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ANTIVIRAL AGENTS

Barrier to Resistance of Dolutegravir in Two-Drug Combinations

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ABSTRACT A major concern when using two-drug anti-HIV regimens is the risk of viral resistance. However, no techniques to evaluate the barrier to resistance of two-drug combinations *in vitro* have been reported. We evaluated the emergence of drug-resistant mutants in a passage study with constant concentrations of two drugs simultaneously. The barrier to resistance of dolutegravir-containing two-drug combinations was higher than the other combinations evaluated in this study.

KEYWORDS barrier to resistance, dolutegravir, human immunodeficiency virus, two-drug regimen

Current antiretroviral regimens consist of one key drug and two nucleos(t)ide reverse transcriptase inhibitors [N(t)RTIs] (1). However, two-drug regimens with similar efficacy and durability to the standard 3-drug regimens would be preferable, as this approach would lessen problems, such as high cost, drug-drug interactions, and long-term side effects, and would reserve a class of drugs for a future treatment option (2). In a study of anti-retroviral therapy (ART)-naive patients, raltegravir (RAL)-ritonavir-boosted darunavir (DRV/r) was inferior to the standard 3-drug regimen in patients with low CD4 counts and high viral loads, and a high emergence of RAL-resistant mutants was seen (3). In another study, patients who switched to ritonavir-boosted atazanavir (ATV/r) and RAL had a higher rate of virologic rebound than those given ATV/r and tenofovir disoproxil fumarate-emtricitabine (TDF-FTC) (4). Therefore, it is important to evaluate the barrier to resistance of two-drug combinations *in vitro* to assess the occurrence of drug-resistant mutants and to support the clinical use of such combinations.

Currently, no methods have been established to evaluate the barrier to resistance of a two-drug combination. Usually, *in vitro* passage studies are used to isolate drug-resistant viruses but are conducted using only one drug. The *in vitro* two-drug combination evaluation with checkerboard method enables researchers to determine whether two drugs act synergistically, additively, or antagonistically, but it cannot provide information on viral resistance (5). In this study, we describe a quantitative method to compare the barrier to resistance of a two-drug combination with that of a single drug or other combinations *in vitro*. Here, we define the barrier to resistance based on the drug concentrations at which drug-resistant mutants emerge. This is a comparative ranking, and if a drug permits the emergence of resistant mutants up to a certain fold of its 50% effective concentration (EC_{50}), but the comparison drug or combination of two drugs does not permit the emergence of drug-resistant mutants at the corresponding fold EC_{50} or combination EC_{50} (cEC_{50}), then the former drug has a lower barrier to resistance than the comparison drug or combination.

Dolutegravir (DTG) was synthesized at Shionogi (Osaka, Japan) (6), and elvitegravir (EVG), lamivudine (3TC), and rilpivirine (RPV) were purchased from Sequoia Research Products (Pangbourne, UK). First, the EC_{50} and 90% effective concentra-

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TABLE 1 EC_{50} and EC_{90} of drugs in the MT-2 MTT assay

Drug	EC_{50} (nM)[a]	EC_{90} (nM)[a]
Dolutegravir	2.1 ± 0.60	5.3 ± 1.3
Elvitegravir	1.4 ± 0.36	4.4 ± 1.3
Rilpivirine	1.2 ± 0.13	2.6 ± 0.49
Lamivudine	3310 ± 325	9055 ± 670

[a]Data represent means and SDs that were calculated from data from three independent experiments.

tion (EC_{90}) of each drug were determined by a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay in MT-2 cells (7) infected with HIV NL-432 (8) as described previously (9). The EC_{50} and EC_{90} of each compound are shown in Table 1. It is well known that the EC_{50} of 3TC varies depending on the cell type (10). We selected MT-2 cells for this study because it is possible to isolate clinically relevant resistant viruses, although the EC_{50} of 3TC in MT-2 is relatively high (11).

Next, we evaluated various drug combinations using a checkerboard method (5). We judged the interaction of each combination of drugs using the combination index (CI) and D values as described previously (5, 12). RPV-RPV was used as a control, as the effect is expected to be additive. The combinations that included 3TC showed very weak synergism. However, all of the combinations in this study displayed additive activity, as demonstrated by their D values (Table 2).

To determine the barrier to resistance of the drugs individually and in combination, we performed passage studies in MT-2 cells. The drug concentrations were kept consistent throughout the study, and the cells were passaged every 3 to 4 days. The cells were passaged with the addition of fresh MT-2 cells if a cytopathic effect (CPE) was observed. We then analyzed the HIV-1 proviral DNA sequence for mutations by PCR using a *Taq* kit (TaKaRa, Shiga, Japan) and specific primers (IN coding region, 5′-AAC AAGTAGATAAATTAGTCAGT-3′ and 5′-TAGTGGGATGTGTACTTCTGAAC-3′; reverse transcriptase [RT] coding region, 5′-GCGGACATAAAGCTATAGGTACAG-3′, and 5′-CACTCCA TGTACCGGTTCTTTTAG-3′). Sequencing was performed by the TaKaRa sequencing service. For comparison, passage studies of the four single drugs were conducted. The starting concentration of each drug was based on its EC_{50}. For RPV, EVG, and DTG, the lowest concentration was half of their EC_{50}s and then 2-fold increments up to 64-fold EC_{50}. For 3TC, 0.5-, 1-, 2-, 64-, 320-, and 640-fold EC_{50} were used because our preliminary results suggested that a higher concentration was necessary for 3TC to inhibit HIV-1 replication completely (data not shown). Only DTG could stop HIV replication above its EC_{90}, and no resistant virus emerged (Fig. 1A). As a single agent, DTG had the highest barrier to resistance, followed by RPV, EVG, and 3TC (Fig. 1B, C, and D).

To specifically determine the barrier to resistance of the drugs in combination, we conducted passage studies with two-drug combinations based on their combination EC_{50} (cEC_{50}). The cEC_{50} of drug *n* (D*n*) was calculated with the EC_{50} and the fractional inhibitory concentration index (FICI) using the following formula: $cEC_{50}Dn = EC_{50}Dn \times FICI$. The FICI was defined as the cross point of $Y = x$ and on an approximate curve of

TABLE 2 Interaction of two-drug combinations[a]

Drug combination	CI value[b]	D values for[c]:		
		Expt 1	Expt 2	Mean
DTG-RPV	0.98	−0.024	0.068	0.022
DTG-3TC	0.86	−0.049	−0.062	−0.055
EVG-RPV	1.02	0.049	0.000	0.024
EVG-3TC	0.86	−0.037	0.016	−0.011
RPV-3TC	0.90	−0.013	−0.028	−0.021
RPV-RPV	1.00	0.032	−0.013	0.010

[a]The interaction of each two-drug combination was determined using the CI values or average D values from two independent experiments. Overall, the interactions were additive.
[b]CI values indicate synergistic (CI, <1), additive (CI, 1), and antagonistic (CI, >1) interactions.
[c]D values indicate strong synergistic (−0.5 to −0.2), weak synergistic (−0.2 to −0.1), additive (−0.1 to 0.1), weak antagonistic (0.1 to 0.2), and strong antagonistic (0.2 to 0.5) interactions.

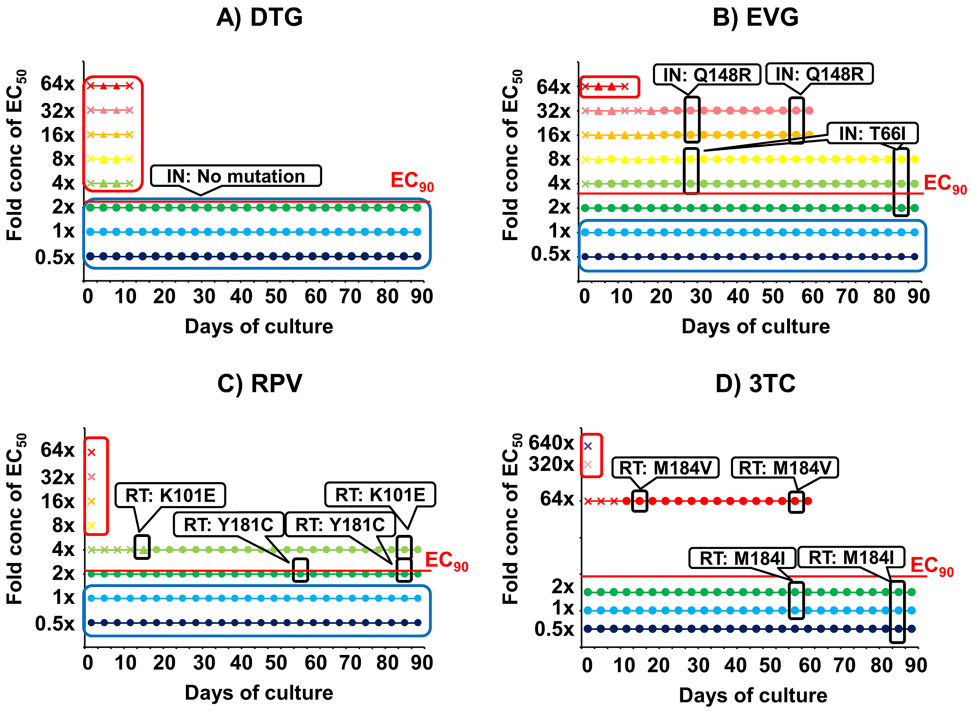

FIG 1 *In vitro* passage studies of single drugs. HIV-1 WT NL-432 was propagated in MT-2 cells in the presence of DTG (A), EVG (B), RPV (C), or 3TC (D). The drug concentrations used were 0.5-fold (blue), 1-fold (light blue), 2-fold (green), 4-fold (yellowish green), 8-fold (yellow), 16-fold (orange), 32-fold (pink), 64-fold (red), 320-fold (lilac), or 640-fold (purple) EC_{50}. A circle indicates that a cytopathic effect (CPE) was observed in more than 80% of the cells. A triangle means that CPE was observed in 30% to 80% of the cells. A cross means that CPE was observed in less than 30% cells. Mutations in the IN or RT coding regions of the virus are indicated in white rounded rectangles at the time points which they emerged. Red rounded rectangles indicate that no virus replicated. The blue rounded rectangles indicate that no mutations were observed in either the IN or RT coding regions despite HIV-1 replication. EC_{90}s are shown in each figure as a red line. Results from one representative well are shown for each drug. The passages of 64-fold EC_{50} of 3TC (D) and both 16- and 32-fold EC_{50}s of EVG (B) were stopped at day 60.

an isobologram of the two-drug combination (12). The cEC_{50} of each drug was roughly equal to half of its EC_{50} (Table 3). These passage studies were done using the same methodology as that of the single-drug passages, with each combination repeated in two independent wells (Fig. 2). The starting drug concentrations were 1-, 2-, 4-, 8-, and 16-fold cEC_{50}s of each combination (Table 3), and each drug was kept at the same fold cEC_{50} concentration throughout the passage experiment. In both wells containing RPV and DTG, wild-type HIV-1 could replicate at both 1- and 2-fold cEC_{50}s and could not replicate at more than 2-fold cEC_{50}s, which were less than their individual EC_{90}s (Fig. 2A). Interestingly, mutations were not observed in either the IN coding region or in the RT coding region, even at the drug concentrations at which HIV-1 replicated. When RPV and DTG were compared to RPV or DTG alone, better resistance profiles were seen, especially for RPV (Fig. 2A versus Fig. 1A and C). In the wells containing 3TC and

TABLE 3 Combination EC_{50} of each drug in two-drug combination

Drug combination	FICI[a]	cEC_{50} D1[b] (nM)	cEC_{50} D2[c] (nM)
DTG-RPV	0.489	1.0	0.60
DTG-3TC	0.428	0.89	1416
EVG-RPV	0.509	0.71	0.63
EVG-3TC	0.431	0.60	1426
RPV-3TC	0.452	0.56	1496
RPV-RPV	0.4999	0.62	0.62

[a]FICI, fractional inhibitory concentration index.
[b]cEC_{50} D1, cEC_{50} of drug 1 in each combination of drug 1-drug 2.
[c]cEC_{50} D2, cEC_{50} of drug 2 in each combination of drug 1-drug 2.

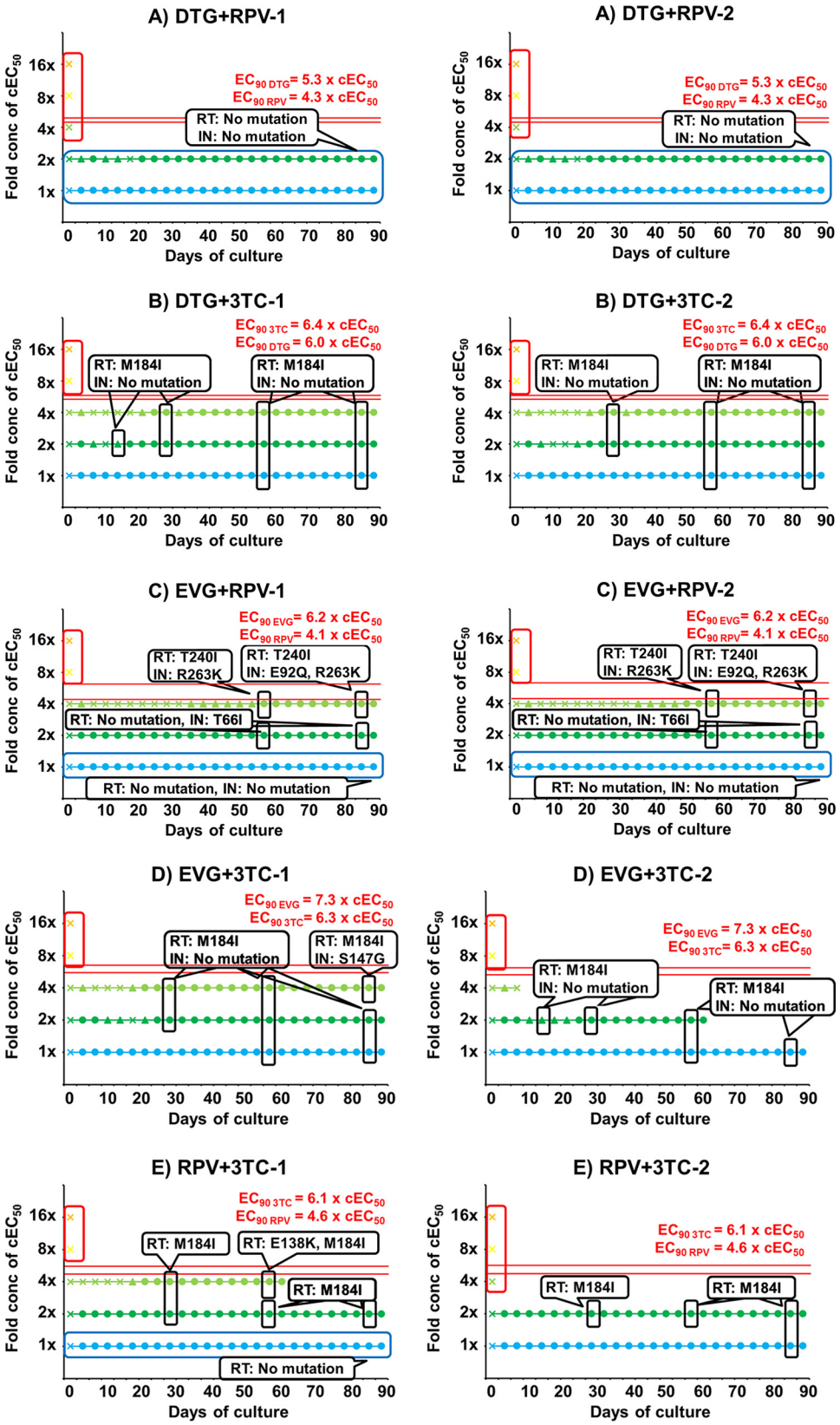

FIG 2 *In vitro* passage studies of two-drug combinations. HIV-1 WT NL-432 was propagated in MT-2 cells in the presence of DTG-RPV (A), DTG-3TC (B), EVG-RPV (C), EVG-3TC (D), or RPV-3TC (E). The drug concentrations are 1-fold (light blue), 2-fold (green), 4-fold (yellowish green), 8-fold (yellow), or 16-fold (orange) cEC_{50}s of each combination. A circle indicates that cytopathic effect (CPE) was observed in more than 80% of the cells. A triangle means that CPE was

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DTG (Fig. 2B), 3TC-resistant virus RT: M184I emerged on day 57 at 1-fold cEC_{50}s, on day 14 or on day 28 at 2-fold cEC_{50}s, and on day 28 at 4-fold cEC_{50}s. No DTG-resistant viruses emerged during the 90-day monitoring period. HIV-1 could not replicate at more than 4-fold cEC_{50}s. Even though 3TC-resistant mutants emerged in the 3TC-DTG combination, the 3TC concentration which permitted viral growth and resistance was decreased more than 32-fold in combination with DTG. This combination effect on 3TC was the highest among the four drugs. Similar combination effects on antiviral activity were seen in the 3TC-RPV, RPV-EVG, and 3TC-EVG combinations (Fig. 2C to E). However, viruses resistant to both drugs emerged in these combinations. RT: K101E (RPV-resistant) and IN: T66I or A (EVG-resistant) were found in the EVG-RPV wells. RT: M184I (3TC-resistant) and IN: S147G (EVG-resistant) emerged in the EVG-3TC wells. In the 3TC-RPV wells, RT: M184I and RT: E138K (RPV-resistant) emerged. These have all been previously reported to be drug-resistant mutations (13–17). Therefore, the barrier to resistance of DTG and 3TC was the second best of the drugs studied. Recently, the 3TC-DTG phase 3 clinical studies Gemini 1 and 2 showed that this combination was not inferior to the standard 3-drug regimen (18). The combination of DTG and RPV, which showed the greatest barrier to resistance, has been approved by the FDA as a two-drug maintenance regimen.

In all the combinations, viruses resistant to one or both drugs occurred at drug concentrations less than the EC_{90}s of each drug, which allowed for some degree of viral replication (Fig. 2). However, for RPV and DTG, no viruses resistant to either drug emerged at 1- or 2-fold cEC_{50}s. This concentration of 2-fold cEC_{50} of RPV plus 2-fold cEC_{50} of DTG is roughly equal to 2-fold EC_{50} of RPV alone or 2-fold EC_{50} of DTG alone. Similarly, no IN mutants emerged at 2-fold EC_{50} when DTG was passaged alone. However, the RT:Y181C mutant emerged at 2-fold EC_{50} (EC_{90}) when RPV was used alone (Fig. 1C). DTG has a high genetic barrier; many of the single IN mutants which can stochastically emerge have similar susceptibility to DTG as the wild-type (WT) virus (9). On the other hand, viral fitness for IN single mutants usually decreases (19), and low selection pressure from DTG does not select for IN single mutants from WT virus (9). At the concentrations of 2-fold EC_{50} for RPV and DTG, viral growth was slow in the initial ~20 days of passage (Fig. 2A). This low replication of virus may correlate with a low chance of the Y181C mutation emerging. Indeed, the emergence of resistance mutations was directly associated with viral growth (Fig. 1 and 2). Some synergistic effect may be seen when RPV and DTG are used in combination.

In our *in vitro* study, the barrier to resistance of 3TC and DTG was second to that of RPV and DTG. It was surprising that high concentrations of 3TC were necessary to prevent viral growth when used alone, but when 3TC was used in combination with DTG, EVG, or RPV, 8-fold cEC_{50} each was enough to prevent viral growth. 3TC and these drugs may have a positive synergistic effect that was not detected in the checkerboard method. Recently, it was reported that the combination of R263K in the IN coding region and M184I/V in the RT coding region decreases HIV-1 replicative capacity (20–22). This effect on viral fitness may be a reason for the higher barrier to resistance of the 3TC-DTG combination. When comparing 3TC resistance *in vitro* and *in vivo*, various factors, such as cell type, whether cells are resting or growing (10), and HIV subtype (23) should be considered.

In conclusion, our data suggest that two-drug combinations *in vitro*, especially with DTG, improve the barrier to resistance compared with each drug alone. The high genetic barrier to resistance of DTG likely contributes to this effect. These results may support future clinical use of the DTG-RPV and DTG-3TC combinations.

FIG 2 Legend (Continued)
observed in 30% to 80% of cells. A cross indicates that CPE was observed in less than 30% of the cells. Mutations in the IN and RT coding regions of the virus are indicated in white rounded rectangles at the time points which they emerged. Red rounded rectangles indicate that no virus replicated, and blue rounded rectangles indicate that no mutations were observed in the IN and RT coding regions despite HIV-1 replication. The EC_{90}s of each drug are shown as red lines. Each passage was conducted in duplicate wells, and the results from both wells are shown as -1 and -2. The passages of 2-fold cEC_{50}s of 3TC-EVG-2 and 4-fold cEC_{50}s of 3TC-RPV-1 were stopped at day 60.

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