The Combined Anti-HIV-1 Activities of Emtricitabine and Tenofovir plus the Integrase Inhibitor Elvitegravir or Raltegravir Show High Levels of Synergy In Vitro

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Highly active antiretroviral therapy (HAART) involves combination treatment with three or more antiretroviral agents. The antiviral effects of combinations of emtricitabine (FTC) plus tenofovir (TFV) plus antiretroviral agents of all the major drug classes were investigated. Combinations of FTC and TFV with a nonnucleoside reverse transcriptase inhibitor (NNRTI) (efavirenz or rilpivirine) or with a protease inhibitor (PI) (atazanavir, lopinavir, or darunavir) showed additive to synergistic activity. FTC-TFV with an HIV-1 integrase strand transfer inhibitor (INSTI) (elvitegravir or raltegravir) showed the strongest synergy. Anti-HIV-1 synergy suggests enhancement of individual anti-HIV-1 activities within cells that may contribute to potent treatment efficacy and open new areas of research into interactions between reverse transcriptase (RT) and integrase inhibitors.

**Highly active antiretroviral therapy (HAART)** is the current standard of care for HIV infection and involves treatment with a combination of three or more antiretroviral agents. Generally, these are combinations of two or more drug classes which target different steps of the HIV-1 replication cycle. The most extensively studied anti-HIV-1 drug combinations are those of nucleoside/nucleotide reverse transcriptase (RT) inhibitors (NRTIs) and nonnucleoside RT inhibitors (NNRTIs). NRTIs are competitive inhibitors of HIV-1 RT that cause chain termination of viral DNA polymerization and form the two-drug backbone of most regimens. The third agents are the different drug classes, consisting of NNRTIs (noncompetitive inhibitors of HIV-1 RT), protease inhibitors (PIs), and integrase strand transfer inhibitors (INSTIs). The first single-tablet regimen containing an INSTI was recently approved and consists of the two NRTIs emtricitabine (FTC) and tenofovir (TFV) disoproxil fumarate (TDF), an oral prodrug of TFV; the INSTI elvitegravir (EVG); and the pharmacoenhancer cobicistat (COBI), which increases EVG concentrations (1).

Combinations of antiviral inhibitors can directly affect the antiviral potency of their counterparts in an additive, antagonistic, or synergistic manner. Determination of the *in vitro* antiviral interactions between inhibitors used together in patients is an important component of the drug development process. Combinations that show antagonism should be avoided, and combinations that show synergy may have added benefit *in vivo*. There have been a number of studies showing that combinations of NRTIs plus NNRTIs inhibit HIV-1 infection more efficiently than the additive effect expected for the individual drugs studied alone, thus demonstrating synergy *in vitro* (2–9). For example, combinations of efavirenz (EFV)-TFV, EFV-FTC, rilpivirine (RPV)-TFV, and RPV-FTC have shown moderate to strong antiviral synergy against HIV-1 in cell culture (3, 10). Studies have also shown that some combinations within a drug class, such as two or more NRTIs, can act synergistically *in vitro* (11–17). In-depth studies have been performed on the combination of FTC and TFV, and these two drugs show synergy (by median-effect analysis, combination index range of 0.52 to 0.56) to strong synergy (by MacSynergy analysis, synergy volumes of 153 to 181 nM2%) against HIV-1 in cell culture (3, 10). This has been partially explained by a positive metabolic interaction between FTC and TFV that leads to higher levels of phosphorylation to the active metabolites when dosed in combination and more efficient trapping of TFV in a dead-end chain-terminated complex (3, 10, 17). Combinations of NRTIs or NNRTIs with INSTIs have also shown additive to synergistic effects *in vitro* (18, 19).

As combination therapies are the standard of care in HIV treatment, it is important to understand how newer inhibitors in different classes work in combination with existing therapies. This study evaluates the *in vitro* anti-HIV activity of three-drug combinations of FTC and TFV plus representatives from all the major drug classes—NNRTIs, PIs, and INSTIs.

**MATERIALS AND METHODS**

**Reagents.** TFV, FTC, EVG, atazanavir (ATV), darunavir (DRV), and COBI were synthesized at Gilead Sciences, Inc. Raltegravir (RAL) was purchased from Naefa Pharmaceutical, Inc. (Edmonton, Alberta, Canada). EFV and lopinavir (LPV) were purchased from Toronto Research Chemicals (North York, Ontario, Canada). RPV was synthesized by Janssen Infectious Diseases BVBA (Beerse, Belgium). Ribavirin (RBV) and zidovudine (AZT) were purchased from Sigma-Aldrich (St. Louis, MO). Stavudine (d4T) was provided by Bristol-Myers Squibb (Princeton, NJ).

**Susceptibility assays.** MT-2 cells were obtained from the NIH AIDS Research and Reference Reagent Program and were maintained as described previously (10). The cells were infected with the HIV-1 strain IIIb virus (Advanced Biotechnologies, Columbia, MD) or xXLA1 virus (20), as described previously (10). TFV, FTC, EVG, RAL, EFV, RPV, ATV, DRV, LPV, RBV, AZT, and d4T were each tested for effective concentrations that inhibited 50% of viral replication (EC50), determined using the

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GraphPad Prism (La Jolla, CA). After a 5-day incubation period at 37°C, the virus-induced cytopathic effect was determined using an XTT [2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide]-based colorimetric cell viability assay (21).

For the three-drug-combination studies, compounds were combined at a 1:1:1 ratio based on their EC₅₀. Eight concentrations of each combination were assayed in triplicate in 96-well plates, using 1.5-fold serial dilutions.

Drug combination data analysis. Three-drug-combination data were analyzed according to the median-effect principle of Chou and Talalay (22) using CalcuSyn software (version 2.0, Biosoft, Cambridge, United Kingdom). The EC₅₀s obtained with single antiviral agents were compared to the EC₅₀s obtained with the combination drugs. When studying RBV, which has no antiviral effect against HIV-1, a negligible dose response from 0% to 0.2% inhibition was entered into the software in order to model the synergy or antagonism effects seen with this drug. Virus inhibition values between 5% and 99% were used in the analysis; extrapolated values were not included. A combination index (CI) was calculated from the data as a measure of the interaction among drugs. CI values of <0.9 indicate synergy, CI values of 0.9 to 1.1 indicate an additive effect, and CI values of >1.1 indicate antagonism. The degree of synergy is proportional to the CI value; key values noted in this study showed CIs ranging from 0.3 to 0.7 (representing synergy), 0.7 to 0.85 (representing moderate synergy), and 3.3 to 10 (representing strong antagonism). The reported CI value for a combination of drugs is the average of the CI values at EC₅₀, EC₇₅, and EC₉₀ from each replicate experiment. To graph the data, all CI points from the replicate experiments were plotted on one graph for each combination, and the mean and 95% confidence interval lines were determined from these complete data sets.

RESULTS

Three-drug combinations of TFV, FTC, and other antiretroviral agents showed antiviral additivity or synergy against HIV-1 in cell culture. Three-drug-combination studies were performed with FTC-TFV plus representative agents of the other major drug classes—NNRTIs, PIs, and INSTIs (Fig. 1). The two NNRTIs studied were EFV and RPV. The EFV–FTC–TFV combination showed synergy as expected, with a CI value of 0.56 ± 0.08 (3). The RPV–FTC–TFV combination showed moderate synergy (CI, 0.73 ± 0.13) that was not statistically different from the EFV–FTC–TFV combination.

The three PIs studied were DRV, ATV, and LPV. The combinations of DRV–FTC–TFV and ATV–FTC–TFV both showed moderate synergy, with CI values of 0.77 ± 0.11 and 0.83 ± 0.19, respectively. The LPV–FTC–TFV combination showed additivity, with a CI value of 0.97 ± 0.10.

The INSTIs studied were Efavirenz and RAL. The Efavirenz–FTC–TFV combination showed synergy, with a mean CI value of 0.47 ± 0.09. The RAL–FTC–TFV combination was tested in parallel and showed synergy, with a mean CI value of 0.52 ± 0.05. The single-tablet regimen containing Efavirenz, COBI, FTC, and TDF also contains a pharmacokinetic enhancer, COBI, which lacks antiviral activity; the in vitro Efavirenz–FTC–TFV combination was tested with an overlay of 25 μM COBI to determine whether COBI altered the antiviral activity of the triple combination. This combination showed synergy comparable to that of the Efavirenz–FTC–TFV combination, with a mean CI value of 0.45 ± 0.06. The Efavirenz–FTC–TFV combination was significantly more synergistic than the RAL–FTC–TFV combination (P = 0.03) but was only numerically more synergistic than the Efavirenz–FTC–TFV combination (P = 0.15). The Efavirenz–FTC–TFV combination was significantly more synergistic than all three of the combinations of FTC–TFV with PIs (P = 0.013 for ATV, P = 0.009 for DRV, and P = 0.002 for LPV). We also carried out the isobologram analysis (23, 24) for the combinations of FTC–TFV with the representative NNRTIs, PIs, and INSTIs, and found similar synergy results (data not shown).

Control experiments combining FTC, FTC, and FTC yielded the expected additivity, with a mean CI value of 0.92 ± 0.06. The d4T–AZT–RBV combination was tested as an antagonism control, since previous reports have shown RBV–d4T to be strongly antagonistic (25, 26), RBV–AZT to be strongly antagonistic (25), and d4T–AZT to be additive to antagonistic (27, 28). The triple combination d4T–AZT–RBV showed strong antagonism, with a mean CI value of >5.9.

Overall, the combination of FTC–TFV and the INSTIs showed the highest level of synergy, with no evidence of antagonism (Fig. 2). The combinations of FTC–TFV with antiretroviral agents from the NNRTI and PI classes showed synergy or additivity, with no evidence of antagonism.

DISCUSSION

This study investigated the anti-HIV-1 activity of three-drug combinations consisting of FTC and TFV plus a third antiretroviral agent from one of the three major drug classes—NNRTIs, PIs, and INSTIs. The three-drug combination of FTC–TFV with the INSTIs EVG and RAL synergized with the NNRTIs EFV and RPV or the PIs DRV and ATV all showed antiviral synergy in cell culture. Additive antiviral activity was found for FTC–TFV and LPV. The strongest synergy observed was with the combinations of FTC–TFV with the INSTIs EVG and RAL. Synergy between the NNRTI backbone FTC–TFV has been attributed to elevations in the levels of the active metabolites of FTC and TFV when dosed together in cells and to enhanced dead-end complex formation of TFV–terminated DNA and HIV–1 RT in the presence of FTC–triphosphate (TP) (3, 17).

Three-drug combinations of FTC–TFV with two agents from the INSTI class showed comparable and high-level synergy. EVG and RAL have a similar mechanism of action of inhibiting integrase (IN) strand transfer activity, which is required for the integration of HIV–1 DNA into the host genome (29). Previous studies have shown additive to synergistic inhibition of HIV–1 infection using a combination of the integrase inhibitor RAL plus FTC or TFV (30) and EVG plus FTC or TFV (19). Therefore, synergy in vitro was not unexpected.

Clinical studies of INSTIs with or without FTC/TDF have shown durable efficacy (31–39). The INSTIs as a class and by themselves elicit a more rapid viral load decline than that observed for any other drug class. Monotherapies with the EVG or RAL INSTI have shown significant drops in viral load over 10 days, with average decreases of about 2.2 log₁₀ HIV–1 RNA copies/ml (31, 32). Further, when dosed in a combination study of treatment-naive subjects receiving tenofovir and lamivudine (3TC) as a background regimen, subjects taking RAL had a more rapid decline in HIV–1 RNA than subjects taking the EVG–3TC–TDF regimen, although both arms reached the same reduction in viral load by week 24 (40). Clinical studies comparing Efavirenz–COBI– FTC–TFV with EVF–FTC–TFV or ATV–ritonavir–FTC–TFV had the similar result of a transiently more rapid viral load suppression (36, 37). The finding in this study that NRTIs with INSTIs showed the highest level of synergy may contribute to the clinical efficacy of INSTIs with FTC–TFD.

Potential underlying mechanisms of rapid HIV–1 RNA decreases with INSTIs have been proposed (41, 42). The most widely
FIG 1 Three-drug combination and control results. Three-drug antiviral synergy plots of the combination index (CI; synergy score) versus the fractional effect (level of inhibition of viral replication). Dotted black line, additivity line indicating a CI value of 1; solid red line, mean synergy curve fit line; dotted red line, 95% confidence interval. CI values were calculated as described in Materials and Methods, using CalcuSyn. These numbers represent the mean and standard deviation of at least three independent experiments.
accepted model is that the INSTIs uniquely block HIV-1 replication in cells that have been infected and have produced the viral double-stranded DNA genome but have not yet undergone integration into the host genome (preintegration latency) (41). The first-phase viral load decay rate is longer for INSTIs than for NNRTIs (43), potentially because they act later in the HIV-1 replication cycle (42). Although INSTIs show a rapid viral load decline in monotherapy studies, and the synergy of FTC with TFV is well established, the finding that INSTIs combined with NRTIs showed the highest levels of synergy does not appear solely attributable to a simple combination of two classes of drugs since the NRTIs with PIs were significantly less synergistic. RT and IN are both present in HIV-1 preintegration complexes (PICs) (44), so there may be an interaction between these two enzymes or the drugs that target them. Studies have shown that the IN protein is required for efficient reverse transcription and may interact physically with RT or other components of the RT initiation complex (45, 46). Other studies with noncatalytic site integrase inhibitors (NCINIs) showed that these inhibitors block viral replication by targeting an IN-dependent step during virus production that subsequently causes a defect in reverse transcription in newly infected target cells (47, 48). Additionally, the INSTI drug to HIV-1 double-stranded DNA target ratio may be increased in the presence of NRTIs leading to better inhibition of the virus with INSTIs. This study supports these observations and hypotheses by showing that combinations of FTC-TFV with a third agent from one of the major drug classes, INSTIs, NNRTIs, or PIs, all showed additive to synergistic anti-HIV-1 activity in vitro. The strongest synergy was seen with the combinations of EVG or RAL with FTC-TFV, and this may contribute to the durable clinical antiviral efficacy observed for these drug regimens, which are both recommended as preferred INSTI-based regimens for ART-naive patients (49).

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REFERENCES


