, 7/12 and 7/12 of HIV isolates with EVG, CAB, DTG, or BIC, respectively (Table 3). With BIC or DTG, the acquisition of solitary resistance mutations, including R263K (n=3), S153Y/F (n=3) or H51H/Y (n=1) negatively impacted on viral fitness, precluding drug-dose escalations beyond 0.005–0.025 µM.

Of note, CAB, an analogue of DTG, showed a lower genetic barrier to resistance than DTG and BIC. Although resistance patterns to CAB was like DTG and BIC for 10 of 12 isolates, two isolates showed a lower barrier to CAB resistance. Two viral variants 5326 and 96USSN20 variants acquired complex L74M/G140S/S147G/Q148K and R263K/S153A/S153Y/G163R resistant species allowing for drug-dose escalations to 1 and 0.5 µM by week 46, respectively (Fig. 1; Table 3). Two other variants, 14947 and 6343, acquired R263K/S153A and S153Y/G163R variants.

In this study, several clinical strains (14514, 10387, 10249, 14624 and 14515) showed a lower propensity to develop resistance to all tested INSTIs. Resistance profiles to DTG, BIC and CAB developed along the R263K or S153F/Y pathway; resistance to EVG was limited to the acquisition of T66I or E92Q at week 46. For the remaining seven isolates, CAB showed a lower barrier to resistance than DTG and BIC, with the acquisition of Q148R/K + 3 mutations in two isolates (Table 3).

EVG showed the lowest genetic barrier with emergent resistance observed in all 12 HIV strains (Table 3). Clinical isolates that failed to develop resistance with DTG, BIC, or CAB, including 14514, 10387, 10249, and 14515, acquired only T66I (n=3) or E92Q (n=1) singleton mutations with EVG at weeks 36 or 46. The other isolates accumulated 2–4 secondary drug resistance mutations along the T66I and E92QV pathways, leading to viral escape and EVG dose escalations to 1–5 µM.

Resistance was only observed in a proportion of the primary isolates. We speculate that this viral strain heterogeneity may explain why most primary viral strains rarely develop resistance to DTG and BIC in clinical

### Table 3 Selection of drug resistance to dolutegravir (DTG), bictegravir (BIC), cabotegravir (CAB) and elvitegravir (EVG) at the final week of passage

| Virus isolate | Subtype | Acquired mutations at final passage (week 46) of selective drug pressure\(^a\) | Acquired mutations (week 26–40)\(^b\) |
|---------------|---------|---------------------------------------------------------------------------------|---------------------------------------------------------------------------------|
|               |         | DTG | BIC | CAB | EVG | RAL |
| 14514 | B | R263K | None | None | T66I | – |
| 10387 | B | None | None | None | T66I | E92Q |
| 10249 | B | R263K | None | None | E92Q | None |
| 14624 | B | None | None | H51HY | T66I | T97A, N155H |
| 14637 | B | R263K | R263K | R263K | T66I, E157Q, R263K | N155H |
| 14947 | B | R263K | R263K | R263K, S153A | T66I, E138EK, S147Q, Q148R | Y143R, L74M, E92V, F121Y, G163R |
| 5326 | B | H51HY | S153Y | L74M, G140S, S147Q, Q148K | R263K, S153A | Y143R, L74M, T97A, E157Q |
| 4742 | C | None | None | R263K | E92Q, R263K | T66K |
| 10947 | C | R263K | R263K | S147Q | E92V, R263K | – |
| 6343 | A | R263K | S153Y | S153Y, G163R | T66I, R263K | – |
| 14515 | AG | None | R263K | None | T66I, H51HY | – |
| 96USSN20 | AG | R263K | S153FS, E157EK | L74M, E138K, Q148R, R263K | T66I, Q146R, S147Q, Q95R | L74M, V79I, E138K, G140A, Q148R |
| pNL4-3 | B | R263K, M50I | R263K, M50I | S153F | T66I, T97A, S147Q, S119R, S153A | T66I, T97A, G163R, D232N |

\(^a\) Genotypic analysis was performed of at weeks 0, 16, 24 and 46. The mutations acquired at final week of passage are listed in the order of their first appearance.

\(^b\) The emerging resistance patterns to RAL determined in previous studies on viral strains are shown for comparative contextual purposes.
settings. Our recent studies showed that select HIV-1 viral strains, including 14637 and 14947, associated with large cluster transmission outbreaks may show accelerated escape from integrase in cell culture compared with viral isolates from singleton/small clusters [25]. Large cluster strains can assist in deducing potential pathways implicated in the development of resistance to DTG, BIC and CAB (Table 3).

Different viral subtypes resulted in similar resistance profiles to INSTIs. In our previous studies, drug selections with the subtype C 4742 strain, the G118R resistance pathway arose with DTG and the Merck investigational MK2048 [35]. MK2048, the Merck investigational integrase inhibitors, showed high potency against most RAL/EVG resistant variants but its clinical development was halted due to poor pharmacokinetics [4]. The development of the G118R resistance was ascribed to a signature natural polymorphism at codon 118 in isolate 4742. In this study, 4742 developed no resistance with DTG and BIC, R263K with CAB, and E92V/R263K with EVG (Table 3).

Resistance to integrase inhibitors using patient-derived recombinant strains

The integrase E157Q substitution has been described as a common natural polymorphism present in 2.3% of HIV-1 viral sequences, including 3.8% and 6.0% of treatment-naive patients with subtype B and subtype CRF02_AG subtype infections, respectively (Los Alamos database, www.hiv.lanl.gov, accessed June 8, 2018). The E157Q has also been observed in several persons failing INSTI-based regimens, including RAL and DTG [36–39]. To date, very few data are available regarding virological response in patients harbouring E157Q-mutated viruses.

To assess the potential impact of E157Q on emergent resistance to INSTIs, recombinant viruses were constructed where patient-derived integrase were inserted on a pNL4-3Δ integrase background. Viruses included recombinant strains with (n=5) and without (n=5) the E157Q substitution (n=5) in integrase.

The progress of viral selections in CBMCS in stepwise increasing concentrations of DTG, BIC, CAB, EVG, and RAL over time is depicted in Fig. 2. It was noteworthy that strains harbouring the E157Q substitution showed a significantly attenuated development of resistance to RAL and EVG. The hypersensitivity of viruses to E157Q suggests that this mutation is a compensatory mutation, commonly selected with INSTIs with minimal effects on drug susceptibility [4, 40]. The E157Q mutation arose in a EVG and RAL selections in recombinant strains E78004 and E78060, respectively (Table 4).

![Drug dose escalations (mean±SEM) reflect the differential emergence of resistance to integrase inhibitors by recombinant strains encoding patient-derived integrase with (n=5) and without (n=5) the E157Q resistance substitution](image-url)
In contrast, patient-derived pNL4-3 recombinant strains showed a high genetic barrier to resistance for DTG, BIC, and CAB, both in the presence or absence of E157Q. All recombinant strains harbouring patient-derived integrase developed resistance to all INSTIs over 36–38 weeks. With DTG, BIC and CAB, the predominant resistance profile included R263K (n = 11), R263K/M50I (n = 7), and S153Y/F (n = 5).

Selection of strain E78004 with CAB resulted in high level resistance along a Q148R/E138K/G140S/L74I pathway (Table 4). This isolate developed Q146R/Q95K with DTG and BIC, a hitherto unreported profile for both drugs. Resistance profiles associated with escape from EVG drug pressure were associated with T66I and the accumulation of major resistance including Q148R/K, E138K, and S147G. Emergent high-level resistance to RAL were associated with the Y143R/G and Q148R pathways.

### Phenotypic drug susceptibility of CAB- and EVG-resistant viral variants

Phenotypic drug susceptibility to DTG, BIC, CAB, EVG and RAL was deduced in PHA-stimulated CBMC using select clinical isolates (Table 5) and recombinant strains with patient-derived integrase (Table 6).

Here, we showed two isolates 5326 and 96USSN20 serially accumulated resistance mutations with CAB, leading to drug dose escalation of 0.5 and 1 µM, respectively. Viruses were amplified at weeks 8, 16, 24 and 46 weeks (Table 5). The first appearance of Q148K as a solitary mutation under CAB pressure in clinical isolate 5326 and recombinant strain E78004 at weeks 18 conferred low-level (< 2–3 fold) resistance to CAB, DTG, BIC and RAL with moderate (12–32-fold) reduced susceptibility to EVG (Tables 5, 6). For isolate 5326, the progressive accumulation of Q148K/G140S/G147GS resulted in increasingly high cross-resistance to CAB, RAL and EVG while retaining susceptibility to DTG and BIC (Table 3). The resistant variant of 5326 amplified at week 48 under selective CAB pressure, harbouring L74M/G140S/S147G/Q148K mutations showed high-level cross-resistance to all INSTIs, including DTG, BIC, CAB, EVG and RAL (Table 5). Similarly, 96USSN20 and E78004 viruses developed resistance along a Q148R pathway leading to L74M/E138K/G148R/R263K and L74I/E138K/G140S/Q148R conferring cross-resistance to all INSTIs.

Phenotypic drug susceptibility assays explored the potential impact of the E157Q substitution drug susceptibility to INSTIs (Table 6). Viral strains E78004 and E78060 acquiring the E157Q under EVG and RAL showed hypersensitivity to DTG, BIC, CAB, consistent with the observed attenuated development of resistance to RAL and EVG of E157Q relative to wild-type recombinant strains.

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**Table 4: Cell culture selections of viral recombinant strains bearing the integrase from patient samples with and without the E157Q polymorphisms**

| Virusa | Codon 157 | Acquired mutations at final passage (week 36–38) of selective drug pressureb |
|--------|-----------|--------------------------------------------------------------------------------|
| DTG    | BIC       | CAB       | EVG       | RAL       |
| pNLWT  | WT        | R263K, M50I | R263K, M50I | S153F | T66I, T97A, S147G, S119R, S153A | ND |
| pNL157Q| E157Q     | R263K, M50IM | Q146R | R263K, M50I | T66I, S147G, Q95K | E92Q |
| E78001 | WT        | S153F      | S153Y      | R263K, M50I | N155H | Q95R, S147G, Q148R | T97A, G163R, V151I, L74M |
| E78003 | WT        | S147G, H51Y | R263K, M50I | S153Y | T66I, Q146R, S230R | Y143R, L74M, V151I |
| E78004 | WT        | Q146R, Q95KQ | Q146R, Q95KQ | Q148R, E138K, G140S, L74I | T66I, Q95K, E157Q, S230R | T66A, A128T, Y143G, G163R, V151I |
| E78005 | WT        | R263K      | S153Y      | S153F | T66A, Q146I | T97A, Y143R, V151I |
| E78060 | WT        | R263K      | R263K, M50I | Q146L | T97A, Q146R, T66I | T97A, E157Q, A128AT, V151I |
| E78110 | E157Q     | R263K, M50I | R263K | R263K | T66I, R263K, M50I | Y143R |
| E102430| E157Q     | R263K      | R263K | R263K, M50I | E138K, Q148K | V151S, L74M |
| E102952| E157Q     | S153F      | R263K, M50I | R263K | T66I, E92Q | Q148R, E138K, G140A, V151V |
| E103211| E157Q     | R263K      | R263K | R263K, H51N | S147G, Q148R, E138K | Y143R |
| E103212| E157Q     | R263K      | R263K | R263K, M50I | H51Y, S147G, T97A | T97A |

The underline refers to the de novo acquisition of E157Q during selection

* Integrase derived from clinical isolates with or without the E157Q substitution were inserted into integrase-depleted pNL4.3 plasmids. Isolated recombinant viruses were serially passaged in escalating concentrations of dolutegravir (DTG), bictegravir (BIC), cabotegravir (CAB), elvitegravir (EVG) or raltegravir (RAL) over the course of 36 weeks.

b Genotypic analysis was performed at weeks 0, 16, 24 and 36–38. The mutations acquired at final week of passage are listed in the order of their first appearance. Mutations highlighted in italics conferred high-level resistance.
Table 5 Phenotypic drug susceptibility of viral strains to integrase strand transfer inhibitors (INSTIs) harvested at the designated week of selection with cabotegravir (CAB) or elvitegravir (EVG)

| Virus selection week drug | Acquired resistance mutations | EC_{50} (nM) in CBMCs (fold-resistance relative to WT control) |
|---------------------------|--------------------------------|---------------------------------------------------------------|
| 6343 No drug              | WT                             | 0.10 (0.6 x) 0.17 0.23 0.21 ND                              |
| 6343 Wk 46 CAB            | S153Y, G163R                   | 0.60 (6 x) 1.33 (8 x) 0.87 (3.2 x) 0.53 (2.5 x) ND          |
| 5326 No drug              | WT                             | 0.77 0.41 0.20 0.253 1.12                                    |
| 5326 Wk 45 EVG (1 µM)     | S263K, S153A                   | 0.52 (0.7 x) 0.53 (1.3 x) 0.19 (1.0 x) 0.53 (2.1 x) 1.12 (1.0 x) |
| 5326 Wk 17 CAB (0.01 µM)  | Q148K                          | 1.57 (2.0 x) 0.90 (2.2 x) 0.61 (3.1 x) 2.96 (11.8 x) 3.57 (3.2 x) |
| 5326 Wk 28 CAB (0.5 µM)   | Q148K, G140S, G147GS           | 3.60 (4.7 x) 0.72 (1.8 x) 8.07 (40 x) 180 (720 x) 66.5 (60 x) |
| 5326 Wk 48 CAB (1.0 µM)   | Q148K, G140S, S147G, L74M      | 125 (162 x) 49.01 (120 x) 139.7 (700 x) 3429 (>1000 x) 1007 (900 x) |
| 96USSN20 No drug          | WT                             | 0.58 0.77 0.29 0.20 1.60                                    |
| 96USSN20 Wk27 EVG (2.5 µM)| T66I, T97A, Q147G              | 0.40 (0.7 x) 0.80 (1 x) 1.20 (4 x) >100 (>500 x) 1.7 (1 x) |
| 96USSN20 Wk 17 CAB (0.025 µM)| Q148R, E138EK, R263KR         | 8.03 (1.4 x) 5.83 (8 x) 2.40 (8.3 x) 22.35 (112 x) 12.01 (7 x) |
| 96USSN20 Wk 27 CAB (0.25 µM)| Q148R, E138K, R263K, L74RM   | 100 (17 x) 9.01 (12 x) >30 (>100 x) >300 (>1500 x) 300 (188 x) |
| 96USSN20 Wk 45 CAB (0.5 µM)| Q148R, E138K, R263K, K74M    | 13.93 (24 x) 13.40 (17 x) 47.8 (165 x) 1612 (8060 x) 568 (355 x) |

*Viruses were harvested at the designated week of selection, amplified in PHA-stimulated CBMCs and genotyped. Viruses were co-cultured in PHA-stimulated CBMCs to deduce drug susceptibility against dolutegravir (DTG), bictegravir (BIC), cabotegravir (CAB), elvitegravir (EVG) and raltegravir (RAL). Samples in italics represent greater than 5-fold reduction in drug susceptibility.

Table 6 Phenotypic drug susceptibility of viral strains to integrate strand transfer inhibitors (INSTIs) harvested at the designated week of selection with cabotegravir (CAB) or elvitegravir (EVG)

| Viral variant-drug selection week (drug) | Acquired resistance mutations | EC_{50} (nM) in CBMCs (fold-resistance relative to WT control) |
|-----------------------------------------|------------------------------|---------------------------------------------------------------|
| pNL4.3                                  | WT                           | 0.71 0.49 0.39 0.13 –                                          |
| pNL4.3-R263K                            | R263K                        | 1.56 (2.2 x) 1.60 (3.3 x) 0.91 (2.4 x) 0.79 (6.1 x) –          |
| pNL4.3-S153Y                            | S153Y                        | 3.34 (4.7 x) 3.40 (7.0 x) 1.01 (2.6 x) <0.3 (2 x) –           |
| pNL4.3-S153F                            | S153F                        | 0.45 (0.6 x) 0.63 (1.3 x) 0.56 (1.4 x) <0.3 – –               |
| E78004 No drug                          | WT                           | 0.49 1.26 0.27 0.65 1.18                                     |
| E78004 Wk18 CAB (0.0025 µM)              | Q95KQ, Q148R                 | 1.28 (2.6 x) 1.17 (0.9 x) 0.67 (2.5 x) 0.90 (32 x) 1.64 (14 x) |
| E78004 Wk26 CAB (0.005 µM)               | Q95KQ, Q148R, E138EK         | 1.60 (3.3 x) 1.77 (1.4 x) 3.04 (11.3 x) 93.57 (144 x) 20.76 (18 x) |
| E78004 Wk36 CAB (0.25 µM)                | Q148R, E138K, L74G, G140GS   | 12.34 (25 x) 6.08 (53 x) 23.6 (87 x) 36.16 (57 x) 3182 (>100 x) |
| E78004 No drug                          | WT                           | 0.66 0.66 0.43 0.79 1.69                                     |
| E78004 Wk18 EVG (0.25 µM)                | T66I, Q95K, E157EQ           | 0.59 (0.9 x) 0.56 (0.9 x) 0.24 (0.5 x) 29.10 (37 x) 14.57 (8.6 x) |
| E78004 Wk26 EVG (0.25 µM)                | T66I, Q95K, E157EQ           | 0.53 (0.8 x) 0.38 (0.6 x) 0.39 (0.9 x) 69.76 (89 x) 7.98 (4.7 x) |
| E78004 Wk36 EVG (2.5 µM)                 | T66I, Q95K, E157EQ, S230R    | 0.06 (0.1 x) 0.01 (0.01 x) 0.03 (0.1 x) 123.10 (156 x) 5.47 (3.2 x) |
| E78060 No drug                          | WT                           | 0.52 0.92 0.45 0.46 2.43                                     |
| E78060 Wk36 RAL (0.5 µM)                 | T97A, A128AT, E157Q, V151I   | 0.31 (0.6 x) 0.30 (0.3 x) 0.22 (0.5 x) 23.79 (52 x) 49.83 (21 x) |
| E102952 Wk18 CAB (0.025 µM)              | WT, E157Q                    | 0.09 0.25 0.13 0.33 0.44                                     |
| E102952 Wk18 RAL (0.025 µM)              | Q148R, E157Q                 | 0.17 (0.8 x) 0.10 (0.4 x) 0.14 (1 x) 0.41 (1.3 x) 0.75 (1.7 x) |
| E102952 Wk38 RAL (0.2 µM)                | Q148R, E138K, G140A, V151V, E157Q | 5.46 (58 x) 2.63 (106 x) 2.13 (163 x) 1255 (>100 x) 1519 (>100 x) |

*Viruses were harvested at the designated week of selection, amplified in PHA-stimulated CBMCs and genotyped. Viruses were co-cultured in PHA-stimulated CBMCs to deduce drug susceptibility against dolutegravir (DTG), bictegravir (BIC), cabotegravir (CAB), elvitegravir (EVG) and raltegravir (RAL). Samples in italics represent greater than 5-fold reduction in drug susceptibility. pNL4.3 recombinant virus are included as controls with R263K and S153Y mutations inserted by site-directed mutagenesis.

One recombinant strain, E78004, acquired a Q148R resistance pathway under selective pressure with CAB. The appearance of Q148R/Q95KQ followed by Q148R/Q95KQ/E138EK resistant strains at weeks 18 and 26 resulted in moderate 2.5- and 11.3-fold resistance to CAB, while retaining susceptibility to DTG and BIC. The
outgrowth of the L74I/E138K/G140GS, Q148R showed 25-, 5.3-, 87-, 57- and >100-fold cross-resistance to DTG, BIC, CAB, EVG, and RAL, respectively.

Switching EVG-resistant strains to DTG, BIC, or CAB
To gain further understanding of the residual efficacies of DTG, BIC, and CAB on EVG-resistant variants, we performed switch experiments. Six EVG-resistant variants and the pNL4.3 recombinant strain showed high-level resistance at week 46, growing in the presence of

| Virus | Initial EVG selection | Drug switch selection | Resistance mutations at week 27 | Lost EVG mutations | Acquired mutations |
|-------|-----------------------|-----------------------|---------------------------------|-------------------|-------------------|
| 6343  | EVG Wk 46             | Pre-switch 0.25        | T66I, R263K                     |                   |                   |
| 6343  | DTG 0.010             | M50MI, R263K          | T66I                            | M50IM             |                   |
| 6343  | BIC 0.050             | R263K                 | T66I                            |                   |                   |
| 6343  | CAB 0.050             | R263K                 | T66I                            |                   |                   |
| 6343  | No drug               | R263K                 | T66I                            |                   |                   |
| 14637 | EVG Wk 46             | Pre-switch 1.0         | T66I, E157Q, R263K              |                   |                   |
| 14637 | DTG 0.010             | E157Q, R263K          | T66I                            |                   |                   |
| 14637 | BIC 0.025             | E157Q, R263K          | T66I                            |                   |                   |
| 14637 | CAB 0.050             | E157Q, R263K          | T66I                            |                   |                   |
| 14637 | No drug               | E157Q, R263K          | T66I                            |                   |                   |
| 5326  | EVG Wk 46             | Pre-switch 1           | S153A, R263K                    |                   |                   |
| 5326  | DTG 0.010             | S153A, R263K          | T66I                            |                   |                   |
| 5326  | BIC 0.010             | S153A, R263K          | T66I                            |                   |                   |
| 5326  | CAB 0.050             | S153A, R263K          | T66I                            |                   |                   |
| 5326  | No drug               | S153A                 | T66I                            |                   |                   |
| 14624 | EVG Wk 46             | Pre-switch 1           | T66I                            |                   |                   |
| 14624 | DTG 0.005             | T66I, L74M, E138K, S147G, M154IM |                   | L74M, E138K, S147G, M154IM |
| 14624 | BIC 0.010             | H51HY                 | T66I                            |                   |                   |
| 14624 | CAB 0.10              | T66I, L74M, G140GS, S147G |                   | L74M, G140GS, S147G |
| 14624 | No drug               | T66I                  |                                 |                   |                   |
| 14947 | EVG Wk 46             | Pre-switch 5           | T66I, E138E, S147G, Q148R       |                   |                   |
| 14947 | DTG 0.005             | T66I, E138E, S147G, Q148R, S230N |                   | S230N             |                   |
| 14947 | BIC 0.025             | T66I, E138E, S147G, Q148R, S230N |                   | S230N             |                   |
| 14947 | CAB 0.100             | T66I, L74M, E138E, S147G, Q148R, S230N |                   | L74M, S230N       |                   |
| 14947 | No drug               | T66I                  | E138E, Q148R                    |                   | S230N             |
| 96USSN20| EVG Wk 46             | Pre-switch 2.5         | T66I, S147G, Q146R, S147G       |                   |                   |
| 96USSN20| DTG 0.100             | T66I, Q146R, S147G, E138E1AEKT, Q148R |                   | Q146R             | E138AEKT, Q148R   |
| 96USSN20| BIC 0.050             | T66I, Q146R, S147G, E138A, T97A, Q148R |                   | Q146R             | T97A, E138A, Q148R |
| 96USSN20| CAB 0.25              | T66I, Q146QR, S147G, E138A, Q148QR |                   | Q146QR             | E138A, Q148QR |
| 96USSN20| No drug               | T66I, S147G           | Q146R                           |                   |                   |
| pNL4.3| EVG Wk 46             | Pre-switch 2.5         | T66I, T97A, S147G, V151I, S153A |                   |                   |
| pNL4.3| DTG 0.025             | T66I, T97A, S147G, V151I, S153A |                   |                   |                   |
| pNL4.3| BIC 0.025             | T66I, T97A, S147G, V151I, S153A |                   |                   |                   |
| pNL4.3| CAB 0.250             | T66I, T97A, S147G, V151I, S153A |                   |                   |                   |
| pNL4.3| No drug               | T66I, T97A, S147G, V151I, S153A |                   |                   |                   |

Patient-derived viral strains, subjected to EVG selective pressure for 46 weeks, were switched to serially increasing concentrations of DTG, BIC, CAB or no drug for 27 weeks. Genotyping was performed at week 27 and re-genotyped to monitor the loss and acquisition of mutations.
1–2.5 μM EVG. These resistant variants were amplified at week 47 and switched to serial drug-dose escalations with DTG, BIC or CAB for a further 27 weeks.

As summarized in Table 7, The EVG-resistant variants retained residual susceptibility to second generation INSTIs. There was however, broader antiviral sensitivity to DTG and BIC than CAB. Drug dose escalations with the latter three drugs were initiated at 0.001 μM. Following passage for 17 weeks, drug-dose escalations reached 0.023±0.012 μM, 0.027±0.006 μM, and 0.121±0.034 at week 27. Drug dose escalations were significantly higher for CAB than DTG or BIC (Bartlett’s statistic = 14.16, p = 0.0008, p < 0.05, post hoc Tukey’s test).

Genotypic analysis showed the switch of EVG-resistant strains (n = 6) to DTG, BIC, CAB or a no drug control for 27 weeks resulted in the loss of the T66I (3/6 selections) or Q146R (1/6) substitutions associated with primary resistance to EVG (Table 7). For isolate 6343, the loss of T66I was accompanied by the acquisition of M50I with DTG (Table 7). The EVG-resistant isolates 6343, 14637 and 5326, harbouring R263K, E157Q/R263K and S153A/R263K, showed residual susceptibility to DTG, BIC and CAB with no further acquisition of resistance mutations at week 27 (Table 7).

The EVG-resistant 14624 T66I variant displayed a higher residual antiviral susceptibility to BIC than DTG and CAB (Table 7). With BIC, T66I was lost and H51HY was acquired. In contrast, the loss of T66I in 14624 was accompanied by the acquisition of L74M/E138K/S147G/M154I/M and L74M/G140S/S147G at week 27 with DTG and CAB, respectively.

The EVG-resistant 14947 variant T66I/E138K/S147G/Q148R variant accumulated S230N with BIC and DTG and L74M/S230N with CAB. The 96USSN20 virus resistant to EVG (T66I, Q146R, and S147G) lost Q146R and acquired E138A and Q148R with DTG, BIC and CAB. The final concentrations at week 27 revealed CAB escape.

Taken together, DTG, BIC and CAB showed broad antiviral efficacies against wild-type and EVG-resistant viruses. BIC and DTG appear to be better able to inhibit viral replication than CAB in several primary HIV-1 isolates and EVG-resistant variants.

Discussion

The findings in this study demonstrated that DTG and BIC showed higher barriers to resistance than EVG. With EVG, resistance was observed in all 12 clinical isolates. In 4/12 and 6/12 selections with DTG and BIC, no resistance mutations arose in long-term passage, respectively. In the remaining selections, the acquisition of singleton R263K, S153Y/F or H51Y substitutions conferred low-level resistance, regardless of viral subtype. Although 4/12 and 6/12 selections with CAB, yielded no resistance or minor resistance, respectively, two selections with CAB (one subtype B and one CRF02_AG), resulted in the acquisition of Q148R/K with multiple secondary resistance substitutions conferring high-level cross-resistance to all INSTIs.

Although there is a high correlation in the genotypic and phenotypic characteristics associated with resistance to DTG and BIC, CAB may show a lower barrier to resistance. In selection of two clinical isolates and a patient-derived recombinant strain, resistance to CAB arose through a Q148R. The first appearance of Q148R showed <3-fold resistance to CAB, DTG and BIC. The sequential accumulation of mutations by these three strains resulted in Q148R/E138K/R263K/L74M, Q148K/G140S/S147G/L74M and Q148R/E138K/L74I/G140S mutational motifs conferring in high-level cross-resistance to all five INSTIs.

BIC and CAB, like DTG displayed antiviral efficacies against viral variants acquiring EVG-resistance mutations. Overall, BIC and DTG were superior to CAB. This is consistent with recent studies modelling the respective binding of CAB, BIC and DTG within the active site of the integrase enzyme [31].

The selection of Q148R in CAB selections is consistent with the observed acquisition of Q148R in two patients in the Latte clinical trials [14, 32]. Molecular models suggest that there is more conformational rigidity with CAB than DTG in metal-chelating scaffold leading to potential steric interactions between CAB and the Q148 locus [41]. The potential steric interactions induced by Q148R at and near the β4–α2 loop may affect binding kinetics of CAB, leading to a decreased dissociative half-life with Q148R between that of DTG and RAL [41].

CAB, an analogue of DTG, has been formulated as an oral tablet (half-life 40 h) and as a long-acting injectable nanosuspension with an intramuscular and subcutaneous half-life of 40 days. CAB/RIL has shown durable viral suppression in patients who are suppressed to less than 50 copies/ml, providing proof of principle for its use in two-drug maintenance therapy, as well as a potential PreP strategy [14, 32, 42]. The observed emergence of resistance through the Q148R pathway may lead to cross-resistance to the entire class of INSTIs. Although in vitro findings may not arise in the clinic, careful attention may be needed to assure drug adherence and prevent tail periods of declining drug in injectable formulations.

It is noteworthy that HIV-1 viral variants may differ in their replicative fitness and their ability to override resistance bottlenecks. In our previous studies, we showed that viral variants associated with large cluster transmission outbreaks may show a facilitated development of resistance to DTG and EVG than viruses leading to singleton
transmission [25]. In this study, isolates 14637 and 14947 were associated with large cluster outbreaks.

This study utilized a panel of clinical isolates reflective of newly-infected treatment-naive persons harbouring CCR5 viruses. Our analysis also included recombinant CXC4-tropic pNL4.3 recombinants constructs encoding patient-derived integrase. Most reported studies to date, have limited their performed in vitro analysis of BIC and CAB using pNL4.3 vector constructs or MT-2 or MT-4 cells [31, 43–45].

In previous studies, subtype C variant, 4742, developed the G118R mutation in cell culture selections with DTG and Merck investigation INSTI, MK2048 [46]. The development of G118R was associated with a rare GGA natural polymorphism at codon 118, facilitating a G to A transition leading to G118R (AGA). In this study, the 4742-viral strain gave rise to no resistance with either DTG or BIC, R263KR with CAB, and E92EG/R263K with EVG.

Taken together, our findings show improved resistance profiles for DTG and BIC in all tested viral strains. The CAB, an analogue of DTG with high potency, may be prone to the development of Q148K/R leading to cross-resistance to the entire class of integrase inhibitors. The high potency of DTG and BIC confirms their suitability for use in resource-limited settings dominated by non-B subtypes. A larger panel of viral isolates are needed to address the potential development of the Q148R/K resistance pathway with CAB [43].

Conclusions
The advent of integrase inhibitors has transformed the management of HIV-1 infection. Although treatment failure with RAL and EVG can result in the emergence of INSTI resistance, DTG and BIC have been quite impervious to the development of resistance in the clinical setting [15, 47, 48]. To date, there have been few reports of virological failure and resistance in treatment-naive persons receiving triple combination DTG-containing regimens [47, 48] and in virologically suppressed patients receiving DTG monotherapy [46, 49]. The present study used in vitro selections using viral isolates from 20 persons to show patterns of resistance to DTG, BIC and CAB that may potentially arise in real-world settings. Our findings indicate that drug resistance monitoring in patients on INSTI-based regimens is essential, despite the high-genetic barriers of these drugs. Although in vitro experiments might not always reflect what, will happen in patients, resistance may be breached when INSTIs are given in monotherapy and in select patients failing INStI-based regimens [50]. As treatment options coalesce around the use of second-generation integrase inhibitors in resource-limited settings, more information is needed on emergent resistance to DTG, BIC and CAB and their potential impact on viral response to later-line treatment options [50].

Methods
Cells and antiviral compounds
BIC and EVG were kindly provided by Gilead Sciences Inc. (Foster City, California). CAB was purchased from Toronto Research Chemicals (Toronto, Canada). DTG was kindly provided by ViiV Healthcare (Research Triangle Park, Inc.). MT-2 and 96USN20 cells were obtained from the NIH AIDS Reagent program, Division of AIDS, NAID, NIH with cell line provided by Dr D Richman and Drs D Ellenberger, P Sullivan and RB Lai, respectively [51, 52]. Cord blood mononuclear cells (CBMCs) were isolated as previously described from non-nominate discarded blood obtained through the Department of Obstetrics, Jewish General Hospital [53]. The CEM-GXR cells and HIV-1 pNL4.3 delta integrase plasmid (Δint) were kindly provided by Dr. Mark Brockman (Simon Fraser University, Burnaby, Canada [54].

Isolation of patient-derived HIV-1 primary isolates and recombinant strains encoding patient-derived HIV-1 integrase
The FRQS-Réseau SIDA supports a representative cohort of newly-infected persons with clinical indication of primary infection. In this study, HIV-1 strains were isolated from seven subjects harboring subtype B infections and four subjects harboring non-B subtype infections. HIV-1 isolates were amplified as previously described through co-culture of patient CD8-depleted peripheral blood mononuclear cells (PBMCs) with CD8-depleted phytohemagglutinin-stimulated CBMCs [53, 55]. Amplified cell-free viral supernatants were tittered and stored at −70 °C until use [53, 55]. In addition, viruses were amplified from the 96USN20 strain (subtype CRF02_AG) and the pNL4.3 subtype B reference clone obtained from the NIH AIDS Reagent program, Division of AIDS, NIAID, NIH.

Clinical plasma samples were also obtained from treatment-naive persons harboring the WT (n = 5) or E157Q (n = 5) substitution in integrase. Patient-derived amplicons encoding the integrase gene (1064 nucleotides long) were inserted into the pNL4-3 recombinant vector as previously described [54]. Briefly, HIV-1 was RT-PCR amplified from plasma HIV RNA using sequence-specific subtype B primers [56, 57]. Second round PCR was performed using Expand™ High Fidelity Enzyme (Roche Diagnostics, Laval, Quebec) with forward (IN4155F-5′- GTACCAGCACACAAAGGATTTGGAG) and reverse primer (IN5219R-5′-CCTAGTGGAATGTGTACCTTCT
GAAC), primers. Recombinant viruses were generated by co-transfecting sequence verified second-round PCR amplicons with linearized Δint-pNL4.3 into CEM-GXR cells via electroporation cells [58]. Transfection cultures were maintained and resulting viruses harvested as described previously [54]. Upon harvest, recombinant viruses were sequence validated.

Amplified infectious clinical isolates and recombinant viral stocks were genotyped and stored at −70 °C. GenBank accession numbers and integrase polymorphisms are indicated in Tables 1 and 2.

**Cell culture-based selection of resistance to integrase inhibitors**

Selections of HIV-1 variants resistant to INSTIs were performed through serial passage of patient-derived clinical isolates (n = 12) or recombinant strains (n = 10) in CBMCs, in the presence of serially escalating concentrations of DTG, BIC, CAB and EVG over the course of 36–46 weeks, as previously described [53, 59–61]. At each passage, aliquots of cell-free supernatant were stored at −70 °C for further analysis.

Stepwise drug-dose escalations were based on weekly reverse transcriptase (RT) enzymatic assays performed for each isolate in the presence and absence of the relevant INSTI [34]. Briefly, 10 µl clarified culture supernatant were incubated in a 50 µl reaction mixture containing 50 mM Tris (pH 8.0), 5 mM MgCl₂, 150 mM KCl, 5 mM dithiothreitol, 0.3 mM glutathione, 0.5 mM EGTA, 0.05% Triton X-100, 50 µg of poly(rA)-oligo(dT)₁₂–₁₈ per ml and 0.1 µCi of [³H]-TPP. After a 4 h incubation at 37 °C, 150 µl of ice-cold 10% trichloroacetic acid (TCA) was added for 30 min at 4 °C to precipitate incorporated [³H]-TTP. The precipitated mixture is transferred onto a Millipore multiscreen Glass Fiber FC plates with 10% TCA, vacuum drained, and washed twice with 10% TCA and once with cold ethanol using a Millipore multiscreen manifold. Scintillation cocktail (30 µl) is added to each well. Radioactivity is measured using a Perkin Elmer MicroBeta Trilux microplate counter.

Residual viral efficacy to DTG, BIC and CAB against EVG-resistant viruses was assessed by switch experiments. EVG-resistant viruses isolated at week 46 were grown in escalating concentrations of DTG, BIC, CAB or no drug control for a further 17 and 27 weeks. Genotyping at week 17 and 27 monitored the loss of EVG-associated resistance mutations and acquisition of mutations to second generation INSTIs.

Genotypic analyses were performed at weeks, 8, 16, 27–30 and 46 weeks to evaluate the acquisition and accumulation of amino acid substitutions that could be associated with reduced susceptibility to antiretroviral drugs (i.e., drug resistance mutations). Sanger (population sequencing of viral RNA extracted from culture supernatants across the integrase coding regions was performed as previously described [60, 62].

Phenotypic susceptibility to DTG, BIC, CAB, EVG and RAL were monitored using a cell-based in vitro assay. Briefly, viruses were amplified from stored cell culture supernatants at designated weeks following in vitro selection. Resistant and wild-type control viruses were infected with serial dilutions of INSTIs. After 7 days, culture supernatants were collected and analyzed for RT activity. The 50% effective concentrations were calculated on the analysis of dose–response curves using GraphPad Prism version 6.07 software.

**Authors’ contributions**

We dedicate this paper in the memory of Mark A. Wainberg, who dedicated his career in the field of HIV/AIDS drug resistance. BB designed and supervised the study and wrote the manuscript. MO and RI performed the drug selections. RI performed the genotyping. TM provided the pNL4.3 with R263K and S153Y introduced by site-directed mutagenesis. J-PR supervised the collection of plasma and cells from persons recruited into the Montreal PHI cohort. All authors read and approved the final manuscript.

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**Competing interests**

The authors declare that they have no competing interests.

**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Consent for publication**

Not applicable.

**Ethics approval and consent to participate**

Blood samples were obtained from participants in the FRSQ-Réseau SIDA primary HIV cohort. The patients provided informed written consent and the protocols were approved by ethics committees of the respective clinics and the Jewish General Hospital in accordance with the Declaration of Helsinki, national and institutional standards [63].

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References
1. Brenner BG, Ibanescu RI, Hardy I, Roger M. Genotypic and phylogenetic insights on prevention of the spread of HIV-1 and drug resistance in "real-world" settings. Viruses. 2017;10:10.

2. Wainberg MA, Zaharatos GJ, Brenner BG. Development of antiretroviral resistance related to the high genetic barrier to drug resistance. Virus Res. 2017;239:1–9.

3. Brenner BG, Wainberg MA. Clinical benefit of dolutegravir in HIV-1 management of patients with multidrug-resistant virus: a phase II randomised controlled trial. Lancet. 2007;369:1261–9.

4. Elzi L, Erb S, Furrer H, Cavassini M, Calmy A, Vernazza P, Gunthardt H, Bernasconi E, Battegay M. Swiss HIV Cohort Study: adverse events of raltegravir and dolutegravir. AIDS. 2017;31:1853–8.

5. Grinsztejn B, Nguyen BY, Katlama C, Gatell JM, Lazzarin A, Vittecoq D, Oliveira M, Delelis O, Morand-Joubert L, Mouscadet JF, et al. Genetic barriers for integration of reverse transcriptase inhibitors in antiretroviral-naive adults with HIV-1 infection (GS-US-380-1489): a double-blind, multicentre, phase 3, randomised controlled non-inferiority trial. Lancet. 2017;390:2063–72.

6. Margolis DA, Gonzalez-Carcia J, Bellohr DA, McDonough EI, Kuhl BD, Faltenbacher VH, Xu H, Michaud V, Sloan RD, Wainberg MA. HIV-1 subtype B and C integrase enzymes exhibit differential patterns of resistance to integrase inhibitors in biochemical assays. AIDS. 2010;24:2171–7.

7. Messpelet T, Quashie PK, Han YS, Oliveira M, Zanichelli V, Wainberg MA. The R263K substitution in HIV-1 subtype C is more deleterious for integrase enzymatic function and viral replication than in subtype B. AIDS. 2015;29:1459–66.

8. Brenner BG, Ibanescu RI, Oliveira M, Roger M, Hardy I, Routy JP, Stiff P, Quinones-Mateu ME, Wainberg MA. Montreal HIV/CNS: HIV-1 strains belonging to large phylogenetic clusters show accelerated escape from integrase inhibitors in cell culture compared with viral isolates from singleton/small clusters. J Antimicrob Chemother. 2017;72:2171–83.

9. Quashie PK, Oliveira M, Veres T, Osman N, Han YS, Hassounah S, Han Y, Liang J, Singhroy DN, Wainberg MA. The R263K substitution in HIV-1 subtype C is more deleterious for integrase enzymatic function and viral replication than in subtype B. Antimicrob Agents Chemother. 2015;59:4975–80.

10. Margolis DA, Gardner-Garcia J, Stellbrink HJ, Eron JJ, Yantrampanan Y, Podzamczer D, Zanichelli V, Wainberg MA. Integrase strand transfer inhibitors in the management of HIV-positive individuals. Ann Med. 2014;46:123–9.

11. Gallant J, Lazzarin A, Mills A, Orkin C, Podzamczer D, Tebas P, Girard PM, Brar I, Daar ES, Woh I, et al. Bictegravir, emtricitabine, and tenofovir alafenamide versus dolutegravir, abacavir, and lamivudine for initial treatment of HIV-1 infection (GS-US-380-1489): a double-blind, multicentre, phase 3, randomised controlled non-inferiority trial. Lancet. 2017;390:1499–509.

12. Margolis DA, Gonzalez-Carcia J, Bellohr DA, McDonough EI, Kuhl BD, Faltenbacher VH, Xu H, Michaud V, Sloan RD, Wainberg MA. HIV-1 subtype B and C integrase enzymes exhibit differential patterns of resistance to integrase inhibitors in biochemical assays. AIDS. 2010;24:2171–7.

13. Brenner BG, Ibanescu RI, Oliveira M, Roger M, Hardy I, Routy JP, Stiff P, Quinones-Mateu ME, Wainberg MA. Montreal HIV/CNS: HIV-1 strains belonging to large phylogenetic clusters show accelerated escape from integrase inhibitors in cell culture compared with viral isolates from singleton/small clusters. J Antimicrob Chemother. 2017;72:2171–83.

14. Osman N, Mesplete T, Quashie PK, Oliveira M, Zanichelli V, Wainberg MA. Dolutegravir maintains a durable effect against HIV replication in tissue culture even after drug washout. J Antimicrob Chemother. 2015;70:2810–5.

15. Sax PE, Poznai A, Montes ML, Koenig E, DeJesus E, Stellbrink HJ, Antinori A, Workowski K, Slim J, Reines J, et al. Coformulated bictegravir, emtricitabine, and tenofovir alafenamide for initial treatment of HIV-1 infection (GS-US-380-1490): a randomised, double-blind, multicentre, phase 3, non-inferiority trial. Lancet. 2017;390:2073–82.

16. Tsang M, Jones GS, Goldsmith J, Mulate A, Hansen D, Kan E, Tsai L, Bham RA, Stepan I, Strey KM, et al. Antiviral activity of bictegravir (GS-9833), a novel potent HIV-1 integrase strand transfer inhibitor with an improved resistance profile. Antimicrob Agents Chemother. 2016;60:7086–97.

17. Smith SJ, Zhao XZ, Burke TR Jr, Hughes SH. Efficacies of cabotegravir and bictegravir against drug-resistant HIV-1 infection mutants. Retrovirology. 2018;15:37.

18. Margolis DA, Brenner BG, Smith GHR, de Vente J, Hagens DP, Eron JJ, Griffith SK, Clair MHS, Stevens MC, Williams PE, et al. Cabotegravir plus rilpivirine, once a day, after induction with cabotegravir plus nucleoside reverse transcriptase inhibitors in antiretroviral-naive adults with HIV-1 infection (LATTE): a randomised, phase 2b, dose-ranging trial. Lancet Infect Dis. 2015;15:1145–55.
33. Sax PE, DeJesus E, Crofoot G, Ward D, Benson P, Dretler R, Mills A, Brinon C, Pelouquin J, Wei X, et al. Bictegravir versus dolutegravir, each with emtricitabine and tenofovir alafenamide, for initial treatment of HIV-1 infection: a randomised, double-blind, phase 2 trial. Lancet HIV. 2017;4:e154–60.

34. Boulierie F, Baur S, Gelezunias R, Lович A, Wainberg MA. High frequency of isolation of defective human immunodeficiency virus type 1 and heterogeneity of viral gene expression in clones of infected U-937 cells. J Virol. 1990;64:1745–55.

35. Brenner BG, Thomas R, Blanco JL, Ibanescu RI, Oliveira M, Mesplede T, Golubkov O, Roger M, Garcia F, Martinez E, Wainberg MA. Development of a G184R mutation in HIV-1 integrase following a switch to dolutegravir monotherapy leading to cross-resistance to integrase inhibitors. J Antimicrob Chemother. 2016;71:1948–53.

36. Ghoor J, Mazet AA, Avettand-Fenoël V, Peytavin G, Wirden M, Delfraissy JF, Chai ML. Rapid selection and archiving of mutation E157Q in HIV-1 DNA during short-term low-level replication on a raltegravir-containing regimen. J Antimicrob Chemother. 2009;64:433–4.

37. Malet I, Delelis O, Valantin MA, Montes B, Soulie C, Wirden M, Tchertanov L, Peytavin G, Reyne J, Mouscadet JF, et al. Mutations associated with failure of raltegravir treatment affect integrase sensitivity to the inhibitor in vitro. Antimicrob Agents Chemother. 2008;52:1351–8.

38. Danion F, Belissa E, Peytavin G, Thierry E, Lanternier F, Scemla A, Lortholary O, Delelis O, Valantin MA, Avettand-Fenoël V, Duvivier C. Non-virological response to a dolutegravir-containing regimen in a patient harbouring a E157Q-mutated virus in the integrase region. J Antimicrob Chemother. 2015;70:1921–3.

39. Charpentier C, Descamps D. Resistance to HIV integrase inhibitors: about R265K and E157Q mutations. Viruses. 2018;10:41.

40. Charpentier C, Malet I, Andre-Garnier E, Storto A, Bocket L, Amiel C, Margolis DA, Boffito M. Long-acting antiviral agents for HIV treatment. In: International workshop on antiretroviral drug resistance, 20–22 Feb 2015, Seattle, WA.

41. Dudas K, Wang R, Margolis D, Demarest J, Griffith S, St Clair MH. Characterization of NRTI and NNRTI resistance mutations observed in a study subject on ral two-drug therapy with 10 mg cabotegravir and 25 mg rilpirvirine. In: International workshop on antiretroviral drug resistance, 21–22 Feb 2015, Seattle, WA.

42. Margolis DA, Boffito M. Long-acting antiviral agents for HIV treatment. Curr Opin HIV AIDS. 2015;10:246–52.

43. Neogi U, Singh K, Aralaguppe SG, Rogers LC, Nzenda DT, Sarafianos SG, Heijdeman B, Sonnerborg A. Ex vivo antiretroviral potency of newer integrase strand transfer inhibitors cabotegravir and bictegravir in HIV type 1 non-B subtypes. AIDS. 2018;32:469–76.

44. Yoshinaga T, Kobayashi M, Seki T, Miki S, Wakasa-Morimoto C, Suyama-Kagintani A, Kawauchi-Miki S, Taishi T, Kavsasii T, Johns BA, et al. Antiviral characteristics of GSX1265744, an HIV integrase inhibitor dosed orally or by long-acting injection. Antimicrob Agents Chemother. 2015;59:397–406.

45. Yoshinaga T, Seki T, Miki S, Miyamoto T, Suyama-Kagintani A, Kawauchi-Miki S, Kobayashi M, Sato A, Stewart E, Underwood M, Fujitawa T. Novel secondary mutations C56S and G149A confer resistance to HIV-1 integrase inhibitors. J Antimicrob Chemother. 2015;59:397–406.

46. Brenner BG, Thomas R, Blanco JL, Ibanescu RI, Oliveira M, Mesplede T, Golubkov O, Roger M, Garcia F, Martinez E, Wainberg MA. Development of a G184R mutation in HIV-1 integrase following a switch to dolutegravir monotherapy leading to cross-resistance to integrase inhibitors. J Antimicrob Chemother. 2016;71:1948–53.

47. Cahn P, Pozniak AL, Mingrone H, Shuldyakov A, Brites C, Andrade-Villanueva JF, Richmond G, Buendia CB, Fourie J, Ramgopal M, et al. Dolutegravir versus raltegravir in antiretroviral-experienced, integrase-inhibitor-naive adults with HIV-1 infection: a randomised, double-blind, non-inferiority SAILING study. Lancet. 2013;382:700–7.