Antiviral and Cellular Metabolism Interactions between Dexelvucitabine and Lamivudine

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Abstract

Studies on cellular drug interactions with antiretroviral agents prior to clinical trials are critical to assess possible drug interactions. Herein, we demonstrated that two 2'-deoxycytidine antiretroviral agents, dexelvucitabine (β-D-2’,3’-didehydro-2’,3’-dideoxy-5-fluorocytidine, DFC, D-d4FC, RVT) and lamivudine (3TC), combined in primary human peripheral blood mononuclear (PBM) cells infected with HIV-1_LAI, resulted in additive to synergistic effects. The cellular metabolism of DFC and 3TC was studied in human T-cell lymphoma (CEM) and in primary human PBM cells to determine whether this combination caused any reduction in active nucleoside triphosphate (NTP) levels, which could decrease with their antiviral potency. Competition studies were conducted by co-incubation of either radiolabeled-DFC with different concentrations of 3TC or radiolabeled-3TC with different amounts of DFC. Co-incubation of radiolabeled-3TC with DFC up to 33.3 µM did not cause any marked reduction in 3TC-triphosphate or any 3TC metabolites. However, at high concentrations of 3TC with radiolabeled-DFC, a reduction in the level of DFC metabolites was noted. DFC-TP levels in CEM and primary human PBM cells decreased by 88% and 94%, respectively, when high concentrations of 3TC (33.3 and 100 µM) were added which may impact on the effectiveness of DFC-5’-triphosphate on the HIV-1 polymerase. The NTP levels remained well above the median inhibitory concentration (IC_{50}) for HIV-1 reverse transcriptase (RT). These results suggest that both β-D- and β-L-2’-deoxycytidine analogs, DFC and 3TC, respectively, substrates of 2’-deoxycytidine kinase, could be used in a combined therapeutic modality. However, it may be necessary to decrease the dose of 3TC for this combination to prove effective.
Introduction

The primary goal of antiretroviral therapy for the treatment of human immunodeficiency virus (HIV) infections is suppression of viral replication to undetectable levels. This goal can be achieved by a combination of highly active antiretroviral therapy (HAART), involving the use of agents from at least two distinct classes such as two nucleoside reverse transcriptase inhibitors (NRTI) and either a non-nucleoside reverse transcriptase inhibitor (NNRTI) or a protease inhibitor (PI) (2, 9). However, to date, the combination of two NRTI has been restricted to those nucleosides that are activated by different kinases in their first phosphorylation step (Fig. 1). Nucleoside combinations approved by US Food and Drug Administration (FDA) include: 1) lamivudine (3TC) and zidovudine (ZDV), 2) emtricitabine (FTC) and tenofovir disoproxil fumarate (TDF), 3) abacavir (ABC) and 3TC, 4) ABC, 3TC and ZDV, and 5) FTC, TDF and efavirenz.

Few combination studies have been conducted using nucleoside analogs that share the same phosphorylation enzyme. Previous in vitro studies with 3TC or FTC with apricitabine (ATC, AVX754), a 2’-deoxycytidine analog formerly known as BCH-10618, (-)-dOTC, or SPD754, demonstrated significant reduction in the active nucleoside triphosphate (NTP) levels of ATC-triphosphate (ATC-TP) in primary human peripheral blood mononuclear (PBM) cells (12). Interestingly, the intracellular levels of 3TC-TP in humans were unaffected by co-administration of ATC, but the levels of ATC-TP were reduced by approximately 6-fold in the presence of 3TC (4, 5). These data reinforce the necessity of ascertaining intracellular NTP levels when assessing nucleoside analog interactions. Similarly, the
combination of two thymidine analogs, ZDV and D4T, is contraindicated in the clinic, since they both use thymidine kinase for activation to their corresponding nucleotides (14).

Dexelvucitabine (β-D-2’,3’-didehydro-2’,3’-dideoxy-5-fluorocytidine, RVT, DFC, D4FC), is currently in Phase 2b clinical trials for the treatment of HIV infections (http://www.aidsmeds.com/drugs/Dexelvucitabine) (8). Preclinical studies indicate that DFC triphosphate (DFC-TP) has a long intracellular half-life and inhibits replication of both wild-type and mutant strains of HIV commonly observed during treatment with ZDV, 3TC and other NRTI (19).

3TC is a (-)-β-2’-deoxycytidine analog approved by FDA for the treatment of HIV and HBV infections and is presently one of the most widely used nucleoside analogs in HAART regimens (11). Since 3TC and DFC are both phosphorylated by 2’-deoxycytidine kinase (dCK), it was anticipated that they might interact with each other (19). However, cellular antiviral assays reported herein by our group demonstrated mostly synergistic or additive antiviral interactions at low concentrations of 3TC relative to DFC. Based on these observations, the cellular metabolism of the combination of these two potent 2’-deoxycytidine analogs was studied, in order to determine whether any reduction in active NTP levels occurs.
Materials and Methods

Chemicals

[5-\textsuperscript{3}H]-3TC (specific activity = 8 Ci/mmol) and [6-\textsuperscript{3}H]-DFC (specific activity = 1 Ci/mmol) were synthesized by Moravek Biochemicals, Inc. (Brea, CA). Tetrabutylammonium phosphate (TBAP) was purchased from Alltech Assoc., Inc. (Deerfield, Ill). Scintillation liquid, EcoLite, was obtained from Valeant Pharmaceuticals (Costa Mesa, CA). The chemical purity of each compound, as determined by high-performance liquid chromatography (HPLC) and spectral analysis was greater than 98%. All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell culture systems

Human T-cell lymphoma (CEM) cells were obtained from the American Type Culture Collection (ATCC) (Rockville, MD) and were maintained in suspension cultures in RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY), supplemented with 1 mM sodium pyruvate, 10% (v/v) FBS and 1 mM penicillin G/streptomycin sulfate. CEM cells were grown at 37°C in a 5% CO\textsubscript{2}, 95% air atmosphere. The media were replenished every three days, and cells were subcultured once a week. Primary human PBM cells were isolated using a histopaque technique from buffy coats derived from healthy donors, obtained from the American Red Cross (Atlanta, GA). After processing, the PBM cells were stimulated by incubating cells for 3 days in medium containing 10 µl/ml phytohemagglutinin (PHA) before use. All the experiments reported in this paper were performed using PHA-stimulated primary human PBM cells and not resting cells, except where indicated.
Competition studies:

CEM and primary human PBM cells (2 x 10^6 cells/per time point) were exposed to either 10 µM [³H]-3TC with unlabelled DFC (1, 33.3 and 100 µM) or 10 µM [³H]-DFC with unlabelled 3TC (1, 33.3 and 100 µM) for 4 hr. Radiolabeled-3TC and radiolabeled-DFC (10 µM) with 50 or 100 µM 2’-deoxycytidine (dCyd) was used as a positive control. All combination studies were performed in triplicate.

At selected times, the cells were centrifuged for 10 min at 350 x g at 4°C, and the pellet was resuspended and washed three times with cold phosphate buffered saline (PBS). Viable cells were counted using a hemocytometer, and the viability was assessed by trypan blue exclusion (viability > 98%). Intracellular DFC, 3TC and their respective metabolites were extracted by incubation overnight at -20°C with 60% methanol/water (1 ml); the supernatants were then collected and centrifuged at 14,000 rpm (Eppendorf Centrifuge Model 5415C) for 5 min. The extracts were dried under a gentle filtered air flow and stored at -20°C, until they were assayed. The residues were resuspended in 100 µl of water, and aliquots were injected into the HPLC system.

Separation of DFC and 3TC metabolites was performed by ion-pairing reverse phase HPLC on a Columbus 5 µm C₁₈ column (250 x 4.6 mm) (Phenomenex, Torrance, CA) using a Varian Pro Star HPLC model 210 with manual injection (Walnut Creek, CA). The mobile phase consisted of buffer A (25 mM ammonium acetate with 5 mM tetrabutylammonium phosphate (TBAP), pH 7.0) and buffer B (methanol). Elution was performed using a multistage linear gradient of buffer B from 0 to 50%. Retention time for DFC-DP-choline (6
min), DFC (20 min), DFC-MP (30 min), DFC-DP (40 min) and DFC-TP (48 min) and for 3TC-DP-choline (6 min), 3TC (25 min), 3TC-MP (32 min), 3TC-DP (44 min) and 3TC-TP (52 min). The limit of detection was approximately 0.01 pmol/10⁶ cells. Radioactivity was quantified using a 2500 TR liquid scintillation analyzer (Perkin Elmer, Life and Analytical Sciences, Wellesley, MA). Based on previous work with other cytidine analogs, incubation with alkaline phosphatase and authentic standards, we identified DFC metabolites (13, 17).

Drug interaction studies in HIV infected primary human PBM cells:

The combined antiviral effect of DFC and 3TC was tested against HIV-1_{LAI} in 3-day primary human PBM cells after 5 days in culture. The concentration of the drugs used was based initially on the ratios of their respective 50% effective concentration (EC₅₀) in human lymphocytes (10:1; DFC:3TC). Studies were also conducted at ratios favoring DFC (3:1, 25:1, and 100:1) since in this cell culture system 3TC is more potent than DFC. The medium contained human recombinant interleukin 2 (HR-IL2, 26.5 units/ml). Virus was added to the cell suspensions 1 hr prior to the addition of drugs. The assays were performed in T25 flasks. One ml of supernatant was centrifuged at 12,000 rpm for 2 hr at 4°C in a Jouan Br43i centrifuge (Thermo Electron Corp., Marietta, OH). Ten μL of the resuspended virus pellet extract was used to determine the amount of HIV-1 reverse transcriptase present, as previously described (10). A semi-automated radioactive detection method was performed using a Packard harvester and direct beta counter. The data were analyzed as previously described (10). Cytotoxicity of the drugs alone and in combinations at different ratios was determined in primary human PBM cells using a commercial colorimetric MTT assay as described previously (19, 21).
Statistical Analyses for the combination antiviral studies:

A robust computer algorithm, previously developed to determine synergy, additivity, and antagonism between drugs was used (3, 7). This method calculated the combination index (CI) for each drug combination together with confidence intervals. Based on this method, a C.I. < 1, equal to 1, or > 1 indicates synergy, additivity or antagonism, respectively. This analysis has been used by numerous investigators working on antiviral combinations (6, 18). T-test (two-sample assuming equal variance) was used to determine statistical significance (p < 0.05).
Results

Anti-HIV-1 combination studies between DFC and 3TC in primary human PBM cells demonstrated additivity or synergy at ratios of 10:1, 25:1, and 100:1 (Table 1). Additive to weak antagonism was noted with DFC:3TC only at high effect levels (90-95% inhibition) for the combination at a 3:1 ratio (Table 1). No cytotoxicity was observed for any combination used at the highest concentration as determined by tetrazolium reduction assays (data not shown). Therefore, a cellular metabolism study was performed to determine whether these two potent 2’-deoxycytidine analogs interfered with phosphorylation to active NTP in CEM and primary human PBM cells.

DFC (10 µM) and 3TC (10 µM) were rapidly bioconverted to their monophosphate, diphosphate (DP), triphosphate forms and to the DP-choline derivative in CEM and primary human PBM cells (Figure 2). DFC-TP and 3TC triphosphate (3TC-TP) reached levels of 5.74 ± 0.46 and 5.70 ± 1.72 pmol/10^6 cells in CEM cells after 4 hr, respectively (Fig. 3). Intracellular concentrations in primary human PBM cells of DFC-TP and 3TC-TP after 4 hr incubation were 1.35 ± 0.02 and 2.01 ± 0.37 pmol/10^6 cells, respectively (Fig. 3). The natural nucleoside dCyd was used as a positive control, since it has been previously shown to inhibit the phosphorylation and anti-HIV activity of 3TC (23). As expected, co-incubation of radiolabeled-3TC with dCyd resulted in no detectable 3TC metabolites (Fig. 4).

Competition studies in CEM cells between [^3]H]-3TC with several concentrations of DFC resulted in no significant reduction (p > 0.05) of 3TC-TP levels up to 100 µM DFC (Fig. 5). Even in the presence of 100 µM DFC, [^3]H]-3TC-TP levels were reduced by 25% from 5.70 ± 1.72 pmol/10^6 cells (no DFC added) to 4.28 ± 1.90 pmol/10^6 cells (Fig. 5). Similar results were observed in primary human PBM cells at concentrations up to 33.3 µM.
DFC. However, a significant 46% inhibition of 3TC-TP levels ($p < 0.05$) was observed with 100 $\mu$M DFC in primary human PBM cells, from $2.01 \pm 0.37$ to $1.09 \pm 0.16$ pmol/10$^6$ cells.

No reduction in any of 3TC metabolites were observed when 1 $\mu$M DFC was added, between 10-26% with 33.3 $\mu$M DFC and 13-46% inhibition with 100 $\mu$M DFC. 3TC-DP-choline do not decreased with the addition of DFC in any of the cell systems used in this study.

In primary human PBM cells, the levels of [$^3$H]-DFC-TP at 4 hr were significantly lower than those found in CEM cells (Fig. 6). However, no reduction in [$^3$H]-DFC-TP was noted with 1 $\mu$M 3TC in CEM cells. Significant reductions in [$^3$H]-DFC-TP levels from 5.74 ± 0.46 pmol/10$^6$ cells to 0.67 ± 0.02 and 0.41 ± 0.16 pmol/10$^6$ cells were noted in the presence of 33.3 and 100 $\mu$M 3TC, respectively in CEM cells (Fig. 6). Similar reductions in DFC-TP levels were observed in primary human PBM cells, at all concentrations tested ($p < 0.05$). Whereas DFC-TP levels in CEM cells remained above the 50% inhibitory concentration ($IC_{50}$, 0.18 $\mu$M) for the HIV-1 reverse transcriptase (RT), in primary human PBM cells, these levels remained below the $IC_{50}$ for the HIV-1 RT when 33.3 or 100 $\mu$M 3TC was presented (Fig. 6) (19). Interestingly, in CEM cells, the addition of 3TC as low as 1 $\mu$M decreased by 74% the formation of the liponucleotide DFC-DP-choline ($p < 0.05$, data not shown).

All DFC metabolites levels decreased to the same range in primary human PBM and CEM cells with: 33.3 $\mu$M 3TC (83-94%), 100 $\mu$M 3TC (92% to below limit of detection). At 1 $\mu$M 3TC, we observed a significant decrease of the DFC-DP-choline, 74% inhibition in CEM, but only 48% inhibition in primary human PBM.
Similar results were observed in resting primary human PBM cells when DFC were incubated with 33.3 and 100 μM 3TC (data not shown). The only difference was observed when 1 μM 3TC was added. In resting PBM cells, 1 μM 3TC did not reduce significantly the level of DFC-TP, while in the PHA-stimulated primary human PBM cells a decrease in the DFC-TP levels was observed (data not shown). This is consistent with the fact that cytidine analogs such as DFC and 3TC are cell cycle independent (19, 20).
Discussion

Clinical studies involving combinations of NRTI that share the same activating enzymes have been contraindicated because of potential antiviral antagonism. Since 3TC (and FTC) are widely used for first line therapy, and DFC is being considered for salvage therapy, the potential drug interaction between these two 2'-deoxycytidine analogs was investigated.

Antiviral assays of the combination of DFC and 3TC in infected human PBM cells with HIV-1_LAI demonstrated additive or synergistic effects at relevant ratios close to their respective EC_{50} values or when the ratio favored DFC (10:1, 25:1 and 100:1), suggesting a possible benefit of coadministration for the treatment of HIV infections (Table 1). However, at levels of 3TC close to that for DFC (3:1 ratio of DFC:3TC) additive to moderate antagonism was noted. Therefore, a study of the cellular metabolism of these drugs alone and in combination was warranted.

Both nucleoside analogs were metabolized to their mono-, di-, and triphosphate metabolites and to the DP-choline derivative in CEM and primary human PBM cells. 3TC and DFC were phosphorylated to their respective 5’-triphosphate metabolites at equivalent concentrations in CEM cells (Fig. 3).

After co-incubation of radiolabeled-3TC with different concentrations of DFC for 4 hr, a modest reduction (p > 0.05) in 3TC metabolites was noted (Fig. 5). Even at 100 µM DFC, a non-physiological concentration (expected C_{max} in elimination = 7.70 ± 1.58 µM, median concentration = 2.15 ± 0.38 µM), only a 25% reduction in 3TC-TP concentration was noted in CEM cells, but a 46% reduction was detected in primary human PBM cells (Fig. 5).
DFC had no marked effect on intracellular 3TC-TP levels at physiological concentrations.

When [³H]-DFC was co-incubated in CEM cells with 33.3 or 100 µM 3TC, a marked reduction (≥ 88%) of DFC-TP levels was noted. Similar results were observed in PBM cells (Fig. 6). Liponucleotide metabolites have previously been described for other cytidine analogs including D- and L-2',3'-dideoxycytidine (ddC) and its 5-fluorinated derivative D- and L-FddC, following incubation in cell culture (1, 17). 5’-Diphosphoethanolamine and diphosphocholine liponucleotides may be associated with peripheral neuropathy observed in individuals treated with ddC, although this has not been confirmed (13). These liponucleotides could also serve as a depot form of the drug, with a long intracellular half-life. Even at 1 µM 3TC, a significant reduction in DFC-DP-choline derivative was noted in CEM cells.

Although 3TC reduced intracellular DFC-TP levels in a concentration dependent manner, DFC-TP levels in CEM cells remained above IC₅₀ for the HIV-1 RT. However, in primary human PBM cells, the levels of the DFC-TP were 6-times lower than the IC₅₀ for the HIV-1 RT. Consistent with the combination anti-HIV assays, 3TC partially prevented the phosphorylation of DFC, but only at high non-physiological concentrations (> 1-3 µM).

These findings are consistent with a previous report by Erickson-Viitanen et al. and support the hypothesis that enzymes involved in the activation of DFC and 3TC are not rate limiting for the production of their 5’-triphosphate metabolites (9). It is likely that drug interaction occurs at the NTP levels with the HIV-RT. Furthermore, it is known that D- and L-nucleoside analogs can be phosphorylated by different enzymes at the nucleoside
diphosphate level (NDP to NTP) (16). Thus, the lack of drug interaction could also result from affinities to different kinases.

A recent Phase 2b clinical study demonstrated that DFC is a powerful drug against HIV-1 resistant viruses containing thymidine analog and/or M184V mutation in the viral polymerase (8). Interestingly, DFC 200 mg orally once a day was highly effective in drug-experienced individuals who were not taking 3TC or FTC (mean reduction in viral load at week 16 was 1.4 and 1.5 log_{10} copies/mL in optimized and non-optimized regimens, respectively), but was less effective when these oxathiolane nucleoside analogs were administered, which supports results presented herein. Thus, in vitro competition studies between nucleoside analogs can provide information that may be extrapolated to humans, especially when physiological relevant concentrations are used.

Taken together, these studies suggest that although 3TC and DFC are cytidine analogs activated by dCK, they may be considered for combination therapy for the treatment of HIV infections, although a dose reduction for 3TC may be needed (9, 20). It should be noted that emtricitabine, a related nucleoside is approved at a dose of 200 mg which is 33% and 50% lower than the approved dose of 3TC for HIV and hepatitis B virus, respectively. Alternatively, since both 3TC and DFC can be given once a day, a future salvage therapy clinical trial should be considered where the drugs are given at different time of the day (e.g., 9 AM and 9 PM). Similar intracellular pharmacokinetic drug interaction studies should be considered with other novel nucleoside analogs to maximize the understanding of potential interactions before they are administered to HIV-1 infected individuals.
Acknowledgements

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RFS receives or will receive royalties from the sale of 3TC and DFC. Parts of this paper were presented at national and international meetings prior to the release of clinical data with the combination of 3TC and DFC (15).
References


Figure legends

Figure 1: Intracellular phosphorylation of nucleosides and their incorporation into HIV-1-RT. AVX754 is also known as SPD754 or (-)-dOTC. NDPK = nucleoside diphosphate kinase; *the nucleoside analog is likely a substrate for this enzyme.

Figure 2: Intracellular concentrations of $[^3H]$-DFC or $[^3H]$-3TC and their respective metabolites after 4 hr incubation in primary human PBM cells [DFC-metabolite (solid), 3TC-metabolites (white)]. Metabolites were separated by ion pairing reverse phase HPLC with a Columbus 5 µm C18 column (Phenomenex, Torrance, CA) using a model Pro Star (Varian, Walnut Creek, CA) with manual injection. The mobile phase consisted of buffer A (25 mM ammonium acetate with 5 mM TBAP, pH 7.0) and buffer B (methanol). Elution was performed using a multistage linear gradient of buffer B. DFC-MP was below limit of detection.

Figure 3: Intracellular concentrations of 10 µM $[^3H]$-DFC-TP or 10 µM $[^3H]$-3TC-TP in CEM and primary human PBM cells at 4 hr [DFC-TP (solid), 3TC-TP (white)]. Metabolites were separated as described in Figure 2. * S.D. for DFC-TP in primary human PBM cells is 0.02 pmol/10^6 cells.

Figure 4: Competition study: radiolabeled 10 µM 3TC with 100 µM 2'-deoxycytidine (dCyd) in CEM cells after 1 hr co-incubation [3TC, 10 µM (solid); 3TC, 10 µM + dCyd,
100 µM (white)]. No nucleoside MP-, DP-, triphosphate or DP-choline metabolites were detected.

Figure 5: Competition study: Intracellular concentrations of [³H]-3TC-TP after co-incubation with radiolabeled 10 µM 3TC (solid) with DFC [1 µM (white), 33 µM (dots), or 100 µM (stripes)] for 4 hr in CEM and primary human PBM cells.

Figure 6: Competition study: Intracellular concentrations of [³H]-DFC-TP after co-incubation with radiolabeled 10 µM DFC (solid) with 3TC [1 µM (white), 33.3 µM (dots), 100 µM (stripes)] for 4 hr in CEM and primary human PBM cells.
**Figure 1**

2'-Deoxycytidine analogs

- **DFC**
  - Deoxycytidine
  - **DFC-MP***
  - **DFC-DP***
  - **DFC-TP**
  - HIV-RT
  - HIV polymerase

- **3TC**
  - **3TC-MP***
  - **3TC-DP***
  - **3TC-TP**
  - HIV-RT
  - HIV polymerase

- **AVX754**
  - **AVX754-MP***
  - **AVX754-DP***
  - **AVX754-TP**
  - Chain termination

Thymidine analogs

- **ZDV**
  - **ZDV-MP***
  - **ZDV-DP***
  - **ZDV-TP**
  - HIV-RT

- **D4T**
  - **D4T-MP***
  - **D4T-DP***
  - **D4T-TP**
  - HIV-RT
  - HIV polymerase
Figure 2

Intracellular concentration (pmol/10⁶ cells ± SD)

- DP-choline
- Nucleoside
- Monophosphate
- Diphosphate
- Triphosphate

DFC-MP
Figure 3

Intracellular concentration (pmol/10^6 cells ± SD)

- CEM cells
- PBM cells
Intracellular concentration (pmol/10^6 cells ± SD)

- 3TC-DP-choline
- 3TC
- 3TC-MP
- 3TC-DP
- 3TC-TP

Figure 4
Figure 5

3TC-TP intracellular concentration (pmol/10^6 cells ± SD)

CEM cells

PBM cells

0%
+10%
-26%
-25%
0%
+20%
-14%
-46%
Figure 6

Comparison of DFC-TP intracellular concentration (pmol/10^6 cells ± SD) between CEM cells and PBM cells. The graph shows a significant decrease in DFC-TP concentration in PBM cells compared to CEM cells, with percentages indicating the reduction.
Table 1: Effect of 3TC and DFC combination against HIV-1\textsubscript{LAI} infected human PBM cells.

<table>
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<th>Treatment (ratio)</th>
<th>EC\textsubscript{50}\textsuperscript{a}</th>
<th>EC\textsubscript{90}\textsuperscript{a}</th>
<th>Combination index (C.I.) +/- S.E. at F\textsubscript{a}\textsuperscript{b} of:</th>
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<td>1.99</td>
<td>0.80 +/- 0.63 1.00 +/- 0.43 1.08 +/- 0.39</td>
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<td>0.77</td>
<td>0.86 +/- 0.58</td>
<td>0.95 +/- 0.41 1.02 +/- 0.32</td>
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<td>DFC + 3TC (25:1)</td>
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<td>1.15 +/- 0.65 0.97 +/- 0.46 0.82 +/- 0.32</td>
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\textsuperscript{a} EC\textsubscript{50} is the median effective concentration, and EC\textsubscript{90} is the effective concentration at 90% inhibition in \( \mu \text{M} \) as determined from the median effect plot.

\textsuperscript{b} C.I. < 1, equal to 1, or > 1 indicates synergy, additivity or antagonism, respectively. F\textsubscript{a} is a component of the median effect equation referring to the fraction of the system (e.g., 0.50 means the C.I. at a 50% reduction of RT activity). C.I. values were determined for a mutually non-exclusive interaction (\textit{values in italics are for mutually exclusive interaction, which is less rigorous}).