Simian-tropic HIV as a model to study drug resistance against integrase inhibitors

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Abstract

Drug resistance represents a key aspect of HIV treatment failure. It is important to develop non-human primate models to study issues of drug resistance as well as the persistence and transmission of drug-resistant viruses. However, relatively little work has been conducted using either simian immunodeficiency virus (SIV) or SIV/HIV recombinant viruses to study resistance against integrase strand transfer inhibitors (INSTIs). Here, we used a T-cell tropic SIV/HIV recombinant virus in which the capsid and vif regions of HIV-1 were replaced with their SIV counterparts (stHIV-1_{SCA,SVIF}) to study the impact of a number of drug resistance substitutions in the integrase coding region at positions E92Q, G118R, E138K, Y143R, S153Y, N155H and R263K on drug resistance, viral infectivity and viral replication capacity. Our results show that each of these substitutions exerted effects that were similar to their presence in HIV-1. Substitutions associated with primary resistance against dolutegravir were more detrimental to stHIV-1_{SCA,SVIF} infectiousness than were resistance substitutions associated with raltegravir and elvitegravir, consistent with data that have been reported for HIV-1. These findings support the use of stHIV-1_{SCA,SVIF} as a useful model with which to evaluate the role of INSTI-resistance substitutions on viral persistence, transmissibility and pathogenesis in a non-human primate model.

Introduction

The advent of highly active antiretroviral therapy (HAART) for HIV infection represents a major accomplishment of modern medicine. Despite this, however, the occurrence of drug resistance mutations (DRMs) can reduce viral susceptibility to antiretroviral drugs (ARVs). Cell culture
experiments have often been predictive of HIV drug resistance pathways (1, 2), but the
development and persistence of DRMs is also governed by complex pharmacologic, viral and
host factors (1, 3, 4). Although animal models may allow for direct study of DRMs and their
effect on treatment success (1), there is no model that recreates all aspects of HIV-1 infection in
humans (5). This notwithstanding, infection of macaques with the simian immunodeficiency
virus (SIV) or simian-human immunodeficiency virus (SHIV) has provided important insights
into HIV pathogenesis. The construction of a simian-tropic HIV-1 (stHIV-1_{SCA,SVIF}) virus,
referred to here as stHIV-1, with an 88% sequence homology to HIV-1 has been accomplished
(5). By replacing the HIV-1 capsid and vif regions with the corresponding regions from SIV (2,
5, 6), this HIV-based chimera is capable of infecting both human and macaque cell lines by
evading TRIM5α and APOBEC3G restrictions (5).

Integrase strand-transfer inhibitors (INSTIs) represent the most recent class of ARVs and are
effective, minimally toxic, and tolerable (7). Until now, all HIV drugs have been susceptible to
the development of DRMs that often follows a similar pattern, whereby primary substitutions,
which confer resistance while reducing viral fitness, are followed by compensatory substitutions
that restore fitness while further increasing levels of resistance. The INSTIs raltegravir (RAL)
and elvitegravir (EVG) are compromised by substitutions at positions Y143, Q148 and N155 (8,
9), and additionally at position E92 for EVG alone (10). In contrast, resistance to the newer
INSTI dolutegravir (DTG) is associated with a substitution at position R263K, and no
compensatory substitution for R263K has yet been identified (11). The lack of such
compensation may help to explain the absence of detectable emerging resistance substitutions in
treatment-naïve individuals failing DTG-based therapy (11-14).
The G118R substitution was reported to emerge during selection studies with DTG as well as with the experimental integrase inhibitor MK-2048 (15, 16). Although this substitution has not been subsequently validated in clinical settings with DTG, it has been reported in an individual failing treatment with RAL (17). Similarly, other substitutions that have been shown to emerge in tissue culture under DTG pressure, i.e. L101I, T124A, S153Y/F, have not been observed in clinical studies (18).

Previous studies have shown that HIV-infected humans and SIV-infected macaques share similar patterns of drug resistance substitutions (1, 19). Furthermore, tissue culture studies have demonstrated that the same substitutions that confer resistance against INSTIs in HIV also do so in SIV. Given the high cost of animal-based research and the need for additional models for the study of drug resistance, there is a need to study other viruses as well in order to validate and extend findings previously obtained with either HIV or SIV.

The active site of the integrase coding sequence of HIV-1 is conserved in stHIV-1. Thus, it is reasonable to speculate that stHIV-1(SCA, SVIF) may be a suitable animal model for the study of INSTI DRMs, and this may help in understanding the impact of resistance substitutions on treatment decisions.

Using site-directed mutagenesis, we therefore introduced key DRMs against dolutegravir (DTG), raltegravir (RAL), and elvitegravir (EVG) into the integrase (IN) region of stHIV-1 to determine if resistance was similar to that seen in HIV. Our results demonstrate that DRMs against INSTIs decrease stHIV-1 infectiousness and confer degrees of resistance against these drugs that are comparable to those reported with HIV-1. DTG-specific DRMs have a greater negative effect on infectiousness compared to DRMs against RAL and EVG.
Materials and methods

Cells and antiviral compounds

For the purpose of infectivity and resistance experiments, TZM-bl cells were obtained through the NIH AIDS Research Reagent Program from Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc (Catalogue number 8129). 293T cells were used for transfection with replication-competent wild-type or mutant stHIV-1 and were obtained from the American Type Culture Collection (CRL-11268). Both cell lines were subcultured every 3-4 days in Dulbecco’s minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin and kept at 37°C under 5% CO₂.

Umbilical cord blood mononuclear cells (CBMCs) were isolated by Ficoll-Hypaque (GE Healthcare) gradient centrifugation from blood obtained through the Department of Obstetrics, Jewish General Hospital, Montréal, Canada. CBMCs were cultured as previously described (20). The stHIV-1\textsubscript{(SCA, SVIF)} plasmid was generously provided by Dr. Theodora Hatziioannou of the Aaron Diamond Research Center, New York, NY. DTG, RAL and EVG were provided by GlaxoSmithKline/ViiV Healthcare, Merck Inc, and Gilead Sciences, respectively.

Mutagenesis of the integrase region of stHIV-1

The stHIV-1\textsubscript{(SCA, SVIF)} plasmid was constructed (5) and kindly provided by Dr. Theodora Hatziioannou of the Aaron Diamond Research Center. The QuickChange II XL site-Directed mutagenesis kit (Stratagene) was used to generate desired mutations in the integrase region of stHIV-1 using the following primers: E92Q (sense: 5' - GCAGAAGTAATTCCAGCACAGACAGGGCAAGAAA - 3' and antisense: 5' - TTTCTTGCCCTGTCTGTGCTGGAATTACTTCTGC - 3'), E138K (sense: 5' - GCAGAAGTAATTCCAGCACAGACAGGGCAAGAAA - 3' and antisense: 5' - TTTCTTGCCCTGTCTGTGCTGGAATTACTTCTGC - 3').
GGCGGGGATCAAGCAGAAATTTGGCATTCCCTA-3' and antisense: 5'-
TAGGGAATGCGCAATTTCTGCTTGATCCCGCC-3'), G118R (sense: 5'-
CCAGTAAAAACAGTACATACAGAATCGCAGCAATTCACC-3' and antisense: 5'-
GGTGAAATTGCTGCGATTGTCTGTATTGTACTTTTACTGG-3'), N155H (sense: 5'-
AAAGTCAAGGGTAAATAGAATCTATGTCAAAAGAATTAAGAAATTATAGGAC-3'
and antisense: 5'-
GTCTATAATTTCTTTAATTCTTTATGCATAGATTCTATTACCCCCTGACTTT-3'),
R263K (sense: 5'-GTAGTGCCAAGAAAAAAAGCAAAGATCATCAGGG-3' and antisense:
5'-CCCTGATGATCTTTGCTTTTTTTCTTGGCACTAC-3'), S153Y (sense: 5'-
TTCTTTAATTCTTTATTCATATATTCTATTACTCCTTGACTTTGGGGATTGTAG-3' and
antisense: 5'-
CTACAATCCCCAAAGTCAAAGATGAAATATATGAAATTTAAAGAAATGTTTTAAGGGATTGTAG-3'),
and Y143R (sense: 5'-CAGGAATTTGGCATTCCCCGCAATCCCCAAAGTCAAGGG-3'
and antisense: 5'-CCCTGACTTTGSGGATTGCGGGGAATGCCAAATTCCTGACTTTGGAATTGTAG-3'). Following
mutagenesis, the plasmids were digested with Dpn I for 4 hr at 37°C and transformed using
Escherichia coli strain XL10-Gold ultracompetent cells, TetΔ(mcrA)183 Δ(mcrCB-hsdSMR-
mrr)173 endA1 supE44thi-1 recA1 gyrA96 relA1 lac [F'proAB lacF'ZAM15Tn10 (Tet') Amy
Cam'] (Stratagene). Plasmid purification was accomplished using the QIAprep MiniPrep Kit
(QIAGEN) and quantified using NanoDrop technology. The presence of the mutations in the
integrase region of stHIV-1(SCA,SVIF) was confirmed by sequencing.

**Generation of replication-competent stHIV-1**

The production of genetically homogeneous stHIV-1 wild-type (WT) or mutated viruses was
accomplished as previously described for pNL4.3 plasmids (n=2 for each virus) (21). Briefly,
12.5 µg of WT and mutated stHIV-1 proviral DNA were transfected into 293T cells using Lipofectamine 2000 (Invitrogen). Fresh medium was added 4 hr following transfection. Culture supernatants were harvested, centrifuged, and passed through a 0.45 µm filter at 48 hr post-transfection. The transfected stHIV-1 viruses were then stored at -80°C for future use. Since HIV-1 pol is conserved in stHIV-1, reverse transcriptase (RT) activity was measured as previously described (20).

**stHIV-1 infectiousness in TZM-bl cells**

Non-competitive short-term infectivity assays with TZM-bl cells (n=6) were used to determine the infectivity of the stHIV-1 wild-type and mutant viruses as previously described for HIV-1 (16). Briefly, 30,000 TZM-bl cells were seeded into 96-well culture plates (Corning) and infected with virus normalized on the basis of RT activity. The luciferase assay system (Promega) and a MicroBeta2 luminometer (PerkinElmer) were used to determine luciferase activity at 48 hr post-infection. The open-source statistics package OpenEpi (http://www.openepi.com/) was used to evaluate significance using the Student’s t-test. A significant difference was defined as p<0.001.

**Susceptibility of stHIV-1 to INSTIs in TZM-bl cells**

Short-term resistance assays with TZM-bl cells were used to determine stHIV-1 susceptibility to DTG, RAL, and EVG, as previously described for HIV-1 (n=9 for DTG, n=6 for RAL and for EVG) (16). In brief, 30,000 cells per well were infected with stHIV-1 WT virus or viruses containing the E92Q, G118R, E138K, Y143R, S153Y N155H, or R263K substitutions in the presence of serial dilutions of DTG, RAL, or EVG in 96-well plates (Corning). The amount of stHIV-1 WT or mutant virus added was normalized based on RT activity (40,000 cpm/well).
48 hr post-infection, luciferase activity was measured using the luciferase assay system (Promega) and a MicroBeta2 luminometer (PerkinElmer) and used to generate data on fold-changes in inhibitory concentrations (EC₅₀) using the sigmoid-dose response function of GraphPad Prism 5.0 software (GraphPad Software, Inc., San Diego, CA).

**Animal and Blood Collection**

Whole monkey blood (obtained from Primus Bio-Ressources Inc., Vaudreuil-Dorion, Québec) was collected from uninfected rhesus macaques (*Macaca mulatta*) and delivered in BD Vacutainer Heparin tubes (Becton, Dickinson and Company, BD). Peripheral blood mononuclear cells (PBMCs) were isolated from donor monkeys by Ficoll-Hypaque (GE Healthcare) gradient centrifugation from rhesus blood. Rhesus PBMCs were cultured as previously described (22).

**stHIV-1 replication capacity in rhesus macaque PBMCs**

Long-term infection assays were used to quantify stHIV-1 replication capacity in rhesus macaque PBMCs by measuring levels of RT activity (counts per minute (cpm)) over time. One million cells per well were added into a 48-well culture plate (Becton, Dickinson and Company, BD) in 1 mL of RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, and 20 U/ml interleukin 2 (IL-2). Cells were infected with the indicated amount of virus normalized on the basis of RT activity (40,000 cpm/well, n=3). Infection was quantified by RT activity as previously described (22).

**stHIV-1 replication capacity in human CBMCs**
CBMCs were subcultured as described for rhesus PBMCs. Long-term infection of CBMCs was similar to that described for rhesus PBMCs. Culture wells were refreshed with volumes of media equivalent to the volumes of supernatants that were removed. stHIV-1 replication capacity was measured by the quantification of RT activity in the cell culture fluids of CBMCs infected with stHIV-WT or mutant viruses over 21 days.

Results

Effect of integrase substitutions on stHIV-1(SCA,SVIF) infectivity

Single-cycle infection assays with TZM-bl cells were used to determine the infectivity of stHIV-1 wild-type (WT) and mutated viruses. The mutant viruses included each of stHIV-1(E92Q), stHIV-1(G118R), stHIV-1(E138K), stHIV-1(Y143R), stHIV-1(S153Y), stHIV-1(N155H), and stHIV-1(R263K). The introduction of the DRMs reduced the infectivity of all mutated viruses compared to WT (Figure 1). All differences were statistically significant (Student’s t-test, p<0.001). Levels of infectiousness were calculated and expressed relative to WT, which was arbitrarily set as 1 (Figure 2). Introduction of the N155H substitution had the smallest effect on stHIV-1 infectivity (3.3-fold decrease in infectiousness compared to WT), whereas R263K had the greatest effect (27.5-fold decrease in infectiousness). Introduction of the E92Q, G118R, E138K, Y143R, and S153Y DRMs resulted in an intermediate effect in regard to reductions in stHIV-1 infectiousness (7.4-, 15.4-, 4.8-, 7.7- and 11-fold, respectively).

Replication capacity of stHIV-1 mutant viruses in rhesus macaque PBMCs
To determine whether the defect in infectiousness of the mutant viruses resulted in an impairment of stHIV-1 replication capacity, we measured the ability of these viruses to grow in rhesus macaque PBMCs over 24 days (Figure 3). These experiments confirmed that all DRM viruses were impaired in replication capacity. Viruses bearing the G118R substitution were the most negatively affected. All substitutions that were tested significantly delayed early (<10 days) growth of stHIV-1. To exclude the possibility of cell type specific effects on viral growth, similar experiments were performed in human CBMCs (Figure 4). Similar to macaque PBMCs, all DRM viruses negatively impacted stHIV-1 replication capacity in CBMCs, and, as for macaque PBMCs, the greatest impact was observed with the G118R substitution. Altogether, long-term replication studies in human CBMCs confirm the results obtained with rhesus macaque PBMCs.

Susceptibility of stHIV-1 mutant viruses to INSTIs

Previous studies with HIV revealed that the insertion of DRMs into the integrase region of pNL4.3 viruses resulted in reduced susceptibility to currently available INSTIs. In order to compare levels of resistance between HIV and stHIV-1, we evaluated the replication capacity of mutated stHIV-1 viruses in the presence of increasing concentrations of RAL, EVG, or DTG using TZM-bl cells. Resistance to RAL was most prominent with the viruses containing E92Q, N155H, and Y143R displaying increases in EC$_{50}$ of 11.5-, 14.7-, and 35.9-fold, respectively, compared to WT (Table 1). Viruses containing G118R conferred low-level resistance to RAL (5.8-fold), whereas the E138K, R263K, and S153Y viruses did not significantly confer resistance to this drug (0.41-, 0.83-, and 1.59-fold, respectively). All mutated stHIV-1 viruses conferred moderate-to-high levels of resistance to EVG, ranging from 16.6-fold for E138K to > 300-fold for E92Q. With respect to DTG, the mutated viruses were associated with low levels of
resistance, with viruses containing G118R conferring the highest level of resistance (6.4-fold).

The R263K substitution did not confer significant levels of resistance against RAL, but did confer low- and moderate-level resistance to DTG (2.5-fold) and EVG (32.6-fold), respectively.

**Discussion**

In an attempt to establish a model to study the effects of DRMs against INSTIs on viral replicative capacity and resistance, we introduced relevant resistance-associated substitutions into stHIV-1$_{(SCA,SVIF)}$. We had earlier performed similar studies with SIVmac239 and showed that SIV and HIV-1 share similar resistance profiles (22). The present study shows that stHIV-1 and HIV-1 also share similarities in regard to the impact of DRMs on resistance against INSTIs and on viral replicative capacity. Our study is important because it expands the number of viral models that might be used to assess retroviral pathogenesis and antiviral effects in animal systems.

Our results show that stHIV-1 and HIV-1 share similar resistance pathway profiles (Table 1). As an example, the E92Q substitution, which has been shown to be prevalent in patients failing EVG-based therapy (23-28), was associated with the highest level of resistance against this drug when introduced into stHIV-1. In agreement with our studies on the role of the G118R substitution in HIV-1 (29), this substitution was the only one that conferred moderate-level resistance in stHIV-1 against DTG (FC=6.4-fold). This is in agreement with observations on the robustness of this drug against most resistance substitutions (7, 30). Similarly, the R263K substitution in integrase resulted in low-level resistance against DTG in stHIV-1 (Table 1) (16, 31). In contrast, introduction of the E138K substitution into stHIV-1 conferred 16.6-fold resistance against EVG whereas this substitution had been shown to be innocuous against this
drug in HIV-1 (18, 32). The resistance levels observed for stHIV-1 and HIV-1 were different for
only some drugs. In particular, S153Y, which was observed during tissue culture selection
experiments with DTG (16, 18) displayed similar levels of resistance against DTG in stHIV-1
and HIV, i.e. fold-changes of 2.2 and 2.5-fold, respectively (Table 1), whereas S153Y conferred
high-level resistance against EVG in stHIV-1 (22.8-fold) and low-level resistance against this
same drug in HIV-1 (4- to 8-fold) (33, 34). Methodological differences may explain observed
disparities in resistance. However, given the similarities between stHIV(SCA,SVIF) and HIV-1, one
cannot exclude that sequence variability in capsid (CA) coding regions may also contribute to the
observed disparities. Although integrase and CA are not known to directly interact, the latter
protein has been shown to contribute to the nuclear import of viral DNA (reviewed in (35)) and
may thus be indirectly important for integrase catalytic activity and integration. Since integrase
also contributes to the nuclear import of the viral genome, viruses with integrase proteins that are
deficient for DNA binding, such as R263K (16), may rely more on CA for nuclear import than
viruses with WT integrase sequences.

The emergence of DRMs in vivo is contingent on the cost on viral replication capacity imparted
by these substitutions (37). One example is the sequential emergence of the M184I and M184V
resistance substitutions in individuals failing 3TC-based therapy (20, 38). Recently, our results
suggested that the high replicative cost associated with the DTG-specific R263K resistance
pathway could help to explain the rarity of DTG-resistance substitutions in treatment-naïve
individuals (11, 21, 30, 31). Therefore, we studied the impact of DRMs against INSTIs on viral
infectiousness and replication capacity (Figure 3). Our results show that stHIV-1 containing
R263K reproduced the decrease in HIV-1 replicative capacity that has been reported with
R263K-containing HIV-1. In particular, the G118R and R263K DTG-specific substitutions were
the most detrimental to stHIV-1 viral infectivity (Figure 1). S153Y was also associated with a high degree of impairment of viral replication. RAL and EVG-specific DRMs, i.e. E92Q, E138K, Y143R and N155H, were less detrimental to stHIV-1 replication capacity than were R263K and G118R and N155H was the least disadvantageous for viral infectiousness.

The HIV-based chimeric virus, stHIV-1, was derived by replacing the capsid and vif regions of HIV-1 by the corresponding regions of SIV and can infect both human primary cells and cell lines (5). We have now further investigated this by examining the impact of DRMs against INSTIs on the replication capacity of stHIV in human CBMCs. We found that the replication profiles of stHIV in CBMCs are similar to those seen in rhesus PBMCs (Figure 3, 4). The G118R-DTG specific mutation was the most detrimental to stHIV-1 viral infectivity in CBMCs (Figure 4), in agreement with what has been reported for G118R-containing HIV-1 and SIV (15, 22). stHIV-1 viruses bearing the Y143R mutation also displayed reduced replication capacity as seen in HIV-1 and SIV (22, 39, 40). Similar to HIV-1 (32), E138K modestly diminished stHIV replication in rhesus PBMC and human CBMCs (Figure 3, 4) and E92Q and N155H HIV-1 bearing-viruses were modestly impaired in replication capacity. Importantly, the correlation between results obtained with human CBMCs and macaque PBMCs suggests that potential reversions in the integrase region of the stHIV-1 variants did not influence the outcome of these experiments.

Together, this study demonstrates that DRMs in stHIV-1 resemble HIV-1 resistance substitutions in regard to diminished drug efficacy and viral replication defects associated with these substitutions. This suggests that stHIV-1 can be used to study the impact of DRMs against INSTIs on viral replicative capacity and expands the number of viral systems that can be used in animals to study the persistence, transmissibility and pathogenicity of INSTI-resistant viruses.
Hopefully, our findings will have relevance for the study of stHIV variants that display resistance against other classes of ARVs as well.

Acknowledgements

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**Figure legends**

**Figure 1.** Effects of integrase substitutions on stHIV-1(SCA,VIF) infectivity in TZM-bl cells. StHIV-1 infectiousness was measured by quantifying luciferase activity in terms of relative luminescent units (RLU) produced by TZM-bl cells at 48h after infection with increasing concentrations of virus as measured by RT activity (X-axis). Means and standard error of the means (SEM) are presented. Lines are fits.

**Figure 2.** Impact of integrase substitutions on drug susceptibility. Relative half maximal effective virus concentrations (EC_{50}s) were calculated and expressed relative to WT, which was arbitrarily set as 1. * indicates significant difference with WT (Student’s t-test, p<0.001).

**Figure 3.** Impact of integrase substitutions on stHIV-1 replication in rhesus macaque PBMCs. Replication was measured on the basis of RT activity in counts per minute (cpm) released by rhesus PBMCs into culture fluids following infection with stHIV-1 viruses. Error bars indicate means and SEM.

**Figure 4.** Impact of integrase substitutions on stHIV-1 replication in human CBMCs. Reverse
transcriptase (RT) activity was measured as counts per minute (cpm) in the culture fluids of CBMCs infected with stHIV viruses.

Tables

Table 1. Effects of drug resistance substitutions on EC\textsubscript{50} for dolutegravir (DTG), raltegravir (RAL), and elvitegravir (EVG).
Table 1. Effects of drug resistance substitutions on EC₅₀s for dolutegravir (DTG), raltegravir (RAL), and elvitegravir (EVG).

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<th>Genotype (stHIV-1)</th>
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<th>EVG Fold-change EC₅₀</th>
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From *Kobayashi, AAC, 2010; **Anstett, submitted for publication; ***Bar-Magen et al, JVI, 2010 and unpublished observations; ****Mesplede et al., JAC, 2014
Figure 1

- Left panels: Infectivity (RLU) vs. logRT (cpm) for stHIV<sub>WT</sub> and stHIV<sub>E92Q</sub>, stHIV<sub>WT</sub> and stHIV<sub>E138K</sub>, stHIV<sub>WT</sub> and stHIV<sub>S153Y</sub>, and stHIV<sub>WT</sub> and stHIV<sub>R263K</sub>.
- Right panels: Infectivity (RLU) vs. logRT (cpm) for stHIV<sub>WT</sub> and stHIV<sub>G118R</sub>, stHIV<sub>WT</sub> and stHIV<sub>Y143R</sub>, stHIV<sub>WT</sub> and stHIV<sub>N155H</sub>.
Figure 2

The figure shows a bar graph with the x-axis labeled as 'WT', 'E92Q', 'G118R', 'E138K', 'Y143R', 'S153Y', 'N155H', and 'R263K'. The y-axis is labeled 'Relative EC50' with values ranging from 0 to 30.

Each bar represents the relative EC50 for the respective mutation, with asterisks (*') indicating significant differences. The bar for WT is the lowest, followed by a gradual increase in EC50 values for the other mutations, with R263K showing the highest EC50 value.
Figure 4