HIV-1 Group O Integrase Displays Lower Enzymatic Efficiency and Higher Susceptibility to Raltegravir than HIV-1 Group M Subtype B Integrase

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HIV-1 group O (HIV-O) is a rare HIV-1 variant characterized by a high number of polymorphisms, especially in the integrase coding region. As HIV-O integrase enzymes have not previously been studied, our aim was to assess the impact of HIV-O integrase polymorphisms on enzyme function and susceptibility to integrase inhibitors. Accordingly, we cloned and purified integrase proteins from each of HIV-1 group O clades A and B, an HIV-O divergent strain, and HIV-1 group M (HIV-M, subtype B), used as a reference. To assess enzymatic function of HIV-O integrase, we carried out strand transfer and 3' processing assays with various concentrations of substrate (DNA target and long terminal repeats [LTR], respectively) and characterized these enzymes for susceptibility to integrase strand transfer inhibitors (INSTIs) in cell-free assays and in tissue culture, in the absence or presence of various concentrations of several INSTIs. The inhibition constant (K_i) and 50% effective concentration (EC_{50}) values were calculated for HIV-O integrases and HIV-O viruses, respectively, and compared with those of HIV-M. The results showed that HIV-O integrase displayed lower activity in strand transfer assays than did HIV-M enzyme, whereas 3' processing activities were similar to those of HIV-M. HIV-O integrases were more susceptible to raltegravir (RAL) in competitive inhibition assays and in tissue culture than were HIV-M enzymes and viruses, respectively. Molecular modeling suggests that two key polymorphic residues that are close to the integrase catalytic site, 74I and 153A, may play a role in these differences.

HIV-1 group O (HIV-O) is one of four main groups of HIV-1. Group O is divided into three clades: 76% of viruses belong to clade A, 7.5% to clade B, and only 5% to clade C. There are also many divergent strains (11.5%) that are not classified into any clade, demonstrating the high genetic diversity of this group (1, 2).

HIV-O presents with distinct molecular and genetic features and a restricted geographical distribution. The majority of HIV-O infections have been diagnosed in Cameroon, with a prevalence of about 1% among persons living with HIV/AIDS (3). France has the largest number of cases outside Cameroon, and a survey (termed RES-O) of group O variants identified 141 patients as of 2014, with the first case having been reported in 1992 (1). A few sporadic cases have also been reported in Europe and North America (4–7).

Due to the scarcity of HIV-O infections, few biological and clinical data are available for this HIV-1 group. Compared to HIV-1 group M (HIV-M), which is responsible for the largest proportion of the current pandemic, HIV-O shows greater genetic diversity. Natural polymorphisms are found in target regions for current antiretrovirals (ARVs), particularly in the protease and integrase regions of HIV-O. Genotypic interpretations of drug resistance using the most common algorithms suggest that these viruses contain natural genotypically defined resistance polymorphisms with relevance for several ARVs, including the protease inhibitor (PI) tipranavir, the fusion inhibitor enfuvirtide, and all nonnucleoside reverse transcriptase inhibitors (NNRTIs) (1, 8). However, polymorphisms in the HIV-O genotype do not necessarily confer the same phenotype as in HIV-M (8, 9). For example, HIV-O viruses that harbor the enfuvirtide (ENF)-associated N42D resistance substitution in gp41 retain *in vitro* susceptibility

to that drug (9). Likewise, some group O patients have demonstrated excellent clinical and virological responsiveness to therapy, despite the fact that treatment failure was predicted by genotypic interpretation algorithms. Although relatively few studies have attempted to establish correlates between HIV-O genotypes and phenotypes and the subsequent emergence of drug resistance (1, 9–13), the analysis of a large number of HIV-O-infected patients has shown that specific polymorphisms can impact on the emergence of resistance-associated substitutions during treatment, consistent with what has been reported for HIV-M subtypes (14–17). To date, only a small amount of information is available in regard to the integrase enzyme of HIV-O viruses.

Integrase, a 35-kDa protein, is a bifunctional enzyme that possesses two distinct activities. First, 3' processing involves the removal of dinucleotides to expose 3' hydroxyls that are attached to invariant CA dinucleotides at the 3' extremities of the viral DNA long terminal repeats (LTR). The second event, termed strand transfer, is a transesterification reaction that inserts reverse-transcribed 3'-processed viral DNA into host cell chromosomal DNA (18).

Despite a certain degree of variability in the integrase region

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TABLE 1 List of the primers used for amplification, sequencing, cloning, and site-directed mutagenesis of HIV-O integrase

Step	Primer ^a	Sequence (5'→3')
SEQ outer PCR	INTOoutU	CCCAACWACACAGAGTGA
	INTOoutL	CATTCTATACTAACCCCATGTCC
SEQ nested PCR	INTOinU	GTATCTTRCATGGGTTCTCTGC
	INTOinL	GGCATYAATCCCCAATATGT
Sequencing	RT02 U*	GCCTCCCACACAAATGAKATAAG
	int-O3 U*	ATAGAYCAGGCACARGAAGATC
	int-O3bis L*	ACCTGCCATCTGTTTTCCATA
	polo 5012 L*	CTACTGCTCCYTCACCTTTCC
Cloning	INO-A F	CTGGTGCCGCGCGGCAGCCATATGTTCTCTAGAAGGAATAGATCAAG
	INO-B F	CTGGTGCCGCGCGGCAGCCATATGTTCTCTGGAAGGAATTGATCAGG
	INO-D F	CTGGTGCCGCGCGGCAGCCATATGTTCTCTAGAGGGAATAGATCAGG
	INO-R	CTTGTTAGCAGCCGATCCTATTATGGTATTTACCAGGCTGTTCC
Site-directed mutagenesis	A6-F185H U	CCCAATCCCCCTTTTCTTTTATGATTGTGAACAAAGACTGCCATT
	A6-F185H L	AAATGGCAGTATTTGTTTACAATCATAAAAGAAAAGGGGGGATTGGGG
	B2-F185H U	CCCAATCCCCCTTTTCTTTTATGATTGTGAACAAATACTGCCATTT
	B2-F185H L	GGAAAACAGATGGCAGGTACTGATAGTGTGGCAAGTAG
	D10-F185H U	CTACTTGCCCACTATCAGTACCTGCCATCTGTTTTCC
	D10-F185H L	AAATGGCAGTATTTGTTTACAATCATAAAAGAAAAGGGGGGATTGGGG
	D10-C280S U	CCCAATCCCCCTTTTCTTTTATGATTGTGAACAAATACTGCCATTT
	D10-C280S U	AATGGCAGTCTTTGTTTACAATCATAAAAGAAAAGGGGGGATTGGG

^a F, forward primer; R, reverse primer. All primers were designed using QuikChange primer design software (Agilent), except for those marked with an asterisk (31).

(19–21), the residues that are involved in enzyme function, including the catalytic triad D₆₄D₁₁₆E₁₅₂ and the zinc-binding site H₁₂H₁₆C₄₀C₄₃, are highly conserved among all HIV variants, including HIV-O. However, a comparison of the HIV-O and HIV-M integrases reveals that 46 of 288 residues are polymorphic (16%) and that this variability rises to >20% if only the residues between positions 51 and 230 (i.e., the catalytic core region) are considered (22, 23). Strikingly, all group O viruses possess four substitutions at positions L74I, S153A, G163Q, and T206S in comparison to HIV-M. The S153A and G163Q substitutions are atypical in that these residues (A for 153 and Q for 163) have not been characterized in regard to resistance against the integrase strand transfer inhibitor (INSTI) family of drugs (1, 2).

The presence of each of the four above-mentioned substitutions should have resulted in a decrease in susceptibility to all currently approved INSTIs based on each of three common resistance algorithms (i.e., ANRS, REGA, and HIVdb) (<http://sierra2.stanford.edu/>). Importantly, two positions that were previously associated with low-level resistance to dolutegravir (DTG), the most recently approved INSTI, i.e., L74 (24) and S153 (22), are mutated in HIV-O.

INSTIs represent a new drug class among ARVs (25, 26) and include raltegravir (RAL), elvitegravir (EVG), and DTG. The latter drug has shown statistical superiority in antiviral activity when used in combination with two nucleoside drugs in comparison with fixed-dose combination involving other compounds (27).

INSTIs are known to be active against different HIV-M subtypes and against HIV-2 (28, 29). Although it was recently shown that 10 heavily pretreated group O patients receiving RAL had good clinical and virological responses (11), the subject of HIV-O integrase enzyme function and susceptibility to INSTIs requires further investigation. This was the aim of our study.

MATERIALS AND METHODS

Viruses. Three viral strains from HIV-O-infected ARV-naïve patients, representative of each clade, and one from HIV-M subtype B were studied: strain 11367 (HIV-O clade A [HIV-O/A]), strain MVP5180 (HIV-O clade B [HIV-O/B]), strain BCF11 (HIV-O divergent strain [HIV-O/Div]), and strain 5326 (HIV-M subtype B [HIV-M/B]). Strains 11367 and 5326 were isolated at the McGill AIDS Center, whereas MVP5180 and BCF11 were obtained from the NIH AIDS Research and Reference Reagent Program (catalog numbers 2878 and 4142, respectively). All these viruses were amplified using cord blood mononuclear cells (CBMCs), as previously described (30). CBMCs were isolated by Ficoll-Hypaque (GE Healthcare) gradient centrifugation from blood obtained through the Department of Obstetrics, Jewish General Hospital, Montréal, QC, Canada. Briefly, 1 million cells were infected with 1 ml of virus aliquot for 2 h, washed to remove unbound virus, and plated in 24-well plates. Fresh cells were added on a weekly basis, and reverse transcriptase (RT) monitoring was carried out weekly.

Amplification of the integrase region. The primers utilized in this study, cited below, are summarized in Table 1. HIV-1 group O RNA was extracted from 140 µl of plasma using the QIAmp viral RNA kit (Qiagen, Toronto, ON, Canada) according to the manufacturer's recommendations. Primers were designed for the amplification of the entire integrase region by nested RT-PCR. For the outer PCR, the primers used were INTOoutU and INTOoutL for the amplification of a 1,235-bp fragment in the *pol* gene region. For nested PCR, the primers used were INTOinU and INTOinL for a 1,120-bp fragment.

RT-PCR. Ten microliters of extracted RNA were amplified in a 50-µl reaction mixture containing 1.2 mM MgSO₄, 20 pmol of each outer primer, and 1 µl of RT/Platinum *Taq* (SuperScript III One-Step RT-PCR system with Platinum *Taq* DNA polymerase; Invitrogen, Burlington, ON, Canada). The reaction was carried out in an Eppendorf thermocycler (Mississauga, ON, Canada) under the following conditions: 50°C for 30 min and 94°C for 2 min, followed by 35 cycles at 94°C for 30 s, 55°C for 30 s, and 68°C for 2 min, and a final extension step at 72°C for 7 min.

FIG 1 Alignment of the amino acid sequences of integrases from the four viruses studied (HIV-O and HIV-M) in comparison with the reference strain HxB2. The key residues of the catalytic site ($D_{64}D_{116}E_{152}$) and the zinc-binding site $H_{12}H_{616}C_{40}C_{43}$ are framed in red. Red arrows highlight the three major resistance-associated mutations for integrase inhibitors (Y143, Q148, N155), and red arrows with an asterisk show the four substitutions found in all HIV-O viruses (L74I, S153A, G163Q, and T206S). The extra 10 C-terminal residues in group O viruses are framed in orange.

Nested PCR. Two microliters of the first-round amplification product was used for the nested PCR in a volume of 50 μ l containing the Phusion high-fidelity (HF) PCR master mix with HF buffer (New England BioLabs Inc., Whitby, ON, Canada). The reaction was run at 95°C for 15 min, followed by 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, with a final extension step at 72°C for 7 min. Amplification products were visualized by 1% agarose gel electrophoresis with SYBR Safe DNA gel staining (Invitrogen, Burlington, ON, Canada).

Sequencing. PCR products were purified using the QIAquick PCR purification kit (Qiagen, Toronto, ON, Canada) and subsequently sequenced on both strands using the same primers as were employed for the nested PCR (i.e., INT-O in F and INT-O in R) and RTO2 U, int-O3 U, int-O3bis L, and polo 5012 L (Table 1) (31). The sequencing reaction was performed with the BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems, Burlington, ON, Canada), according to the manufacturer's recommendations, in an automated capillary 3130xl Genetic Analyzer sequencer (Applied Biosystems, Burlington, ON, Canada). Sequencing results were analyzed using SeqScape software version 2.5. DNA sequences were compared with the HIV-1 group M reference strain HxB2 (Fig. 1) using BioEdit software for alignment (BioEdit sequence alignment editor v7.1.3, copyright 1997-2011, Tom Hall). Baseline sequences were obtained from the NIH database (strains MVP5180 BCF11) or from genotypes performed on plasma samples (isolates 11367 and 5326). After amplification, each virus was resequenced and compared to the reference sequence.

Cloning of the integrase coding region. We used the bacterial expression plasmid pET-15b, containing HIV-M integrase, as previously described (32); we replaced the wild-type HIV-M subtype B integrase with each of several HIV-O integrase coding regions; pET-15b with integrase from pNL4-3 (NIH catalog number 114) was used as a reference (32). Cloning was performed using plasmid after a double restriction enzyme

digestion (BamHI and NdeI) and dephosphorylation by CIP (calf intestinal alkaline phosphatase), according to the manufacturer's instructions (New England BioLabs Inc., Whitby, ON, Canada). We generated a His-tagged integrase using the following cloning primers: INO-A F (11367), INO-B F (MVP5180), and INO-D F (BCF11). INO-R was used as the reverse primer for all the clades (Table 1).

After a double digestion with BamHI and NdeI, PCR products were purified using the QIAquick PCR purification kit (Qiagen, Toronto, ON, Canada) and subsequently introduced into the previously prepared pET-15b plasmid at a 2:1 ratio and using T4 DNA ligase, as recommended by the manufacturer (New England BioLabs Inc., Whitby, ON, Canada).

In order to increase protein solubility, the F185H and C280S substitutions were introduced by site-directed mutagenesis as previously described (33, 34) using *Pfu*Turbo DNA polymerase (Agilent Technologies, Mississauga, ON, Canada) as follows: 100 ng of each plasmid was used in a 50- μ l mix containing 5 μ l of 10 $\times$ cloned *Pfu* DNA polymerase reaction buffer, 1 μ l of deoxynucleoside triphosphates (dNTPs) (10 μ M), 1 μ l of each primer (10 μ M), and 2 μ l of dimethyl sulfoxide (DMSO). The mutagenesis reaction was run for 5 min at 95°C, followed by 22 cycles at 95°C for 30 s, 57°C for 30 s, and 68°C for 7 min and 30 s, and a final step at 68°C. Of note, HIV-O integrases from clades A and B naturally bear the C280S solubility substitution. Primers for site-directed mutagenesis were designed using QuikChange primer design software (Agilent). Each plasmid was amplified in *Escherichia coli* XL10-Gold ultracompetent cells [Tet^r Δ (*mcrA*)183 Δ (*mcrCB*-*hsdSMR*-*mrr*)173 *endA1* *supE44* *thi-1* *recA1* *gyrA96* *relA1* *lac* *Hte* F' *proAB* *lacI*^q Δ M15 Tn10 (Tet^r) Amy Cam^r] (Stratagene, Agilent Technologies, Mississauga, ON, Canada). Successful mutagenesis was confirmed by sequencing (Genome Québec), and the sequences thereby derived were compared with the reference sequence for each virus.

Expression and purification of HIV-O integrase. Plasmids encoding the integrase proteins of the different HIV-O clades were expressed using *Escherichia coli* BL21(DE3) Gold cells [$F^- ompT hsdS_B(r_B^- m_B^-) gal dcm \lambda(DE3)$] (Stratagene, Agilent Technologies, Mississauga, ON, Canada). Bacterial cultures were grown at 37°C in 500 ml Luria-Bertani (LB) broth supplemented with 100 µg/ml ampicillin until they reached an optical density at 600 nm of 0.4 to 0.6. Protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h at 37°C with agitation at 200 rpm. The cultures were then centrifuged at 7,000 rpm for 10 min, and the cell pellets were stored at -80°C until use. The purification of integrase recombinant proteins was performed as previously described for His-tagged integrase (32, 35). For optimization of results, an Mg^{2+} - and Mn^{2+} -enriched buffer was used for resuspension (36), followed by an additional 1 h of incubation at 4°C prior to the second centrifugation. HIV-M integrase containing the F185H/C280S solubility substitutions was expressed and purified following the same methods as described above.

Strand transfer assay. Strand transfer reactions were carried out using Costar DNA-bind 96-well plates (Corning) as previously described (35, 37). The LTR of HIV-M and HIV-O share only 66% sequence homology, and the α4 helix (residues 150 to 163) involved in LTR-specific binding displays significant polymorphism in HIV-O. Therefore, we designed specific unprocessed donor LTR DNA for HIV-O using LTR-O sense (5AmMC12-ATCACGTAGACTGAAGCAGAAAATCTCTAGCAGT-3') and antisense (5'-ACTGCTAGAGATTTTCTGCTTCAGTCTACGTGAT-3') primers, where 5AmMC12 refers to a reactive amino group attached to the 5' end of the oligonucleotide using a 12-carbon linker. These assays were also performed using unprocessed LTR-M in comparison with LTR-O and biotinylated target DNA, in the presence of 30 mM $MnCl_2$, as previously described (35). To produce functional DNA duplexes, equimolar amounts of sense and antisense oligonucleotides were mixed and annealed by heating for 10 min at 96°C and slowly cooled to room temperature over a period of 4 h. Eighty microliters of unprocessed donor DNA duplex, appropriately diluted in phosphate-buffered saline (PBS; pH 8.5) (Bioshop), was added to the 96-well plates, followed by a 48-h incubation at 4°C. The plates were then blocked with blocking buffer (0.5% bovine serum albumin [BSA], 20 mM Tris-HCl [pH 7.8], and 150 mM NaCl) for at least 30 min at 4°C.

3' processing. 3' processing activity was determined using 3'-biotinylated DNA duplex LTR-O 3P sense DNA (5'-AmMC12-ATCACGTAGACTGAAGCAGAAAATCTCTAGCAGT-BioTEG-3'), where BioTEG refers to biotin attached to the 3' end of the oligonucleotide by a linker, and LTR-O antisense DNA (described above), covalently linked to Costar DNA-bind plates under the same conditions as for the strand transfer assay described above (38). Purified integrase was added and incubated for 2 h at 37°C. After the 3'-biotinylated ends cleaved by integrase from the plate were washed away, 3' processing activity was assessed as previously described (38).

Competitive inhibition of strand transfer by the integrase inhibitors RAL, EVG, and DTG. To assess the susceptibility of HIV-O integrase to INSTIs, we conducted competitive inhibition assays using RAL, EVG, and DTG as previously described (39). Purified integrase proteins were studied in the presence of various concentrations of each drug (0 to 3,200 nM) and DNA target (16, 32, 64, and 128 nM). Inhibitory constants (K_i) for each drug were calculated using GraphPad Prism, as previously described (39). To compare the susceptibility of each integrase tested to these various INSTIs, we determined fold change (FC) values in each case (i.e., the ratio of the K_i value for HIV-O integrase to the K_i value for HIV-M/B integrase).

Phenotyping. To determine the susceptibility of HIV-O to different INSTIs *in vitro*, we performed a cell culture-based phenotypic assay using CBMCs as previously described (30). One million cells were infected with each virus (multiplicity of infection [MOI] = 0.1 to 1, determined by viral titration on CBMCs) for 2 h, washed to remove unbound virus, plated in duplicate into 96-well plates in the presence of increasing concentrations

of drug (0.0256 to 400 nM), and then incubated at 37°C. At day 3, cells were fed with fresh medium containing appropriate drug dilution, and RT activity was measured at day 7. The 50% effective concentration (EC_{50}), obtained from 3 independent experiments in duplicate, was then calculated using GraphPad Prism.

***In silico* molecular analysis.** Molecular modeling was performed *in silico*: briefly, the structures of HIV-O integrases were generated by homology with the structure of the integrase from the prototype foamy virus (PFV) in complex with target DNA, as previously reported (18, 40), using an I-TASSER three-dimensional (3D) protein prediction server (41).

In order to reliably model HIV integrase bound to INSTI, the PFV structure PDB identifier (ID) 3S3M (42) was used as the lead template. Dimeric HIV intasome structures were obtained by alignment of monomeric models with the PFV structures, and active-site residues were optimized as previously described (39). Apparent binding poses and energies of the DTG, RAL, and EVG INSTIs in the active site of HIV-O were obtained by *in silico* docking simulations performed with the AutoDock Vina program (43). The HIV-M subtype B integrase was used for comparison. The viral DNA mimic, cations, and active-site water molecules from 3S3M were retained in equivalent positions within the HIV active sites to aid in INSTI binding. Prior to docking, ligands and receptor proteins were processed using AutoDock tools (44). All subsequent image processing and analysis were performed in PyMOL (<http://pymol.org>; The PyMOL Molecular Graphics System version 1.3; Schrödinger, LLC).

Statistical analysis. Results were expressed as means ± standard errors. To determine if differences between the integrase proteins of group O and group M were significant, we performed both chi square (percentage) and Student *t* tests (mean), with *P* values of <0.05 defining a significant difference. One-way analysis of variance (ANOVA) with Dunnett's posttest was performed using GraphPad Prism to compare the means of the four groups studied.

RESULTS

Sequence comparison of HIV-O and HIV-M integrase. In order to compare the amino acid sequences of HIV-O and HIV-M, we performed an alignment with the reference strain HxB2, using BioEdit software (Fig. 1). The HIV-O integrase contains 298 amino acids with 10 extra residues in the C-terminal region. Residues that are involved in enzyme function, including the catalytic triad D₆₄D₁₁₆E₁₅₂ and the zinc-binding site H₁₂H₁₆C₄₀C₄₃, are highly conserved among viruses. Furthermore, all HIV-O integrases bear the L74I, S153A, G163Q, and T206S signature substitutions relative to HIV-M (Fig. 1).

LTR titration for strand transfer activity. The various HIV-O integrase proteins were purified (Fig. 2A). In order to determine the optimum LTR concentration for study, we performed strand transfer assays using various concentrations of LTR-O or LTR-M (0 to 2,400 nM), as shown in Fig. 2B. The highest level of enzyme activity was attained in the presence of 300 nM LTR-O or LTR-M, and the calculated apparent K_m (K_m') was similar for each enzyme studied, using either LTR-O or LTR-M (data not shown).

Protein titration for strand transfer activity. Using a protocol similar to that described above, we also determined the optimum concentration of the different purified integrase proteins by titration (0 to 1,600 nM), as shown in Fig. 2C. The highest rate of strand transfer activity for all integrases tested was observed using 400 nM integrase protein.

Determination of enzymatic activity. Next, we carried out strand transfer assays using fixed concentrations of LTR (300 nM) and integrase (400 nM) and various concentrations of substrate, in this case the target DNA. Two-fold dilutions of substrate were employed (0.9375 nM to 120 nM), as shown in Fig. 3A. These

FIG 2 Titration of the DNA donor and integrase concentrations for the strand transfer assay. (A) Purification of HIV-O recombinant integrase proteins from clades A (IN-O/A) and B (IN-O/B) and a divergent strain (IN-O/Div) and HIV-M recombinant integrase protein (IN-M/B). SDS-PAGE analysis showing that the integrase proteins resolved predominantly in a high-density band (35.6 kDa); the two bands at the bottom represent nonspecifically cleaved forms of the protein. (B) Calibration of the DNA donor mimicking LTR-O using various concentrations, ranging from 0 to 2,400 nM; (C) calibration of the different HIV-O integrases, IN-O/A (clade A), IN-O/B (clade B), and IN-O/Div (divergent strain), and the HIV-M integrase, IN-M/B (HIV-M subtype B), using various concentrations, ranging from 0 to 1,600 nM.

FIG 3 Enzymatic activity of the different HIV-O integrases from clade A (IN-O/A), clade B (IN-O/B), and divergent strain (IN-O/Div) in comparison with HIV-M integrase (IN-M/B). (A) Strand transfer activity: these data were generated using various concentrations of DNA target (0 to 120 nM); (B) 3' processing activity: the data were generated using various concentrations of LTR-O (0- to 40 nM).

findings suggest that the apparent strand transfer activities of the HIV-O enzymes tested are lower than those of HIV-M/B.

We next wished to assess HIV-O integrase 3' processing activity using the LTR-O mimic donor DNA as the substrate and various concentrations of LTR (0 to 40 nM) (Fig. 3B). Each of the HIV-O integrases appeared to display 3' processing activity similar to that of HIV-M.

Competitive inhibition of strand transfer activity by DTG, RAL, and EVG. Integrase enzyme susceptibilities to RAL, EVG, and DTG were analyzed by determining the inhibition constant (K_i) for each drug and calculating fold change (FC) values (i.e., the ratio of the K_i for each drug for HIV-O integrase to the K_i for each drug for HIV-M integrase) (Fig. 4). Despite a trend toward higher K_i values for HIV-O, no statistically significant differences were noted among the four enzymes tested, with the exception of RAL, for which the fold change/ K_i for HIV-O integrase (regardless of clade) was less than that of HIV-M integrase, i.e., FC of 0.4 for HIV-O clade A integrase (IN-O/A) and 0.5 for HIV-O clade B integrase (IN-O/B) and for HIV-O divergent strain integrase (IN-O/Div) ($P < 0.0001$) (Fig. 4A and D).

Susceptibility to INSTIs in tissue culture. We used CBMCs to measure susceptibility to INSTIs in tissue culture and found that HIV-O was susceptible to all three INSTIs tested. In general, results were consistent with the biochemical data described above. Compared to HIV-M, EC_{50} s were marginally lower for RAL, with the exception of HIV-O clade B: 0.27 ± 0.01 nM for HIV-O/A,

FIG 4 HIV-O integrase susceptibility to the integrase inhibitors RAL (A), EVG (B), and DTG (C), as analyzed by the competitive inhibition model, in comparison with HIV-M. The calculated values for the inhibition constant (K_i) are expressed as fold changes (FC) in comparison with HIV-M integrase (FC = 1). The table in panel D shows FC and relative V_{max} values for each HIV-O integrase in comparison with HIV-M (FC = 1 and V_{max} = 100%). Columns represent the means and standard errors from at least three independent experiments. *, $P < 0.05$.

0.49 ± 0.12 for HIV-O/B, 0.32 ± 0.03 nM for HIV-O/Div, and 0.68 ± 0.16 nM for HIV-M/B ($P < 0.05$) (Fig. 5A). The fold change for RAL ranged from 0.4 (HIV-O/A) to 0.7 (HIV-O/B).

For EVG, the EC_{50} s for HIV-O viruses were 0.38 ± 0.2 nM and

0.32 ± 0.4 nM for HIV-O/B and HIV-O/Div, respectively. HIV-M/B displayed about 2-fold-higher EC_{50} s, i.e., 0.73 ± 0.4 nM.

For DTG, EC_{50} s ranged between 1.23 nM (HIV-O/Div) and 2.23 nM (HIV-O/B), and the FC was between 0.6 to 1.

FIG 5 HIV-O susceptibility *in vitro* to the integrase inhibitors RAL (A), EVG (B), and DTG (C) in CBMCs in comparison with HIV-M. EC_{50} is expressed in nM. Columns represent the means and standard errors from at least three independent experiments. *, $P < 0.05$.

FIG 6 Molecular modeling of HIV-O integrase (clade A) based on the PFV structure. (A) To mimic a functional HIV-O dimeric intasome (chain A, green; chain B, pink), the modeled HIV-O monomer was overlaid with the intasome structure of PFV (chain A, yellow; chain B, blue) (PDB ID [3S3M](#)). (B) DNA and cations were modeled by direct overlay with the PFV structure. (C) The major polymorphic residues for HIV-O lie within the enzyme catalytic active site and within the viral DNA binding interface. The three catalytic residues ($D_{64}D_{116}E_{152}$) are shown as red sticks. The amino acid residues of HIV-O integrase are in yellow (I74, A153, Q163, and S206). The Mg^{2+} divalent cations are shown as spheres overlaid with the corresponding residues in the PFV structure.

No statistically significant difference was found among the different viruses for EVG and DTG.

***In silico* molecular analysis.** In comparison with HIV-M integrase, HIV-O proteins contain 10 additional residues at the C terminus. In regard to specific polymorphic positions, two of these, i.e., 74 and 153, are close to the catalytic core, as described in the legend to [Fig. 6](#).

In an effort to understand the structure of HIV-O integrases, a homology model of HIV-O integrase was created using the 3D modeling server known as I-Tasser ([41](#)) ([Fig. 6A](#)). A dimer of the HIV-O integrase intasome was created by direct structural alignment with the crystal structure of the PFV integrase intasome bound to DTG (PDB ID [3S3M](#)) ([42](#)) based on the previously published HIV-M homology model ([35](#)) ([Fig. 7](#)). To facilitate drug docking simulations, each of viral DNA, Mg^{2+} , Zn^{2+} , and key water molecules from [3S3M](#) were retained in an equivalent position in the HIV intasome ([Fig. 6B](#)). Both of the HIV models varied from the PFV template with a root mean square deviation (RMSD) of ~ 2 Å, indicating good homology for the portions of the enzymes that were modeled. The four polymorphic positions implicated in INSTI resistance, i.e., 74, 153, 163, and 206, were all found to be located in the active site in close proximity to the viral DNA binding site and catalytic residues ([Fig. 6B](#) and [C](#)). When DTG, RAL, and EVG were docked into the HIV-O active site, all three INSTIs bound to HIV-O in similar poses to those of PFV and HIV group M integrase ([Fig. 7A](#) to [D](#)). Of note, the apparent binding affinity of the various INSTIs to HIV-O integrase, as cal-

culated by AutoDock Vina ([43](#)), was consistently tighter than that for HIV-M integrase for all three drugs ([Fig. 7](#)), with EVG appearing to bind to HIV-O integrase almost twice as well (-13.7 kcal/mol) than to HIV-M integrase (-7.5 kcal/mol).

DISCUSSION

This work presents the first analysis of the enzymatic function of HIV-O integrase proteins from three different viral clades (i.e., clades A and B and a divergent strain). We analyzed global enzymatic activity by using an integration assay that includes both 3' LTR processing and strand transfer ([Fig. 3](#)). To assess relative differences among enzymes for each step, we also conducted a 3' processing assay. We show that HIV-O integrase during the strand transfer assay appears to be less efficient than HIV-M/B ([Fig. 3A](#)).

Despite 70% homology among amino acids in the *pol* gene, discrepancies have been reported between enzymes derived from HIV-O and HIV-M. Recent biochemical experiments comparing the activity of recombinant HIV-M RT (subtype B) to that of an HIV-O RT confirmed that the latter enzyme had higher thermostability as well as 2.5-fold higher efficiency ([45](#)).

This lower efficiency of HIV-O integrase in regard to strand transfer activity is not reflected in 3' processing activity, for which the HIV-O and HIV-M integrase enzymes show similar levels of activity ([Fig. 3B](#)). This suggests that any discrepancies between levels of integrase activity between HIV-O and HIV-M enzymes were due to the strand transfer step of integration.

No clade-specific effect was noticed in regard to integrase ac-

FIG 7 Simulation of INSTI binding to HIV-O (clade A) and to HIV-M (clade B) integrase intasomes. The INSTI molecules DTG (A, C), RAL (B, D), and EVG were docked into the INSTI binding pocket of HIV-O (A, B) and HIV-M (C, D) using AutoDock Vina (43); the crystallized position of the INSTIs in the respective structures, PDB ID 3S3M, PDB ID 3OYA, and PDB ID 3L2U, were used as positive controls for properly docked orientations (represented as black structures). The key active-site residues implicated in drug resistance and/or binding are shown as sticks. (E) The table shows the apparent binding energy in kcal/mole, calculated by AutoDock Vina (43).

tivities. Further experiments, including the cloning of full-length viruses, will be needed to assess the hypothesis that the decreased function of the HIV-O integrase enzyme might be partially responsible for the fact that HIV group O has been restricted geographically and has not spread as efficiently as group M viruses. It has been shown previously through competitive *in vitro* experiments that the replicative fitness of HIV-O is lower than that of HIV-M (46). However, the extent to which HIV-O integrase polymorphisms account for viral fitness is unknown. Direct quantification of HIV-O virus integration in cell culture through Alu-mediated PCR will help to answer this question.

Competitive inhibition assays showed similar susceptibilities for group O integrases and HIV-M/B integrase for EVG and DTG. In regard to RAL, a lower fold change/ K_i was observed, suggesting that HIV-O integrases may be more susceptible than group M integrases to this inhibitor (Fig. 4). This result was confirmed by phenotyping studies that employed CBMCs (Fig. 5). It should be noted that discrepancies in FC among mutant variants of different subtypes in HIV-M have also been reported (32). Our data support the notion that genetic polymorphisms can impact drug susceptibility.

For HIV-O, it is known that all integrase proteins contain poly-

morphisms that include L74I, S153A, G163Q, and T206S. These substitutions are all at known INSTI resistance positions that are associated with RAL and EVG (L74M, G163RK) and DTG (S153Y), all of which can cause a modest decrease in drug susceptibility. The L74I substitution, in combination with Q148 substitutions, may be a good predictor of treatment outcome with DTG in patients with resistance to RAL or EVG: only 45% of patients with this mutational combination attained an undetectable viral load at day 8 and week 24 following initiation of DTG therapy (24). Because HIV-O integrase already bears L74I, the acquisition of Q148 substitutions during RAL treatment might especially compromise the use of DTG in group O viruses, and more information on this topic is needed. Until now, however, RAL-based therapy has been effective for HIV-O-infected patients, and no resistance-associated substitutions have been reported in the integrase coding regions of HIV-O patients failing treatment with INSTIs (11, 47).

As stated, the structure of HIV-O integrase differs from that of HIV-M by some key residues that are close to the catalytic site, i.e., I74 and A153. These residues might impact on tertiary structure and play a role in the lower overall efficiency of HIV-O enzymes.

The overall structure of HIV-O integrase appears to be very similar to that of HIV-M integrase. It has been well documented that the duration of integrase bound “residence time” of INSTIs can be ordered as follows: DTG > RAL > EVG (48). Residence time is also directly correlated with INSTI binding affinity and levels of resistance attributable to the N155H, Y143R, E92Q, and Q148R substitutions. The fact that DTG, RAL, and EVG, in our simulation, appear to have high and almost equal affinity for HIV-O may mean that HIV-O integrase is more efficiently inhibited than HIV-M wild type, even in the presence of INSTI-associated resistance substitutions. This might also explain the lower FC/EC₅₀ values observed for RAL in both our competitive inhibition and tissue culture assays (Fig. 4A and 5A) as well as the virological successes observed with HIV-O-infected patients treated with RAL (11). Furthermore, HIV-O may have a higher genetic barrier for INSTI resistance than HIV-M, at least for RAL. Studies to address this issue are now under way.

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