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Comparative replication capacity of raltegravir-resistant strains and antiviral activity of the new-generation integrase inhibitor dolutegravir in human primary macrophages and lymphocytes

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Objectives: To evaluate the replication capacity and phenotypic susceptibility to dolutegravir and raltegravir of wild-type and raltegravir-resistant HIV-1 strains in several cellular systems.

Methods: The antiviral activities of dolutegravir and raltegravir were evaluated in human primary monocyte-derived macrophages (MDMs), peripheral blood mononuclear cells (PBMCs) and C8166 T lymphocytic cells. The following raltegravir resistance mutations were analysed: N155H, Y143C, N155H+Y143C and G140S+Q148H.

Results: In the absence of drug, the replication capacity of raltegravir-resistant viruses was strongly reduced compared with wild-type in all cellular models analysed. In MDMs and PBMCs, a dramatic decrease in viral replication was observed for the double mutants N155H+Y143C and G140S+Q148H (ranging from 0.1% to 2.5% compared with wild-type). In MDMs, dolutegravir exhibited high potency, with EC_{50} and EC_{90} values of 1.1 ± 0.9 and 5.5 ± 3.4 nM, respectively (comparable to raltegravir). These values (particularly for EC_{90}) were significantly lower than those observed in PBMCs (EC_{50}: 2.7 ± 1.5 nM; EC_{90}: 14.8 ± 0.9 nM) and C8166 cells (EC_{50}: 5.5 ± 0.8 nM; EC_{90}: 64.8 ± 5.8 nM). In all cellular models analysed, dolutegravir showed full efficacy against N155H and Y143C mutants (dolutegravir fold-change resistance ranging from 0.1 to 1.4; raltegravir fold-change resistance ranging from 0.1 to 10.3). In C8166 (the only cell model in which replication capacity was sufficient to perform the test) dolutegravir showed full efficacy against mutations N155H+Y143C (dolutegravir fold-change resistance: 0.6) and a slightly lower activity against G140S+Q148H (dolutegravir fold-change resistance: 2.1).

Conclusions: Dolutegravir is effective in different HIV cellular targets and against raltegravir-resistant mutants. The high efficacy of dolutegravir in MDMs (cells with limited metabolism) has relevant clinical implications in light of the role of MDMs in the transmission of HIV infection and dissemination in different body compartments.

Keywords: HIV infection, primary cells, inhibition of viral replication

Introduction

One of the most exciting advances in HIV-1 pharmacotherapy was the approval by the US FDA in October 2007 of the first integrase inhibitor, the pyrimidine-carboxamide raltegravir, with high potency and tolerability.[1–7] Two further integrase strand transfer inhibitors, elvitegravir (GS-9137) and dolutegravir (S/GSK1349572), were recently approved for clinical use in August 2012 and August 2013, respectively.[8–11] Raltegravir, elvitegravir and dolutegravir, although structurally diverse, bind to a common D64-D116-E152 motif in the integrase catalytic domain (amino acids 51–212), causing it to disengage from the viral DNA. Resistance to raltegravir arises from three major mutation sites, at positions 155 (N155S/H), 143 (Y143C/R) and 140+148 (G140S+Q148 K/R/H double mutant).[12–15] Additionally, specific secondary mutations (at positions 66, 74, 92, 97 and 138) are also commonly associated with each primary mutation. These mutations serve to significantly enhance resistance and/or viral replication capacity and may reverse fitness defects caused by primary mutations.[16,17] There is extensive cross-resistance between raltegravir and

elvitegravir,[18] which precludes their sequential use. In particular, the double mutation G140S+Q148H confers a high degree of resistance to both raltegravir and elvitegravir.[12,19]

Fortunately, dolutegravir partially overcomes these mutations[20] and could therefore be used as salvage therapy for raltegravir- or elvitegravir-resistant viruses. Knowledge about dolutegravir resistance mutations derives mostly from *in vitro* work in cell lines exposed for months to suboptimal concentrations of the drug.[20–26] Marginal information is available *in vivo* and none has been reported so far *in vivo* that supports the higher genetic barrier for dolutegravir compared with raltegravir. It is well known that CD4+ T cells and monocyte-derived macrophages (MDMs) are the primary target cells for HIV *in vivo*, and antiretroviral drugs can vary in their ability to inhibit the infection of these different cell types.[27] Fundamental differences in cellular composition, metabolism and viral replication kinetics can lead to differences in the efficacy of antiviral drugs in T cells and MDMs.[28] It has long been known that nucleoside analogue reverse transcriptase inhibitors typically work more effectively in MDMs than in T cells,[29] whereas protease inhibitors are less effective in chronically infected MDMs than in T cells.[30,31] Data on the efficacy of integrase inhibitors in MDMs is limited, but crucial in order to improve therapeutic approaches against HIV infection.

For this reason, the aim of our work was: (i) to analyse the replication capacity of HIV-1 wild-type and raltegravir-resistant strains; and (ii) to compare the activity of dolutegravir and raltegravir in both MDMs and lymphocytes.

Materials and methods

Cells

Human T lymphocytic C8166 cells were obtained from ATCC (Manassas, VA, USA). Human primary peripheral blood mononuclear cell (PBMCs) and MDMs were prepared and purified as described in published procedures.[30–32]

Viruses

The CCR5-using HIV-1$_{p81.A}$ plasmid (p81A), kindly provided by Dr A. Cara (Istituto Superiore di Sanità, Roma), was used in all the experiments involving primary cells (PBMCs and MDMs). The characteristics and genomic sequence of this strain have previously been described.[33] The CXCR4-using HIV-1$_{HXB2}$ was used to infect C8166 T cells. It was obtained from an acutely infected H9 CD4+ T lymphocyte cell line. Cell-free virus present in the supernatants was collected, ultracentrifuged, filtered (0.22 μM) and stored at −80°C. Raltegravir-resistant viruses, containing the mutations N155H, Y143R, Y143C, N155H+Y143C and G140S+Q148H, were generated by site-directed mutagenesis (using HXB2 backbone for T cells and p81.A for MDMs and PBMCs; QuikChange II XL Site-Directed Mutagenesis Kit, Agilent) using the manufacturer's procedure. Plasmid DNA was isolated using the QIAprep Spin Midiprep Kit (Qiagen).

Cell culture and transfection

HEK-293T cells were grown in 6-well plates in a 37°C humidified atmosphere containing 5% CO_2, using Dulbecco's modified Eagle's medium (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA). The expression vectors were transfected using the FuGENE 6 transfection reagent (Roche) according to the manufacturer's specifications. At 3 days post-transfection the viruses were harvested, filtered and the aliquots stored at −80°C.

Compounds

Dolutegravir, synthesized under good manufacturing practice conditions, was obtained from GlaxoSmithKline. Raltegravir, synthesized under good manufacturing practice conditions, was obtained from Merck & Co. As a drug control, the non-nucleoside reverse transcriptase inhibitor efavirenz was obtained from the NIH, USA.

HIV-1 infection

For the analysis of drug activity, all the cells analysed (MDMs, PBMCs and C8166 cells) were pre-treated with several doses of raltegravir and dolutegravir for 20 min, and then challenged with viral stock at a concentration of 10000 pg/mL HIV-1 p24. In accordance with our previous paper[30] the raltegravir doses used were: 0.1, 1, 10, 100, 1000 and 10000 nM. The dolutegravir doses used were 0.1, 1, 10, 100 and 1000 nM. As control, efavirenz was used at a concentration of 1 μM, known to be active against HIV-1 replication.[34] After 2 h of incubation the cells were extensively washed to remove the excess virus, and then complete medium containing the same concentration of drugs was added, where required.

Evaluation of viral replication

The viral replication of raltegravir-resistant strains in MDMs and PBMCs was compared with wild-type strains by measuring HIV-1 gag-p24 production (HIV antigen ELISA kit, Bio-Rad, France). In C8166 T cells, used as the cellular control of our system, the cytopathic effect was also analysed by optical microscopy at day 5 and 7 post-infection. The experiments were performed in triplicate in cells from at least two donors.

Evaluation of antiviral activity

The antiviral activity of dolutegravir, raltegravir and efavirenz in infected C8166 T cells was assessed by analysing the cytopathic effect at days 5 and 7 after HIV infection. The EC_{50} and EC_{90} were calculated on the basis of the effective drug concentrations that inhibited 50% and 90% of syncytia formation and cellular aggregation, respectively. Syncytia were evaluated by two independent observers using a semi-quantitative scoring system.[30] The experiments were performed at least twice in sextuplicate.

The activity of dolutegravir and raltegravir against wild-type and raltegravir-resistant viruses was also tested on MDMs and PBMCs, and was expressed as EC_{50} and EC_{90} (effective drug concentrations that inhibited 50% and 90% of HIV p24 production). Supernatants of infected PBMCs (collected at 6 days) and infected MDMs (collected at 14 and 21 days) were assessed to determine virus production in the presence or absence of drugs, by measuring HIV-1 gag-p24 production using a commercially available HIV antigen ELISA kit (Bio-Rad, France). The experiments were performed in triplicate in at least two donors. The geometric mean of p24 production in replicates of each experiment was used to determine the EC_{50} and EC_{90}, by linear regression of the log of the percentage HIV-1 p24 production (compared with untreated controls) versus the log of the drug concentration. Student's *t*-test was used to assess statistically significant differences in EC_{50} and EC_{90} values among the different cellular systems analysed. The reduction in drug susceptibility in the presence of the mutants was expressed as fold-change (FC) resistance. In particular, using the HXB2 or p81A HIV-1 wild-type virus as reference, FC resistance values were calculated by dividing the mean EC_{50} for a recombinant virus by that of the HXB2 or p81A reference strain. The value of this ratio is commonly referred to as the viral susceptibility to a drug. FC>1 indicates that the virus is less susceptible than the reference virus, whereas FC<1 indicates that the virus is more susceptible.

Drug toxicity

Drug toxicity was assessed in the absence of viral infection and in the presence of all the drugs used, as previously described.[30]

Ethics

Ethics approval was not necessary since this study did not involve patients.

Results

Evaluation of raltegravir-resistant virus replication in C8166 cells, PBMCs and MDMs

The replication capacity of raltegravir-resistant viruses was assessed in the absence of drugs in human primary PBMCs and MDMs, as well as C8166 T cells (used as a cell model to set some experimental conditions for the infection). The following raltegravir-resistant viruses were analysed: N155H, Y143C, N155H+Y143C and G140S+Q148H.

In C8166 cells, raltegravir-resistant viruses showed a reduced mean replication capacity, ranging from 53.5% for Y143C to 32.4% for N155H+Y143C (considering wild-type as 100%; Figure 1a).

The impairment in viral replication capacity was observed also in primary PBMCs (Figure 2a) and MDMs (Figure 3a), despite the variability in viral replication due to the use of primary cells obtained from different donors (as already detailed in the literature).[35–38] Indeed, the replication capacity of N155H virus (compared with wild-type) was $57.0 \pm 26.9\%$ and $45.9 \pm 14.4\%$ in PBMCs (Figure 2a) and MDMs (Figure 3a), respectively, while that of Y143C virus was $58.4 \pm 24.6\%$ and $32.7 \pm 10.8\%$, respectively. The decrease in viral replication capacity was even more dramatic for the double mutants (N155H+Y143C and G140S+Q148H), which showed a replication capacity ranging from 0.1% to 2.5% (Figures 2a and 3a). Taken together, the marked replicative defect of N155H and Q148H supports their rapid disappearance as major variants after raltegravir withdrawal *in vivo*.

Comparative activity of dolutegravir and raltegravir in C8166 cells, PBMCs and MDMs against wild-type and raltegravir-resistant viruses

The antiviral activity of dolutegravir and raltegravir was then analysed in PBMCs, MDMs and C8166 cells. For these experiments we used the wild-type viruses as well as the raltegravir-resistant strains.

In MDMs, dolutegravir exhibited high potency, with EC_{50} and EC_{90} values of 1.1 ± 0.9 and 5.5 ± 3.4 nM, respectively. These values were significantly lower than those observed in PBMCs
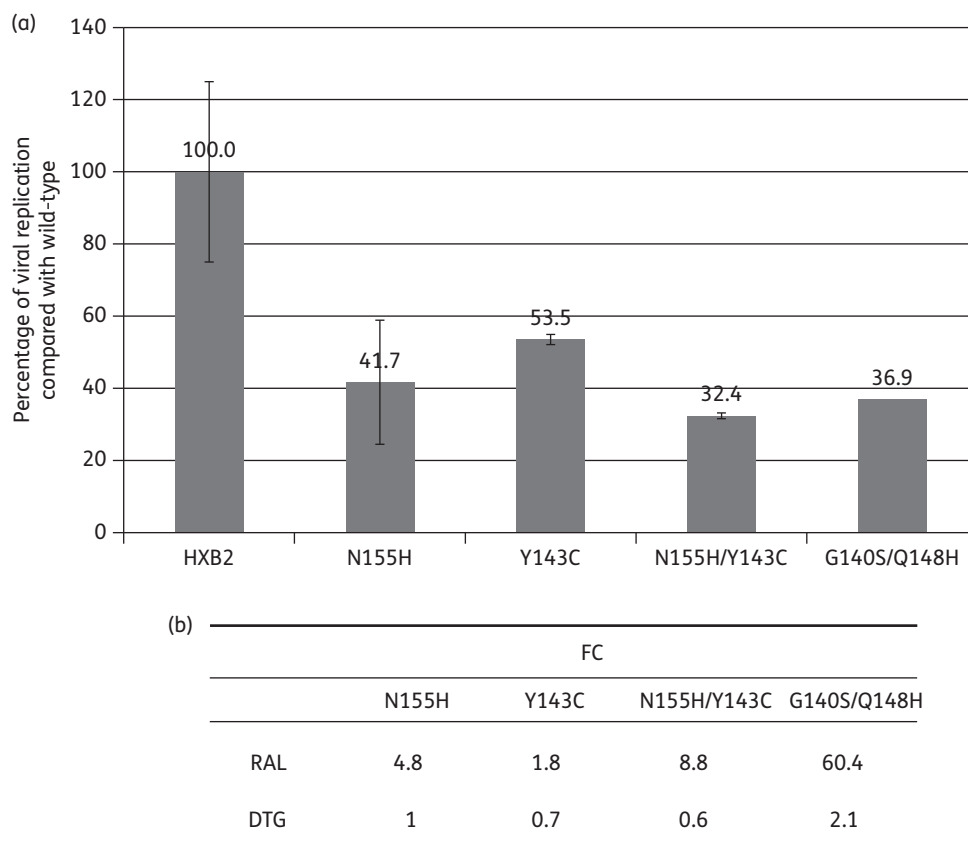
Figure 1. (a) Replication capacity of raltegravir-resistant N155H, Y143C, N155H+Y143C and G140S+Q148H viruses after 5 days of infection in C8166 T cells. All the values obtained were compared with HXB2 virus (100%) corresponding to an HIV p24 production of 284 554 pg/mL for HXB2 in C8166 cells. The replication capacity of N155H was $41.7 \pm 17.2\%$ and that of Y143C was $53.5 \pm 1.4\%$. Regarding the double mutants, the replication capacity of N155H+Y143C was $32.4 \pm 0.8\%$ and that of G140S+Q148H was $36.9 \pm 0.0\%$. The experiments were performed at least twice in sextuplicate. The results are presented as mean values with standard deviations. (b) The table reports the FC values in C8166 cells, calculated by dividing the mean EC_{50} for a recombinant virus by that for the HXB2 reference strain. RAL, raltegravir; DTG, dolutegravir.
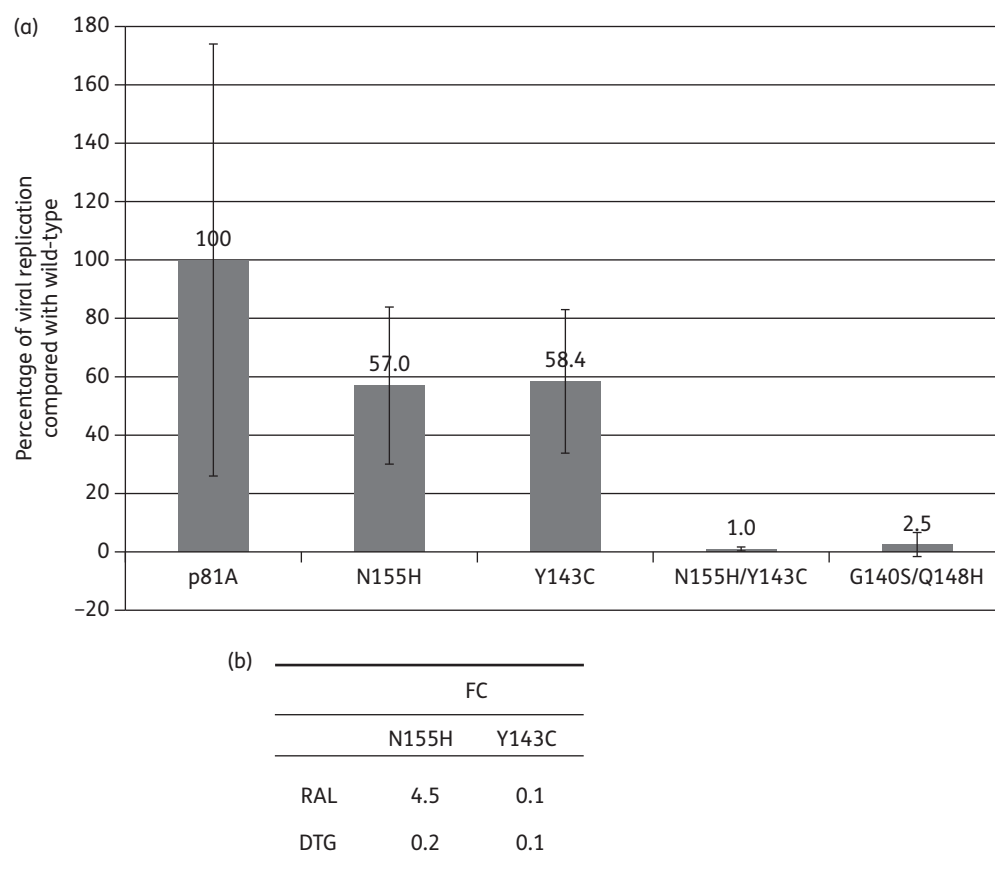
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Figure 2. (a) Replication capacity of raltegravir-resistant N155H, Y143C, N155H+Y143C and G140S+Q148H viruses after 6 days of infection in PBMCs. All the values obtained were compared with p81A virus (100%) corresponding to an HIV p24 production of 169 651 pg/mL for p81A in PBMCs. The replication capacity of N155H was $57.7 \pm 26.9\%$ and that of Y143C was $58.4 \pm 24.6\%$. Regarding the double mutants, the replication capacity of N155H+Y143C was $1.0 \pm 0.7\%$ and that of G140S+Q148H was $2.5 \pm 4.1\%$. The experiments were performed in triplicate in at least two donors. The results are presented as mean values with standard deviations. (b) The table reports the FC values in PBMCs, calculated by dividing the mean EC_{50} for a recombinant virus by that for the p81A reference strain. RAL, raltegravir; DTG, dolutegravir.

and C8166 cells (Table 1). In the case of the EC_{90}, we observed 11.8-fold and 2.7-fold decreases compared with C8166 cells and PBMCs (P values: 0.0005 and 0.016), respectively. In the case of the EC_{50}, we observed a 5-fold decrease compared with C8166 cells (P value: 0.002).

The antiviral activity of dolutegravir was also compared with raltegravir. In MDMs there was a trend toward a lower EC_{50} and EC_{90} of dolutegravir against wild-type viruses compared with raltegravir (although not reaching statistical significance; Table 1).

The second step of our study was to evaluate the EC_{50} and EC_{90} of dolutegravir in different cellular systems against raltegravir-resistant strains.

In MDMs (Figure 3b) and PBMCs (Figure 2b), dolutegravir showed full efficacy against resistant strains N155H and Y143C, with FC values ranging from 1.4 to 0.1, indicating a dolutegravir activity similar or even higher than that observed against wild-type viruses. In C8166 cells, in which both the single and double mutants replicate, dolutegravir showed full efficacy against the resistant strains with mutations N155H, Y143C and N155H+Y143C (dolutegravir FC ranging from 0.6 to 1; raltegravir FC ranging from 1.8 to 8.8). A slight decrease in dolutegravir susceptibility was observed against the

double mutant G140S+Q148H (dolutegravir FC 2.1; raltegravir FC 60.4; Figure 1b).

In the majority of cases the FC for dolutegravir against raltegravir-resistant strains was <1, indicating an EC_{50} lower for raltegravir-resistant strains than for wild-type.

Overall, our results showed that dolutegravir is fully effective against raltegravir-resistant viruses with mutations at positions 155 and 143, in all cell types tested.

Drug toxicity

Treatment of MDMs, C8166 T cells and PBMCs with concentrations of dolutegravir up to 10 000 nM showed no decrease in cell number, thus suggesting the absence of toxicity at all tested concentrations. Thus, the antiviral activity observed in our experiments can be attributed only to the drug inhibitory effect and not to alteration of cellular metabolism or cellular death.

Discussion

Our study showed: (i) dramatic impairment of replication capacity of raltegravir-resistant strains in both PBMCs and MDMs; (ii) strong
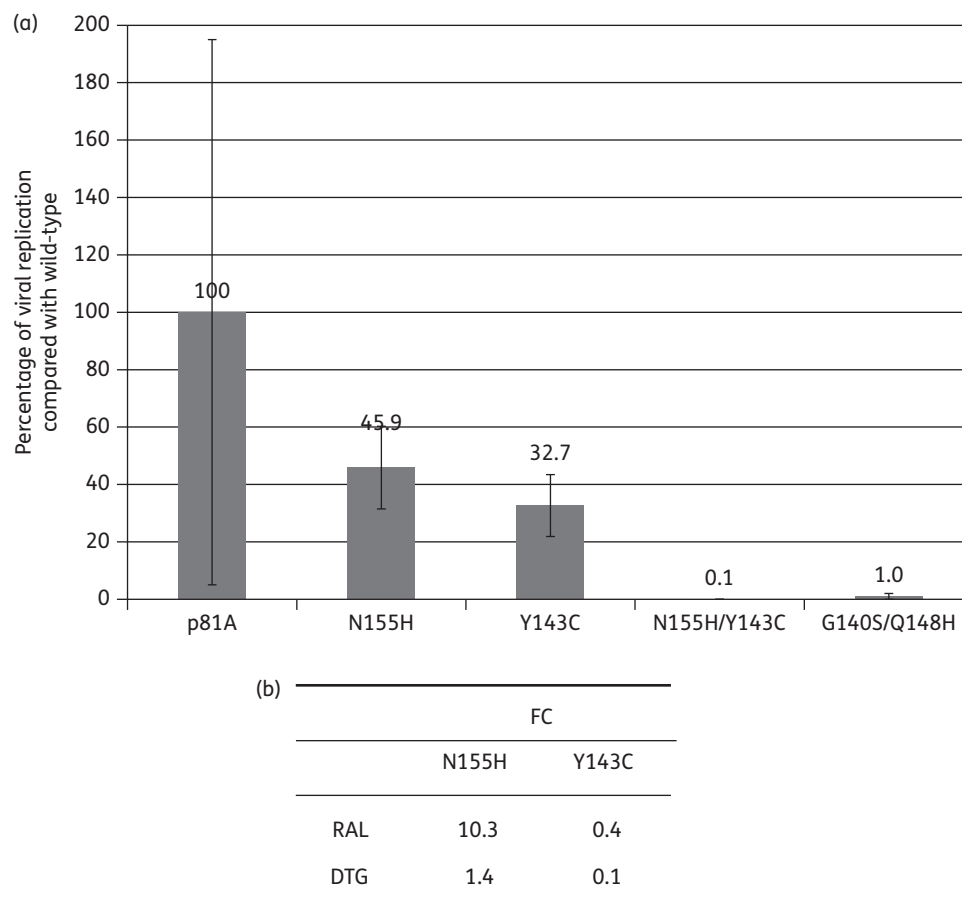
Figure 3. (a) Replication capacity of raltegravir-resistant N155H, Y143C, N155H+Y143C and G140S+Q148H viruses after 14 days of infection in MDMs. All the values obtained were compared with p81A virus (100%) corresponding to an HIV p24 production of 22 495 pg/mL for p81A in MDMs. The replication capacity of N155H was $45.9 \pm 14.4\%$ and that of Y143C was $32.7 \pm 10.8\%$. Regarding the double mutants, the replication capacity of N155H+Y143C was $0.1 \pm 0.0\%$ and that of G140S+Q148H was $1.0 \pm 0.9\%$. The experiments were performed in triplicate in at least two donors. The results are presented as mean values with standard deviations. (b) The table reports the FC values in MDMs, calculated by dividing the mean EC_{50} for a recombinant virus by that for the p81A reference strain. RAL, raltegravir; DTG, dolutegravir.

Table 1. Comparative anti-HIV activity of dolutegravir and raltegravir in different cellular models

	C8166 T cells (day 5)		PBMCs (day 6)		MDMs (day 14)	
	EC_{50} (nM)	EC_{90} (nM)	EC_{50} (nM)	EC_{90} (nM)	EC_{50} (nM)	EC_{90} (nM)
Dolutegravir	5.5 ± 0.8	64.8 ± 5.8	2.7 ± 1.5	14.8 ± 0.9	1.1 ± 0.9	5.5 ± 3.4
Raltegravir	7.7 ± 0.5	66.3 ± 7.4	3.5 ± 0.6	17.8 ± 12.1	2.1 ± 1.4	8.4 ± 1.5

EC_{50}, effective drug concentration required to inhibit syncytia formation in HIV-1 HXB2-infected C8166 T cells by 50% or to inhibit p24 production in HIV-1 p81A-infected PBMCs or MDMs by 50%.
EC_{90}, effective drug concentration required to inhibit syncytia formation in HIV-1 HXB2-infected C8166 T cells by 90% or to inhibit p24 production in HIV-1 p81A-infected PBMCs or MDMs by 90%.

efficacy of dolutegravir in PBMCs and even more in MDMs; and (iii) full efficacy of dolutegravir against raltegravir-resistant strains in both PBMCs and in MDMs.

The decreased replication capacity of raltegravir-resistant strains is in line with previous *in vitro* studies based on

pseudoviruses or recombinant viruses,[39] and with clinical studies showing the rapid disappearance of raltegravir-resistant mutants after raltegravir withdrawal *in vivo*.[40]

The impaired replication capacity of raltegravir-resistant viruses suggests that PBMCs and MDMs may act as reservoirs

mainly for wild-type viruses compared with drug-resistant viruses. This concept is in line with a recent study showing (using 454 ultra-deep pyrosequencing) that plasma and PBMCs hosted drastically different HIV populations even after prolonged exposure to raltegravir selection pressure. In particular, the authors found that the intra-patient prevalence of raltegravir resistance mutations ranged from 78% to 100% in plasma and from 0% to 36% in PBMCs.[41]

In our study, we also found that the EC_{50} and EC_{90} of dolutegravir seem to be lower than those (already low) observed for raltegravir. This is in line with the results obtained in the SAILING study,[42] showing that once-daily dolutegravir has a greater virological effect than twice-daily raltegravir in treatment-experienced patients (71% of patients on dolutegravir versus 64% of patients on raltegravir with undetectable viraemia).

Our study also showed high efficacy for dolutegravir in MDMs. This observation has relevant clinical implications, in light of the role of MDMs in the dissemination and transmission of HIV infection. In particular, it has been amply demonstrated that MDMs cause several pathological abnormalities in the CNS, which can have a clinical appearance in AIDS dementia complex. HIV-1-infected MDMs can release compounds toxic for neurons and astrocytes, which are not directly infected by the virus but die from HIV-1-mediated effects.

Recently, the distribution and antiviral activity of dolutegravir in CSF was investigated, and it was observed that dolutegravir is able to achieve an optimal therapeutic concentration in CSF. Thus, the ability of dolutegravir to enter the CNS, coupled with its high efficacy in MDMs, supports its use in preventing the onset of neurological disorders and AIDS dementia complex.

In addition, due to the pivotal role of MDMs in the dissemination of HIV infection after transmission, the full efficacy of dolutegravir in MDMs provides the rationale for testing the use of this drug in HIV post-exposure prophylaxis regimens, as recently proposed for raltegravir.[43]

The high efficacy of dolutegravir in MDMs (observed also for raltegravir in our previous paper)[30] could be related to the peculiar characteristics of HIV-1 replication in MDMs, which is quite different from that observed in CD4+ T lymphocytes.[44-49] Indeed, MDMs are resting, terminally differentiated cells that undergo replication only in case of very particular conditions and situations.[50] This makes the entire integration phenomenon more difficult, as it usually occurs during the replication cycle of cells.[51] Therefore, it is conceivable that HIV integration, occurring more slowly in MDMs owing to the lower cycle metabolism, should be more easily perturbed by concentrations of integrase inhibitors (dolutegravir or raltegravir) even lower than those effective in replicating cells.[51]

Finally, our study showed full efficacy of dolutegravir against raltegravir-resistant strains with the mutations N155H and Y143C, in both PBMCs and MDMs.

These results provide the biological explanation for the data obtained in clinical Phase IIb studies regarding the ability of dolutegravir (administered twice daily) to be effective in patients with raltegravir resistance mutations at positions 143 and 155.[52,53] The slight reduction in dolutegravir activity against the double mutant G140S+Q148H supports the clinical observation showing a decreased percentage of patients achieving virological success when mutations at position 148 are present at baseline.[54]

This observation entails the necessity of performing genotypic testing in raltegravir-failed patients to analyse the presence of mutations that have different susceptibility to antiviral therapy.

Conclusion

A measurable advantage in the replication capacity of wild-type virus compared with raltegravir-resistant strains in all tested cell systems suggests that MDMs and PBMCs might act as reservoirs more for wild-type virus than for resistant/low-fitness virus. Dolutegravir efficiently reduces HIV-1 replication in MDMs, PBMCs and C8166 cells, with the potential to be effective in different HIV cellular targets, and against raltegravir-resistant strains harbouring the Y143 and N155 mutations.

Transparency declarations

F. C.-S. has received funds for attending symposia, speaking, organizing educational activities, grant research support, consultancy and advisory board membership from Abbott, Merck Sharp & Dohme, Gilead, Janssen-Cilag, Roche, Bristol-Myers Squibb and ViiV. C. F. P. has received funds for attending symposia, speaking, organizing educational activities, grant research support, consultancy and advisory board membership from Abbott, Boehringer Ingelheim, Bristol-Myers Squibb, Gilead, Merck Sharp & Dohme, Janssen-Cilag, Pfizer, Tibotec, Roche and ViiV. V. S. has received funds for attending symposia, speaking and organizing educational activities from ViiV, Merck and Gilead, has received grant research support from Bristol-Myers Squibb and has received funds for attending an advisory board meeting from ViiV. All other authors: none to declare.

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