Potency of Nonnucleoside Reverse Transcriptase Inhibitors (NNRTIs) Used in Combination with Other Human Immunodeficiency Virus NNRTIs, NRTIs, or Protease Inhibitors

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Efavirenz and a series of related quinazolinone nonnucleoside inhibitors of the human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) were evaluated in a series of two-drug combinations with several nucleoside RT inhibitors (NRTIs), nonnucleoside RT inhibitors (NNRTIs), and protease inhibitors (PIs). These combinations were tested in an established HIV-1 RT enzyme assay and a cell-based yield reduction assay with HIV-1 (replicative form [RF])-infected MT-2 cells. Synergy, additivity, and antagonism were determined in the two different assay systems by the method of Chou and Talalay (T.-C. Chou and P. Talalay, Adv. Enzyme Reg. 22:27-55, 1984). Efavirenz, DPC082, DPC083, DPC961, and DPC963 used in combination with the NRTIs zidovudine and lamivudine acted synergistically to inhibit RT activity in the HIV-1 RT enzyme assay and additively to slightly synergistically to inhibit HIV-1 (RF) replication in the yield reduction assay. The five NNRTIs in combination with the PI nelfinavir acted additively in the yield reduction assay to inhibit HIV-1 replication. Interestingly, efavirenz in combination with a second NNRTI acted additively to inhibit HIV-1 RT function in the enzyme assay, while it acted antagonistically to inhibit HIV-1 (RF) replication in the yield reduction assay. These data suggest that antiretroviral combination regimens containing multiple NNTRIs should be given thorough consideration before being used.

An essential step in the replication of human immunodeficiency virus (HIV) is the reverse transcription of viral RNA to proviral DNA by the virus-encoded reverse transcriptase (RT) (2). Inhibition of the function of this enzyme by either genetic or chemical means results in the suppression of virus replication (23). In fact, several nucleoside RT inhibitors (NRTIs) and nonnucleoside RT inhibitors (NNRTIs) have shown in vivo efficacy in the clinic when used in combination with other anti-HIV agents and are recommended for the treatment of individuals infected with HIV (10).

Combination therapy is necessary because monotherapy with any of the currently approved anti-HIV drugs results in the rapid selection of preexisting, drug-resistant variants from the genetically diverse population of virus present in the infected individual (23). Long-term highly active antiretroviral therapy has led to prolonged improvements in such surrogate disease markers as CD4-cell counts and HIV RNA titers in plasma (25, 26). In addition, combinations that consist of multiple NRTIs and an NNRTI may have synergistic effects on the inhibition of viral replication exerted by one or more of the drugs that make up the combination. For example, zidovudine (AZT)-resistant variants of HIV that acquire resistance to lamivudine (3TC) in response to selection with 3TC may regain susceptibility to AZT. Likewise, AZT-resistant variants that become resistant to NNRTIs may, in some cases, regain AZT sensitivity (17, 18). Demonstration of this synergistic effect on the RT at the enzymatic or cellular level may be de-

monstrable by one or more of the several available in vitro assays (3, 15, 16, 19).

Efavirenz, a potent NNRTI, has been approved for use in combination with other antiretroviral drugs for the treatment of HIV type 1 (HIV-1) infection. We have used two different in vitro systems to determine the effects that other antiretroviral drugs have on the potency of efavirenz when they are used in combination to inhibit HIV-1 replication or RT activity. The first system is a yield reduction assay in which we determine the abilities of several anti-HIV compounds, alone and in combination, to inhibit the replication of HIV-1 (replicative form [RF]) in MT-2 cells (21, 22). The second system is an in vitro RT enzyme assay that has been used to identify inhibitors of HIV-1 RT activity (6). This enzyme assay allows measurement of interactions against the target enzyme without the confounding contributions of intracellular processes (i.e., intracellular compartmentalization or interactions with proteins other than the target protein). Furthermore, nucleoside analogs may be compared in the enzyme assay as the preactivated triphosphate (TP) forms, precluding competition for cellular kinases, which may influence the outcome of the cell-based antiviral assay.

With the increasing number of antiretroviral agents available for use in combination-therapy regimens for the treatment of HIV-1 infection, it is becoming imperative that these agents be tested in a system that will predict if a particular combination of antiretroviral agents adversely affects the compounds' expected potencies. For example, data from in vitro enzyme- and cell-based systems have led to the prediction that the combination of AZT and stavudine (d4T) may be less efficacious than either compound alone due to the antagonistic effect that the combination has on the cellular thymidine kinase (13, 20, 28). Recently, this was found to be true in the

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TABLE 1. Potencies and conformity parameters for NNRTIs, NRTIs, and NFV against HIV-1 RT activity and HIV-1 (RF) replication[a]

Compound group and compound	HIV-1 (RF) replication		HIV-1 RT activity	
	IC_{50} (μM)[b]	r^2	IC_{50} (μM)[b]	r^2
NNRTIs				
Efavirenz	0.0007 ± 0.0004	0.966	0.041 ± 0.012	0.981
DPC082	0.0004 ± 0.0001	0.966	0.054 ± 0.020	0.993
DPC083	0.0003 ± 0.0001	0.950	0.041 ± 0.0009	0.976
DPC961	0.0004 ± 0.0003	0.926	0.024 ± 0.004	0.973
DPC963	0.0004 ± 0.0002	0.967	0.019 ± 0.003	0.979
NVP	0.018 ± 0.003	0.938	2.34 ± 0.21	0.985
NRTIs				
AZT	0.022 ± 0.014	0.973	0.0029 ± 0.0004	0.990
3TC	0.153 ± 0.023	0.987	1.45 ± 0.53	0.991
PI, NFV	0.005 ± 0.001	0.959	ND[c]	ND

[a] All values are derived from a minimum of 10 independent experiments for each compound in each assay.
[b] Values are means ± standard deviations.
[c] ND, not determined.

clinical setting, in which HIV-1-infected patients responded better to monotherapy with d4T than to combination therapy with d4T and AZT (11).

Previously, it was reported that when efavirenz was used in combination with AZT-TP in an in vitro HIV-1 RT enzyme assay, these two compounds acted synergistically to inhibit HIV-1 RT activity when activity was measured in the 92 to 97% fractional inhibitory concentration (FIC) range but additively when activity was measured in the 80 to 88% FIC range (3). We have extended this study and found that efavirenz and four related quinazolinone derivatives of efavirenz, DPC082, DPC083, DPC961, and DPC963, used in combination with the NRTIs AZT and 3TC (or AZT-TP and 3TC-TP in the HIV-1 RT enzyme assay) acted synergistically in both the cell-based and enzyme systems.

Interestingly, when DPC083, DPC961, and DPC963 were used in combination with efavirenz, they acted additively to slightly antagonistically to inhibit RT activity in the HIV-1 RT enzyme assay but acted antagonistically to inhibit HIV-1 (RF) replication in MT-2 cells. Furthermore, DPC082 and nevirapine (NVP) in combination with efavirenz acted antagonistically in both assays.

MATERIALS AND METHODS

Chemical reagents. Efavirenz, DPC082, DPC083, DPC961, and DPC963 were synthesized as described previously (6). AZT, d4T, and the triphosphorylated forms of AZT and d4T were purchased from Sigma Chemical Co. (St. Louis, Mo.). 3TC and 3TC-TP were generous gifts from Raymond Schinazi (Emory University, Atlanta, Ga.). Nelfinavir (NFV) and NVP were chromatographically isolated from the corresponding marketed products.

Recombinant poly(I) [poly(rI)] and oligo(dC)$_{15}$ were purchased from Sigma Chemical Co. Poly(rA)–oligo(dT)$_{12\text{-}18}$, TTP, and dCTP were obtained from Amersham Pharmacia Biotechnology. Recombinant HIV-1 RT, [^{3}H]TTP, and [^{3}H]dCTP were purchased from New England Nuclear (Boston, Mass.). Ninety-six-well DEAE filtration plates were produced by Millipore.

Virus and cell lines. HIV-1 (RF), a cell-culture-adapted, limited-passage virus, was obtained from Robert Gallo (National Institutes of Health, Bethesda, Md.) as infected cultures of H9 cells. Virus titers were quantified by plaque assay on MT-2 cells as described previously (21, 22, 25). MT-2 cells, human lymphoblastoid cells transformed with human T-cell lymphotrophic virus type 1, were obtained from David Montefiori (Vanderbilt University, Nashville, Tenn.). The cells were maintained in RPMI 1640 plus 5% (vol/vol) fetal bovine serum and gentamicin (5 μg/ml) at 37°C in a 5% CO_2 atmosphere.

HIV-1 RT in vitro enzyme assay. HIV-1 RT (0.14 or 0.28 nM) and various combinations and concentrations of the RT inhibitors were mixed and incubated for approximately 6 min at room temperature. For the combination experiments involving nucleoside analogs, the triphosphorylated forms were used. At the conclusion of this incubation period, a template-primer mixture consisting of poly(rA)–oligo(dT)$_{12\text{-}18}$ or poly(rI)–oligo(dC)$_{15}$ (final concentrations, 2.5 and 8 μg/ml, respectively) and [^{3}H]TTP or [^{3}H]dCTP (5 μM) were added, and the mixture was incubated at 37°C for 45 min. The nucleic acid was captured on a DEAE ion-exchange membrane. RT activity was quantified by measuring the level of incorporation of [^{3}H]deoxyribonucleotide into the growing DNA strand (6).

HIV yield reduction assay. MT-2 cells were infected with HIV-1 (RF) at a multiplicity of infection of 0.02 in the presence of various combinations and concentrations of several anti-HIV compounds and were incubated at 36°C in a 3% CO_2 atmosphere. Three days after infection, the progeny virus were collected, clarified by high-speed centrifugation, and stored at −70°C (21, 22). The concentration of virus in each sample was determined by plaque assay on MT-2 cell monolayers (24).

Combination experiments. The compounds were combined at concentrations near their experimentally determined 50% inhibitory concentrations (IC_{50}s). The mixture was then serially diluted 32-fold by use of 2-fold dilutions. This dilution set was designated the 1:1 combination. Ratios of drug combinations of 3:1 and 1:3 were also used.

Combination indices (CIs) were calculated by using the model of Chou and Talalay (5) for drug combinations and the CalcuSyn software package (BioSoft). Each experiment with drug combinations was performed between two and five times, with each combination being tested in duplicate, according to the design of the experiment. The CIs between experiments for identical combinations varied less than 15%, and the CIs between internal duplicates varied less than 10%. CIs <0.8 denote synergy, CIs >0.8 but <1.2 denote additivity, and CIs >1.2 denote antagonism.

RESULTS

Effects of NNRTIs, NRTIs, and PIs on HIV-1 replication and HIV-1 RT activity. In the combination experiments described below, the ratios of the compounds used were based on the IC_{50} of each compound determined in the HIV-1 RT enzyme assay and the HIV-1 (RF) yield reduction assay. For example, in the experiments in which the ratio of the compounds is 1:1, the first dilution contains each compound at its respective IC_{50}. Likewise, for the 3:1 and 1:3 ratios, the first dilution contains one compound at its IC_{50} and the other at one-third the concentration of its IC_{50}.

We established the IC_{50}s of the individual compounds under investigation in both the HIV-1 RT enzyme assay and the yield

TABLE 2. CIs[a] of combinations of NNRTIs and AZT (or AZT-TP) in two-compound combinations against HIV-1 RT activity and the replication of HIV-1 (RF)

Compound tested with AZT	Ratio[b]	CI against HIV replication at:			CI against RT activity at:		
		IC_{50}	IC_{75}	IC_{90}	IC_{50}	IC_{75}	IC_{90}
Efavirenz	1:3	0.77	0.59	0.45	0.84	0.71	0.61
	1:1	0.76	0.64	0.54	0.71	0.61	0.54
	3:1	0.71	0.64	0.59	0.72	0.61	0.52
DPC083	1:3	0.81	0.72	0.63	0.55	0.53	0.44
	1:1	0.81	0.58	0.51	0.55	0.41	0.35
	3:1	1.04	0.96	0.92	0.56	0.45	0.35
DPC961	1:3	0.82	0.72	0.58	0.70	0.55	0.43
	1:1	0.74	0.76	0.76	0.63	0.50	0.39
	3:1	0.84	0.76	0.69	0.82	0.55	0.37
DPC963	1:3	0.90	0.65	0.53	1.16	0.75	0.49
	1:1	0.88	0.73	0.65	0.64	0.53	0.44
	3:1	0.78	0.72	0.66	0.57	0.53	0.49

[a] CIs were calculated by using the model of Chou and Talalay (5) for drug combinations and the CalcuSyn software package (BioSoft). CIs <0.8, >0.8 but <1.2, and >1.2 indicate-synergy, additivity, and antagonism, respectively.
[b] The ratio represents the biologically relevant IC_{50} ratio in which the two compounds were combined according to their individual IC_{50}s determined by the HIV-1 RT and yield reduction assays.

reduction assay (Table 1). All compounds inhibited HIV-1 RT activity and HIV-1 (RF) replication in dose-dependent manners.

Efavirenz and related NNRTIs in combination with NRTIs. Treatment with efavirenz has been demonstrated to result in potent, durable suppression of HIV-1 replication in combination regimens with AZT and 3TC (26). We thus examined combinations of efavirenz or the quinazolinone NNRTIs with AZT and 3TC in vitro. Our results with the combination of efavirenz and AZT-TP in the HIV-1 RT assay agreed with those previously described by Carroll et al. (3), in that these two compounds acted synergistically to inhibit the activity of HIV-1 RT (Table 2). We also found similar results for the combination of efavirenz and 3TC-TP (Table 3). Furthermore,

these two combinations also acted synergistically to inhibit HIV-1 (RF) replication in infected MT-2 cells.

Like efavirenz, the quinazolinone NNRTIs DPC083, DC961, and DPC963 acted synergistically with AZT-TP to inhibit RT activity in the HIV-1 RT enzyme assay and virus replication in the yield reduction assay (Table 2). In combination with 3TC-TP, the three quinazolinone derivatives of efavirenz acted synergistically to inhibit the activity of the HIV-1 RT in the enzyme assay, and the combination of DPC083 and 3TC acted synergistically to inhibit HIV-1 (RF) replication (Table 3).

Efavirenz and related NNRTIs in combination with NFV. One strategy used to minimize the risk of selection of drug-resistant variants of HIV-1 during therapy is to use an antiret-

TABLE 3. CIs[a] of combinations of NNRTIs and 3TC (or 3TC-TP) in two-compound combinations against HIV-1 RT activity and the replication of HIV-1 (RF)

Compound tested with 3TC	Ratio[b]	CI against HIV replication at:			CI against RT activity at:		
		IC_{50}	IC_{75}	IC_{90}	IC_{50}	IC_{75}	IC_{90}
Efavirenz	1:3	0.69	0.71	0.78	0.68	0.47	0.34
	1:1	0.63	0.67	0.77	0.66	0.43	0.33
	3:1	0.59	0.61	0.61	0.63	0.51	0.44
DPC083	1:3	0.63	0.86	0.79	0.46	0.54	0.52
	1:1	0.60	0.67	0.76	0.43	0.42	0.43
	3:1	0.46	0.53	0.61	0.57	0.44	0.42
DPC961	1:3	ND[c]	ND	ND	0.51	0.40	0.33
	1:1	ND	ND	ND	0.41	0.38	0.36
	3:1	ND	ND	ND	0.51	0.44	0.39
DPC963	1:3	ND	ND	ND	0.60	0.61	0.60
	1:1	ND	ND	ND	0.51	0.48	0.49
	3:1	ND	ND	ND	0.53	0.49	0.46

[a] CIs were calculated by using the model of Chou and Talalay (5) for drug combinations and the CalcuSyn software package (BioSoft). CIs <0.8, >0.8 but <1.2, and >1.2 indicate synergy, additivity and antagonism, respectively.
[b] The ratio represents the biologically relevant IC_{50} ratio in which the two compounds were combined according to their individual IC_{50}s determined by the HIV-1 RT and yield reduction assays.
[c] ND, not determined.

TABLE 4. CIs[a] of combinations of NNRTIs and NFV in two-compound combinations against the replication of HIV-1 (RF)

Compound tested with NFV	Ratio[b]	CI at:			r^2
		IC_{50}	IC_{75}	IC_{90}	
Efavirenz	1:3	1.15	0.81	0.58	0.99
	1:1	0.98	0.66	0.44	0.99
	3:1	1.38	0.81	0.49	0.99
DPC082	1:3	0.93	0.99	1.05	0.99
	1:1	0.77	0.63	0.52	1.00
	3:1	0.73	0.58	0.47	0.99
DPC083	1:3	1.48	0.86	0.53	1.00
	1:1	1.19	0.67	0.41	0.99
	3:1	1.62	0.96	0.59	0.96
DPC961	1:3	0.77	0.54	0.40	0.96
	1:1	0.28	0.47	0.80	0.96
	3:1	0.86	0.65	0.47	0.99
DPC963	1:3	1.08	0.99	0.94	0.98
	1:1	1.01	1.00	0.91	0.97
	3:1	0.99	0.89	0.84	0.99

[a] CIs were calculated by using the model of Chou and Talalay (5) model for drug combinations and the CalcuSyn software package (BioSoft). CIs <0.8, >0.8 but <1.2, and >1.2 indicate synergy, additivity, and antagonism, respectively.
[b] The ratio represents the biologically relevant IC_{50} ratio in which the two compounds were combined according to their individual IC_{50}s determined by the yield reduction assays.

roviral drug regimen that affects as many different antiviral targets as possible. Thus, treatment regimens may include a drug from each of the NNRTI, NRTI, and PI classes. To ascertain the effect on total antiviral activity when efavirenz or any of the quinazolinone NNRTIs is used in combination with a PI, we determined the changes in the expected potencies, if any, of the NNRTIs and the PI NFV when they were used in combination to inhibit HIV-1 replication in MT-2 cells (Table 4). We found that, as expected, the combination of any of the NNRTIs with NFV acted additively to synergistically to inhibit HIV-1 replication.

Efavirenz combination with NNRTIs. Because of their potent antiviral activities and low pill burdens, dual NNRTI therapy represents an attractive treatment option. To ascertain the antiviral activities of two NNRTIs used in combination, we examined the effects of efavirenz in combination with the quinazolinone NNRTIs DPC082, DPC083, DC961, and DPC963 as well as NVP (Table 5). Efavirenz in combination with DPC083, DPC961, and DPC963 acted additively in the HIV-1 yield reduction assay but additively to slightly antagonistically in the HIV-1 RT enzyme assay. The combination of efavirenz and DPC082 was additive to slightly antagonistic in the HIV-1 (RF) yield reduction assay but was antagonistic in the HIV-1 RT enzyme assay. Finally, the combination of efavirenz and NVP was consistently antagonistic in both assays.

DISCUSSION

Efavirenz, DPC082, DPC083, DPC961, and DPC963 are highly potent and selective inhibitors of HIV-1 RT activity and HIV-1 replication in cell culture (6). However, because of the rapid selection of drug-resistant variants and the greater success of multidrug regimens in the clinic, these compounds would not be used as initial monotherapy but instead would be used as part of multidrug combinations. The success of a multidrug regimen in the clinic is dependent not only on pharmacokinetic and pharmacodynamic parameters but also on any effect that the drugs that make up the regimen may have on each other at the enzymatic and cellular levels.

In vitro drug interaction studies have shown that NNRTIs can act synergistically with an NRTI to inhibit HIV-1 RT

TABLE 5. CIs[a] of combinations of NNRTIs in two-compound combinations against HIV-1 RT activity and the replication of HIV-1 (RF)

Compound tested with efavirenz	Ratio[b]	CI against HIV replication at:			CI against RT activity at:		
		IC_{50}	IC_{75}	IC_{90}	IC_{50}	IC_{75}	IC_{90}
DPC082	1:3	1.90	2.18	2.53	1.90	2.22	2.60
	1:1	1.09	1.17	1.28	1.45	1.68	1.96
	3:1	1.36	1.10	0.90	1.26	1.33	1.43
DPC083	1:3	3.55	3.38	3.44	0.81	0.92	1.08
	1:1	2.47	2.39	2.35	1.12	1.18	1.28
	3:1	4.07	3.16	2.47	0.88	1.02	1.23
DPC961	1:3	4.03	2.69	2.06	1.07	0.98	0.90
	1:1	3.39	3.57	4.47	1.12	1.16	1.21
	3:1	3.96	2.31	1.56	1.10	1.20	1.31
DPC963	1:3	4.74	2.49	1.69	1.05	0.95	0.86
	1:1	4.44	2.88	2.17	1.14	1.13	1.13
	3:1	4.78	2.85	1.97	1.45	1.15	0.91
NVP	1:3	1.28	2.83	6.92	1.50	1.89	2.52
	1:1	1.24	2.25	4.26	1.51	1.60	2.67
	3:1	3.17	2.76	2.42	1.62	1.89	2.52

[a] CIs were calculated by using the model of Chou and Talalay (5) for drug combinations and the CalcuSyn software package (BioSoft). CIs <0.8, >0.8 but <1.2, and >1.2 indicate synergy, additivity, and antagonism, respectively.
[b] The ratio represents the biologically relevant IC_{50} ratio in which the two compounds were combined according to their individual IC_{50}s determined by the HIV-1 RT and yield reduction assays.

activity and/or HIV-1 replication (1, 3, 4). In clinical studies, combinations of an NNRTI with one or more NRTIs result in significant improvements in patients' clinical markers (7, 9, 26). However, the apparent efficacies of the combinations in the clinical setting cannot be necessarily attributed to synergistic effects at the enzymatic or cellular level since other factors such as pharmacological synergy, decreased selection of drug-resistant variants, and genotypic changes in the virus population in the presence of multiple drugs may also affect their potencies.

We found that efavirenz, DPC083, DPC961, and DPC963 acted synergistically to inhibit RT activity when they were used in combination with the NRTIs AZT-TP or 3TC-TP in the HIV-1 RT enzyme assay. The synergism seen with the combination of efavirenz and AZT-TP in the RT assay agreed with data previously published by Carroll et al. (3). Moreover, we also observed that the synergism of these combinations increased (as determined by decreased CIs) as the levels of inhibition of RT activity or HIV-1 replication increased. Similar results were obtained with the quinazolinone derivatives of efavirenz and AZT-TP. Furthermore, combinations of the four NNRTIs and 3TC-TP also acted synergistically to inhibit HIV-1 RT activity. Although the mechanism for the synergy seen by use of these NNRTIs plus AZT and 3TC is not known, it is conceivable that the interaction of the NNRTI with the RT at the NNRTI binding site, which has been shown to cause conformational distortion of the catalytic aspartate triad (8), may allow improved incorporation of the NRTI into the growing DNA molecule, leading to more efficient chain termination or decreased rates of phosphorolytic removal of the terminator. Any and all of these effects would result in apparent synergy in the overall level of inhibition observed.

In the virus replication assay, all NNRTI-NRTI combinations acted additively to slightly synergistically. Since the combinations were less synergistic in the cell-based system than in the enzyme system, the cellular environment must, in some way, affect the interaction of these two classes of compounds. One possible explanation for this observation is that the cellular environment mitigates some of the molecular rearrangement in the HIV-1 RT that occurs in the "test tube" environment. Alternately, the cell could affect the intracellular concentrations of drug such that the concentrations or ratios are in the range at which they act more additively to inhibit enzyme function. This could be through alteration of the amount of drug taken up by the cell, the intracellular half-life, or the rate of elimination.

Since it is not possible to determine the effect that combinations of NNRTIs and PIs have on each other's potencies in a single enzyme assay, these combinations were analyzed only by the yield reduction assay. As expected, we found that the combinations of the NNRTIs efavirenz, DPC082, DPC083, DPC963, and DPC963 and the PI NFV acted additively to synergistically to inhibit HIV-1 (RF) replication in MT-2 cells. Synergy between inhibitors of the HIV-1 RT and protease may be the result of effects on reduced virus levels in later rounds of infection in in vitro assays caused by agents that act early instead of late in the virus replication cycle.

We found that the combination of efavirenz and NVP was the only NNRTI-NNRTI combination that consistently acted strongly antagonistically in both the HIV-1 RT enzyme assay and the virus replication assay. Since antagonism was seen in both assays, we can assume that the effect observed occurred at the level of the enzyme and was not a result of an effect on a cellular process. It is possible that this antagonism could be a result of very different affinities of the HIV-1 RT enzyme for the two compounds. A similar explanation has been forwarded to explain the antagonism seen with the combination of AZT and d4T. In vitro, mitogen-activated thymidine kinase, which is responsible for converting these two compounds to their monophosphate forms inside the cell, has a 600-fold greater affinity for AZT than d4T (12). This results in a very low conversion of d4T to d4T-TP and a lower than expected level of activated AZT, leading to a weaker total antiviral effect than that of either compound alone.

We observed that efavirenz in combination with any of the related NNRTIs acted antagonistically in the yield reduction assay, while it acted additively to slightly antagonistically in the RT enzyme assay. While it is predictable that these combinations should have acted additively to inhibit HIV-1 RT in the enzyme assay, it is not easily explained why these compounds acted antagonistically in the cellular environment. Since there was no change in the expected and observed potencies of the drug combinations in the RT enzyme assay, the antagonism seen in the virus assay must be a consequence of a cellular process that influences the potencies of these compounds. As with the NNRTI-NRTI combinations in the virus replication assay, we speculate that these drug combinations may affect such cellular processes that influence compound uptake, localization, intracellular half-life, and/or elimination. For example, NNRTIs of the benzoxazenone and quinazolinone classes are highly protein bound (6); equilibration with nonspecific intracellular binding sites may be altered with multiple competing inhibitors.

Interestingly, a recent study claimed that the combination of efavirenz and NVP acted synergistically to inhibit HIV-1 replication in fresh peripheral blood mononuclear cells (15). We do not know the reason for such a disconnect in their results and those that we report here, but it may be because vastly different biological models (i.e., clinical isolates in fresh peripheral blood mononuclear cells versus HIV-1 [RF] in MT-2 cells) and different experimental procedures (e.g., the method used to treat the virus with drug, the method used to detect viable virus, and the model used to calculate CIs) were used in the two studies. Others have previously shown that the concentrations and ratios of compounds used can greatly affect the interactions of two compounds used in combination in vitro (12; D. P. Merrill, T.-C. Chou, and M. S. Hirsch, Reply, J. Infect. Dis. **174:**672, 1996). In our own studies with two NNRTIs used in combination, we found that by greatly altering the compound ratios, we could affect the final CIs (data not shown).

To attempt to make our model as relevant as possible, we have incorporated the following. First, we have set up our compound concentrations and ratios to be what we believe are biologically relevant. For instance, in our 1:1 ratio, the initial dilution contains each compound at it IC_{50}, whereas the 1:3 and 3:1 ratios contain one compound at its IC_{50} and the other compound at one-third its IC_{50}. In all three cases, the initial dilution is then serially diluted 32-fold by use of 2-fold dilutions. Second, we have determined that our results are in agreement with those that have been published previously. For

example, with the combination of efavirenz and AZT-TP, we have obtained results similar to those obtained by Carroll et al. (3) (Table 2); and we found that the combination of AZT and d4T acted additively in the HIV-1 RT enzyme assay and antagonistically in the virus replication assay (data not shown), in agreement with the results of Hoggard et al. (13) and Zhu et al. (28). Finally, we tested efavirenz in combination with itself and found that it acted, as expected, additively to inhibit both HIV-1 RT function in the enzyme assay and HIV-1 replication in the yield reduction assay (data not shown).

It is unknown if our in vitro observations of the antagonistic action of efavirenz plus another NNRTI would translate to antagonistic action in the clinical situation; however, there is precedence for these in vitro models to be predictive of the clinical situation (11, 13). The best example is the combination of AZT and d4T. Although there is some disagreement in the literature whether this combination acts additively or antagonistically in an in vitro cell-based system to inhibit HIV-1 replication (13, 20, 28), this combination has been shown to interact antagonistically with the cellular thymidine kinase (13). The different results seen in the two HIV-1 replication systems are believed to be due more to differences in the concentrations and the ratios of the compounds tested than to a difference in the cell types or virus strains used (12; Merrill et al., reply). In the clinic, the combination of AZT and d4T was found to be inferior to d4T alone (11). HIV-1-infected patients who were treated with the combination showed greater declines in CD4-cell count and lesser decreases in plasma HIV-1 RNA levels from the baseline levels than patients who were treated with d4T. Thus, in this case, the in vitro model was predictive of the clinical outcome.

The results of this study suggest that the effectiveness of antiretroviral treatment regimens that include efavirenz or one of the related quinazolinone NNRTIs in combination with NRTIs or PIs should not be adversely affected. However, antiretroviral combination regimens that may contain efavirenz together with another NNRTI may result in less than expected antiviral activity. This may be especially true when drug-naive HIV-1 is the target virus population. Interestingly, HIV-1-infected individuals who were undergoing cotreatment with efavirenz and NVP had lower levels of exposure to efavirenz than HIV-1-infected individuals who were treated only with efavirenz (29). Nevertheless, results from a preliminary clinical study in which efavirenz, NVP, and didanosine were used in combination to treat HIV-1-infected individuals showed that a double NNRTI and NRTI combination may be as effective as a regimen containing an NNRTI, a PI, and an NRTI (W. Jordan, R. Jefferson, F. Yemofio, L. Tolber, V. Conlan, H. Carroll, D. C. Green, A. Green, and R. Green, Abstr. XIII Int. AIDS Conf., 2000). Additional studies to determine the clinical effectiveness of dual NNRTI therapy are ongoing (clinical study 2NN, sponsored by Boehringer Ingleheim).

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