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Short Communication

Dead-end complexes contribute to the synergistic inhibition of HIV-1 RT by the combination of rilpivirine, emtricitabine, and tenofovir

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ABSTRACT

The single tablet regimen of the nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) tenofovir disoproxil fumarate (TDF), emtricitabine (FTC), and the non-nucleoside reverse transcriptase inhibitor (NNRTI) rilpivirine (RPV) is approved for the treatment of HIV-1 infection in treatment-naïve adults. Previous studies have shown that two-drug combinations of these drugs show additive to synergistic HIV-1 antiviral activity in cell culture. In this study, two-drug combinations of tenofovir (TFV) + FTC, RPV + TFV, and RPV + FTC inhibited HIV-1 replication in cell culture with strong synergy and no evidence of antagonism. The triple drug combination of RPV + FTC + TFV displayed moderate synergy comparable to efavirenz (EFV) + FTC + TFV. The formation of dead-end complexes (DEC) of HIV-1 reverse transcriptase (RT), NRTI chain-terminated primer/template, and the next complementary nucleotide or NNRTIs was studied using gel mobility shift assays. DEC formation was seen with TFV-terminated DNA primer/template, HIV-1 RT, and FTC-triphosphate (TP) in addition to the natural nucleotide dCTP, thus stabilizing chain-termination. The NNRTI RPV also formed DEC-like complexes with TFV- and FTC-monophosphate (MP)-terminated DNA primer/templates and HIV-1 RT, and stabilized chain-termination by both NRTIs. Overall, the combinations of RPV, FTC, and TFV inhibit HIV-1 replication with moderate to strong synergy. This may be partially explained by enhanced DEC formation of NRTI chain-terminated DNA primer/template and HIV-1 RT in the presence of the other drugs in the combination, leading to more stable chain-termination and replication inhibition by NRTIs.

Single-tablet regimens (STRs) of antiviral drugs are important therapy options for HIV-infected people and have many benefits, including improved potency, better adherence, and a higher barrier to resistance development (Llibre and Clotet, 2012; Thompson et al., 2012). Two non-nucleoside reverse transcriptase inhibitor (NNRTI)-containing STRs are currently available: the NNRTI efavirenz (EFV) with the two nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) emtricitabine (FTC) and tenofovir disoproxil fumarate (TDF) (Atripla®, EFV/FTC/TDF) and the NNRTI rilpivirine (RPV) with FTC/TDF (Complera®, Eviplera®, RPV/FTC/TDF). A key component of antiviral potency is the interaction of the antiviral drugs in cells, which is the focus of the current study. Combinations of two NRTIs or NRTIs plus NNRTIs have demonstrated synergy *in vitro* (Brennan et al., 1995; Buckheit et al., 2001; Carr et al., 1996; Chong and Pagano, 1997; Feng et al., 2009; King et al., 2002; Pauwels et al., 1994; Richman et al., 1991; Schader et al., 2011). FTC + TFV show antiviral synergy partially due to a positive metabolic interaction resulting in higher levels of phosphorylation to their active metabolites (Borroto-Esoda et al., 2006; Feng et al., 2009).

The antiviral effects of two-drug combinations of TFV, FTC, and RPV were analyzed using MacSynergy II (version 1.0, Ann Arbor, MI), which results values of additivity (−25 to 25 nM^2%), synergy (25 to >100 nM^2%), and antagonism (<−100 to −25 nM^2%) (Prichard et al., 1993; Prichard and Shipman, 1990). The combinations of TFV + FTC, RPV + TFV, and RPV + FTC showed strong synergy with volumes of 153 nM^2%, 156 nM^2%, and 135 nM^2%, respectively (Fig. 1), and were similar to results with TFV, FTC, and EFV (Feng et al., 2009). Antagonism volumes for these combinations were between 0 and −14 nM^2%. Control experiments yielded strong synergy for ribavirin (RBV)+ddI (250 nM^2%); RBV + d4T showed strong antagonism (−493 nM^2%) (Baba et al., 1987; Margot and Miller, 2005); and RPV + RPV showed additivity (synergy/antagonism volumes of 15/−2.6 nM^2%). Similar synergy results were found using isobologram analysis (data not shown).

Three-drug combination studies were analyzed using median-effect analysis in CalcuSyn, which calculates a combination index

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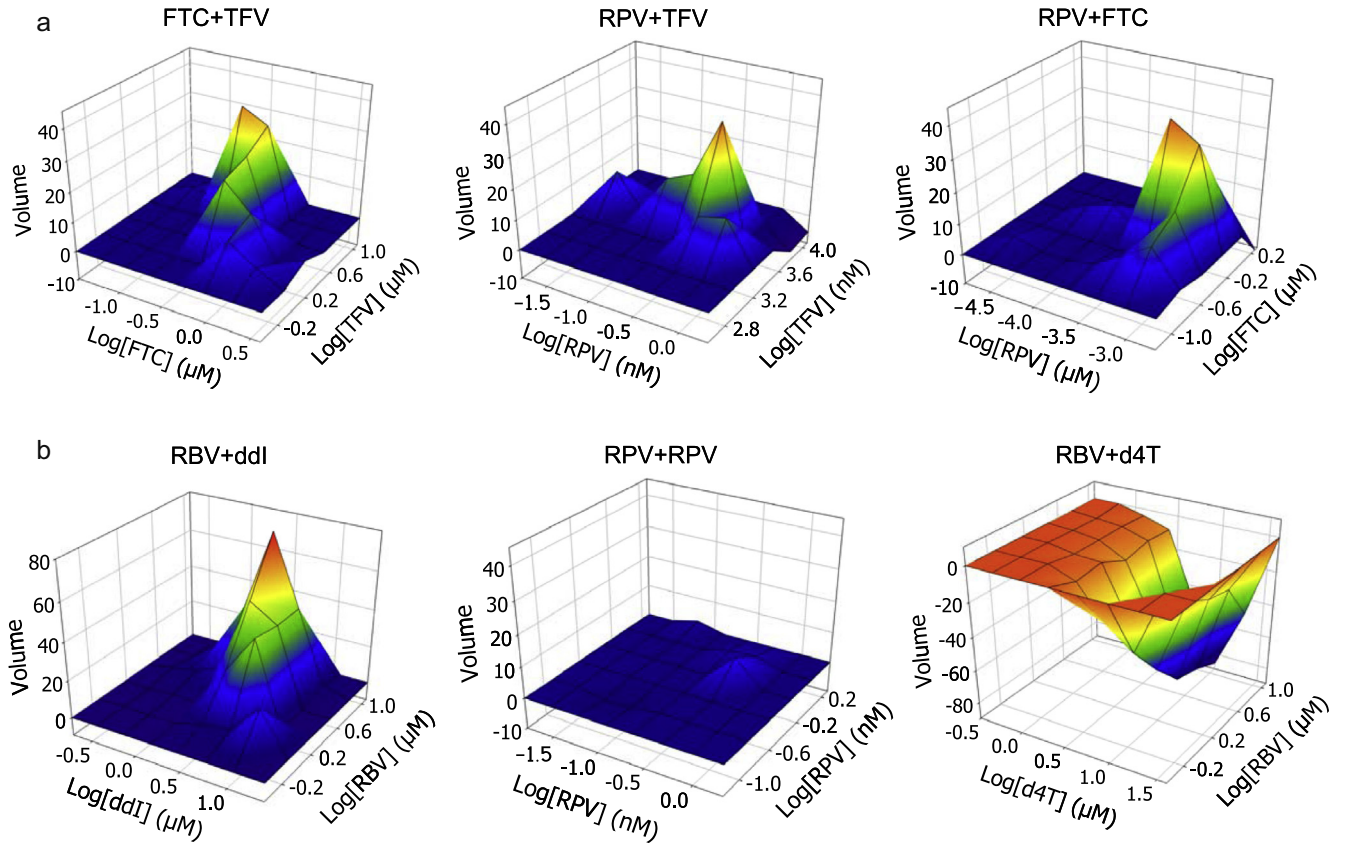

Fig. 1. Anti-HIV-1 two-drug combinations of RPV, TFV, and FTC. Experiments were repeated at least 3 times (2 times for controls) and representative two-drug combination plots of the concentration of each drug vs. the synergy/antagonism volumes are shown. The EC_{50} values for TFV, FTC, and RPV were 3.3 μM, 0.47 μM, and 0.25 nM, respectively. MT-2 cells were infected with HIV-1 strain IIIb in a 5-day assay. (a) The combinations of FTC + TFV, RPV + TFV, and RPV + FTC all showed strong synergy (mountains). (b) The two-drug combination controls all yielded expected results: RBV + ddI showed strong synergy; RPV + RPV was additive (flat); and RBV + d4T showed strong antagonism (valley). The green to red colors in (a) and (b) for RBV + ddI represent increasing synergy, blue represents additive inhibition, and antagonism in (b) for RBV + d4T increases from orange to blue.

(CI) to measure synergy (CI < 0.9), additivity (CI between 0.9 and 1.1), and antagonism (CI > 1.1) (Chou and Talalay, 1984). The combination of RPV + FTC + TFV showed moderate synergy (CI = 0.73 ± 0.13) (Fig. 2). EFV + FTC + TFV showed synergy (CI = 0.56 ± 0.08) similar to that of RPV + FTC + TFV ($p = 0.09$). Controls showed FTC + FTC + FTC was additive (CI = 0.92 ± 0.06) and d4T + AZT + RBV was strongly antagonistic (CI > 5.9).

A mechanism of synergy is the formation of the dead-end complex (DEC), a structure composed of HIV-1 RT, NRTI chain-terminated primer, and DNA template in the presence of the next complementary dNTP (Gao et al., 2000; Tong et al., 1997). These complexes are salt-stable and resistant to being disrupted in the presence of excess template, poly rA•p(dT). DEC stabilize NRTI chain-termination and inhibit NRTI excision (Isel et al., 2001; Meyer et al., 1999). DECs with TFV-terminated primer/template/RT and the next complementary dNTP, FTC-TP, or EFV (referred to as DEC-like complexes) were reported (Feng et al., 2009).

Here, DEC formation using TFV-terminated DNA primer/template and HIV-1 RT was assessed in the presence of the next correct nucleotide dCTP or FTC-TP, or EFV or RPV (Table 1, Fig. 3). DEC formation was quantified using three kinetic constants: K_d, the dissociation constant, B_{max}, the maximum percentage of DNA primer/template forming a DEC, and B_{max}/K_d, a measure of the efficiency of DEC formation. TFV-terminated and ddAMP-terminated DNA formed DEC with RT in the presence of dCTP and FTC-TP. The efficiency of DEC formation using the next complementary nucleotide dCTP was greater for primers terminated with ddAMP compared to TFV, which may be explained by the more favorable translocation equilibrium of ddAMP to the post-translocation position required for DEC formation compared to TFV (Marchand et al., 2007). ddAMP-terminated primer/template plus RT formed DEC greater than 10 times more efficiently with dCTP than with FTC-TP, while TFV-terminated primer/template plus RT had similar DEC formation with FTC-TP and dCTP.

RPV formed DEC-like complexes with TFV-terminated DNA/RT ($B_{max}/K_d = 950$), notably >200-fold more efficiently than dCTP or FTC-TP (Table 1). EFV formed DEC-like complexes with TFV-terminated DNA/RT (Feng et al., 2009) highly efficiently (B_{max}/K_d = 2380), >500-fold more efficiently than dCTP or FTC-TP, and was not statistically different than RPV ($p = 0.18$). EFV and RPV did not form DEC-like complexes with ddAMP-terminated DNA/RT at the highest concentrations tested (20 μM). Experiments with a second TFV-terminated primer/template, L31/WL50-33G, showed DEC formation with dCTP, FTC-TP, RPV, and EFV, but at lower efficiencies than those seen for the D25/D50 primer template (data not shown).

DEC formation with FTC-MP-terminated DNA showed low efficiency with dATP or TFV-DP. In contrast, both EFV and RPV formed DEC-like complexes with higher efficiencies (B_{max}/K_d = 130 and 32,

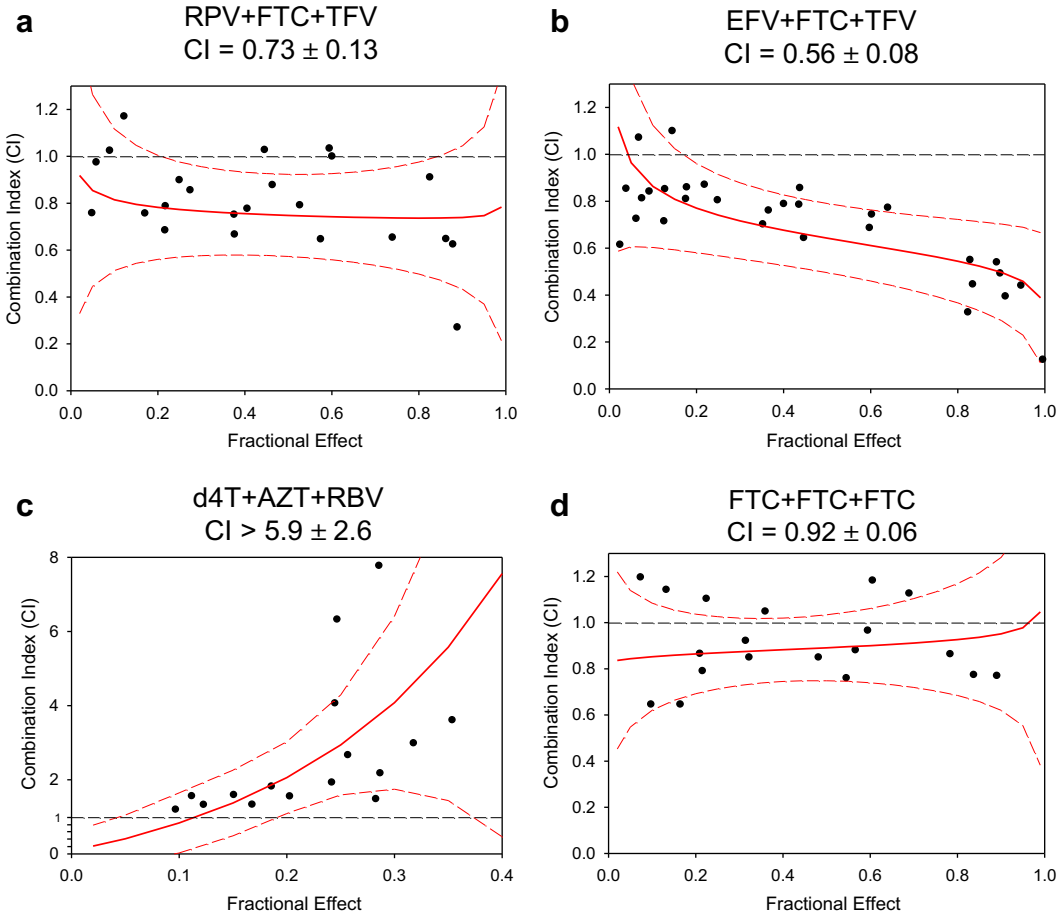

Fig. 2. Anti-HIV-1 three-drug combinations of RPV + FTC + TFV and EFV + FTC + TFV. Experiments were performed in MT-2 cells infected with HIV-1 strain xxLAI in a 5-day assay (Shi and Mellors, 1997). Experiments were repeated at least 3 times and the combined three-drug antiviral synergy plots of the CI (synergy score) vs. the fractional effect (level of inhibition of viral replication) are shown. Additivity line of CI = 1 (dotted black line), mean synergy curve fit line (solid red line), 95% confidence intervals (dotted red lines). The three-drug combinations of RPV + FTC + TFV (a) and EFV + FTC + TFV (b) showed moderate and strong synergy, respectively. The control for antagonism, d4T + AZT + RBV (c), showed strong antagonism, and the additivity control, FTC + FTC + FTC (d), was additive.

Table 1
Formation of dead-end complexes.[a]

Terminator	Next compound	K_d (μM)	B_{max} (%)	B_{max}/K_d
TFV	dCTP	12 ± 4.4	31 ± 7.3	2.6
TFV	FTC-TP	9.2 ± 1.6	41 ± 2.3	4.5
TFV	RPV	0.05 ± 0.02	52 ± 5.1	950
TFV	EFV	0.02 ± 0.01	36 ± 5.4	2380
ddAMP	dCTP	0.61 ± 0.15	36 ± 12	58
ddAMP	FTC-TP	6.8 ± 0.38	38 ± 6.6	5.5
ddAMP	RPV	No DEC[b]	No DEC	No DEC
ddAMP	EFV	No DEC	No DEC	No DEC
FTC-MP	dATP	14 ± 2.0	20 ± 5.5	1.4
FTC-MP	TFV-DP	540 ± 260	9.4 ± 5.6	0.02
FTC-MP	RPV	0.26 ± 0.04	8.3 ± 0.91	32
FTC-MP	EFV	0.05 ± 0.02	6.0 ± 0.01	130
ddCMP	dATP	0.38 ± 0.18	25 ± 8.7	66
ddCMP	TFV-DP	7.2 ± 3.1	22 ± 8.0	3.1
ddCMP	RPV	No DEC	No DEC	No DEC
ddCMP	EFV	No DEC	No DEC	No DEC

[a] Dead-end complex formation was repeated at least 2 times and quantified using three kinetic constants: K_d, the dissociation constant, B_{max}, the maximum percentage of DNA primer/template forming a dead-end complex, and B_{max}/K_d, a measure of the efficiency of dead-end complex formation (Feng et al., 2009).

[b] No DEC = less than 1% dead-end complex formation was detected at the highest drug concentration tested.

respectively). ddCMP-terminated DNAs formed DEC with dATP and TFV-DP, but neither EFV nor RPV formed DEC-like complexes at the highest concentrations tested (20 μM). Background levels of a ddCMP-terminated DNA/RT complex decreased with increasing amounts of EFV or RPV (data not shown). Experiments with a second FTC-MP-terminated primer/template showed DEC formation with dATP, TFV-DP, RPV, and EFV, although again at lower efficiencies (data not shown). Interestingly, neither EFV nor RPV were able to form DEC-like complexes with either ddCMP-terminated DNA tested in this study.

In summary, the combinations of TFV + FTC, RPV + TFV, RPV + FTC, and RPV + FTC + TFV all showed strong to moderate synergy against HIV-1 in cell culture. This may be due in part to enhanced DEC or DEC-like complex formation of TFV-terminated DNA primer/template plus HIV-1 RT in the presence of FTC-TP or RPV, and by FTC-MP-terminated DNA primer/template and HIV-1 RT in the presence of RPV, as well as increased levels of the active metabolites TFV-DP and FTC-TP in cell culture when the drugs are dosed together. These results suggest that the components of the two approved single tablet regimens of RPV/FTC/TDF and EFV/FTC/TDF show synergy by similar mechanisms unique to the NRTI/NNRTI classes of RT inhibitors.

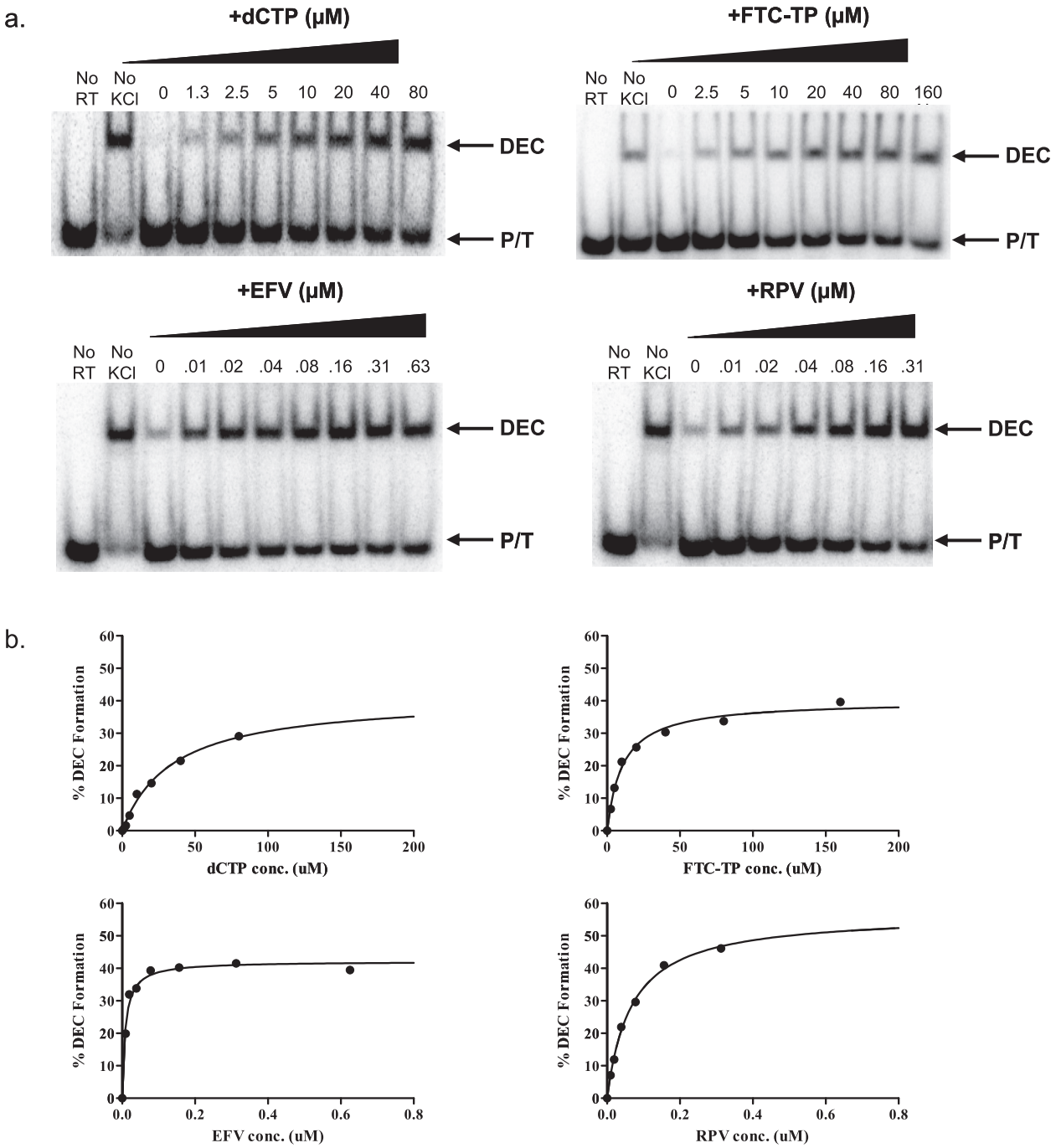

Fig. 3. Dead-end complex formation by TFV-terminated primer D25/D50 template and HIV-1 RT. Experiments were repeated at least 2 times and representative gels are shown. Dead-end complex formation assays were performed as previously described with modifications (Cruchaga et al., 2005; Feng et al., 2009; Isel et al., 2001; Tong et al., 1997). Two sets of primer/templates, D25/D50 and D26/D50 and L31/WL50-34G and L31/WL50-33G were used (Feng et al., 2009; Meyer et al., 1998, 2000; White et al., 2004). γ-^{32}P–ATP-labeled primer/template was chain terminated with ddATP, TFV-DP, ddCTP, or FTC-TP and then the next complementary nucleotide or the NNRTIs EFV or RPV was added for 15 min at room temperature, followed by the addition of 5 units/mL poly rA•p(dT)$_{12-18}$ and 66.7 mM KCl at 37 °C for 5 min. The DNA was separated and analyzed as described (Feng et al., 2009). (a) DEC formation of ^{32}P-labeled, TFV-chain terminated D25/D50 template (P/T), RT and the next complementary nucleotide dCTP or FTC-TP, or the NNRTIs EFV or RPV. With no RT, only P/T is visible as the lower band on the gel. With no KCl, a complex of HIV-1 RT plus P/T is visible as the higher band on the gel. Dead-end complexes were formed with increasing concentrations of the next nucleotide or EFV or RPV and are stable in KCl. (b) The amounts of free P/T and dead-end complexes were quantified, and the percentage of dead-end complex formed was plotted against the concentration of drug added. The data was fit using the single ligand binding equation $y = (B_{max} \times [\text{ligand}])/(K_d + [\text{ligand}])$.

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