Activities of the Human Immunodeficiency Virus Type 1 (HIV-1) Protease Inhibitor Nelfinavir Mesylate in Combination with Reverse Transcriptase and Protease Inhibitors against Acute HIV-1 Infection In Vitro

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Nelfinavir mesylate (formerly AG1343) is a potent and selective, nonpeptidic inhibitor of human immunodeficiency virus type 1 (HIV-1) protease that was discovered by protein structure-based design methodologies. We evaluated the antiviral and cytotoxic effects of two-drug combinations of nelfinavir with the clinically approved antiretroviral therapies zidovudine (ZDV), lamivudine (3TC), didanosine (ddI), stavudine (d4T), stavudine (d4T), didanosine (ddI), indinavir, saquinavir, and ritonavir and a three-drug combination of nelfinavir with ZDV and 3TC against an acute HIV-1 strain RF infection of CEM-SS cells in vitro. Quantitative assessment of drug interaction was evaluated by a universal response surface approach (W. R. Greco, G. Bravo, and J. C. Parsons, Pharm. Rev. 47:331–385, 1995) and by the method of M. N. Prichard and C. Shipman (Antiviral Res. 14:181–206, 1990). Both analytical methods yielded similar results and showed that the two-drug combinations of nelfinavir with the reverse transcriptase inhibitors ZDV, 3TC, ddl, d4T, and ddC and the three-drug combination with ZDV and 3TC resulted in additive to statistically significant synergistic interactions. In a similar manner, the combination of nelfinavir with the three protease inhibitors resulted in additive (ritonavir and saquinavir) to slightly antagonistic (indinavir) interactions. In all combinations, minimal cellular cytotoxicity was observed with any drug alone and in combination. These results suggest that administration of combinations of the appropriate doses of nelfinavir with other currently approved antiretroviral therapeutic agents in vivo may result in enhanced antiviral activity with no associated increase in cellular cytotoxicity.

To date, several antiretroviral drugs have been approved for use in the treatment of individuals infected with human immunodeficiency virus (HIV). These include the nucleoside analogs and the nonnucleoside reverse transcriptase (RT) inhibitors zidovudine (ZDV), stavudine (d4T), zalcitabine (ddC), lamivudine (3TC), and nevirapine, which target RT, an enzyme which functions early in the HIV life cycle. More recently, a second class of antiretroviral agents has been approved. These agents target the viral protease, an enzyme which functions at a late stage of the virus life cycle. The HIV protease inhibitors approved to date include indinavir (MK-639), ritonavir (ABT-538), saquinavir (Ro 31-8959), and, most recently, nelfinavir (AG1343). Although all of these agents have been shown to have antiviral activity in patients when administered as monotherapy regimens, their clinical benefit has been limited by the emergence of drug-resistant virus strains associated with continuing viral replication (5, 14, 26). These observations indicate that combination therapy will be required. Recently, the superiority of treating patients with combinations of either RT inhibitors or RT and protease inhibitors has emerged (for a review, see reference 24). These data are consistent with the results of in vitro studies, in which additive to synergistic interactions have been demonstrated for combinations of antiretroviral agents that interact with the same or different targets (6–8, 15–17, 20, 22, 25, 27, 33, 34).

Nelfinavir mesylate is a potent and selective, nonpeptidic inhibitor of HIV type 1 (HIV-1) protease (\( K_i = 2 \) nM) that was discovered by protein design methods. In vitro, nelfinavir is effective at inhibiting the replication of laboratory, clinical, and RT inhibitor-resistant strains of HIV-1 and -2 with 50% effective concentrations (EC\(_{50}\)) ranging from 9 to 60 nM and a mean 95% effective concentration of 59 nM (29). In phase I and II and phase III controlled clinical trials, nelfinavir has produced significant reductions in HIV RNA levels in plasma and marked increases in CD4\(^+\) cell counts and has generally been well tolerated when used alone or in combination either with d4T or with ZDV and 3TC (4, 9, 12, 19, 23, 30). In addition, genotypic characterization of the protease gene coding regions in the genomes of HIV-1 variants isolated from in vitro selection studies and from patients treated with nelfinavir have identified a previously undescribed amino acid substitution from an aspartic acid (D) to an asparagine (N) at residue 30 (28, 29). In vivo, amino acid substitutions at residues described for other protease inhibitors have not been observed (e.g., residues 48, 82, and 84) or have been observed only rarely (e.g., residue 90). These observations, together with preliminary data which describe the sensitivity of nelfinavir-resistant isolates to other protease inhibitors, may provide an additional rationale for the use of nelfinavir in sequential or combination therapeutic regimens with other inhibitors from this class (28).

In the current studies, we have evaluated the antiviral efficacy and cytotoxicity resulting from use of the combination of nelfinavir in two- and three-way drug regimens with the RT inhibitors ZDV, 3TC, ddI, d4T, and ddC, as well as the protease inhibitors indinavir, saquinavir, and ritonavir, in an acute HIV-1 infection model. Quantitative assessment of drug interaction was evaluated by a universal response surface approach (URSA) implemented by Greco et al. (11), which fits the...
experimental data to a concentration-effect surface by nonlinear regression. Data from drug combination experiments were also analyzed by the technique of Prichard and Shipman (32). That technique determines the difference in antiviral activity between the observed combined effects and those expected if the drug combination had no interactive effect.

Both analyses indicated that the interaction of nelfinavir with the RT inhibitors resulted in additive to statistically significant synergistic interactions. In contrast, the combination of nelfinavir with other protease inhibitors ranged from slightly antagonistic (indinavir) to additive (ritonavir and saquinavir). Little cytotoxicity was observed with either drug alone or in combination. These results support the administration of nelfinavir in combination-therapy regimens with other clinically approved antiretroviral therapeutic agents.

MATERIALS AND METHODS

Compounds. Nelfinavir mesylate was synthesized at Agouron Pharmaceuticals, Inc. Other HIV protease inhibitors including indinavir, ritonavir, and saquinavir were kindly provided by Merck Research Laboratories, Abbott Laboratories, and Roche Research Centre, Welwyn Garden City, Herts, England, respectively. d4T was kindly provided by Bristol-Myers Squibb Pharmaceutical Research Institute, Wallingford, Conn. ZDV, ddI, ddC, and 3TC were kindly provided by Southern Research Institute, Birmingham, Ala.

Cells and virus strains. The CEM-SS human T-cell line and HIV-1 strain RF were obtained from the AIDS Research and Reference Program, Division of AIDS, National Institutes of Health, and the National Institute of Allergy and Infectious Diseases.

Cell protection assay. The inhibitory effects of combinations of compounds on HIV-1 replication were measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction method (1). Compounds were dissolved in dimethyl sulfoxide to a final concentration of 40 mg/ml and were then diluted 1:200 in medium. A total of 50 μl of each compound was added to the wells of a 96-well plate, and successive twofold dilutions were prepared. CEM-SS cells were infected with HIV-1 RF at a multiplicity of infection of 0.09. Following a 4-h adsorption period, 100 μl of infected or uninfected cells was added to the drug-containing microtiter plate to yield a final concentration of 104 cells/well. Six days after infection, MTT (5 mg/ml) was added to the test plates and the amount of formazan produced was quantified by measuring the absorbance of light at 570 nm. Percent inhibition for all drug combinations was calculated as the amount of formazan produced in the wells containing drug-free, uninfected cells. The EC50 was calculated as the concentration of drug that increased the percentage of formazan produced by infected, drug-treated cells to 50% of that produced by uninfected, drug-free cells. Percent cytotoxicity was calculated for all combinations as the concentration of drug that increased the percentage of formazan produced by infected, drug-treated cells to 50% of that produced by uninfected, drug-free cells. The EC50 was calculated as the concentration of drug that increased the percentage of formazan produced in wells containing drug-free, uninfected cells.

Antiviral effects of two-way drug combinations of nelfinavir and RT inhibitors. The antiviral efficacy and cytotoxicity of nelfinavir alone and in combination with the RT inhibitors ZDV, 3TC, ddC, d4T, and ddI were evaluated against an acute HIV-1 infection of CEM-SS cells in vitro. In these and subsequent experiments, five successive twofold dilutions of nelfinavir were evaluated alone and in all possible combinations with five successive twofold dilutions of a second drug. The highest concentrations of each drug used in each experiment were as follows: nelfinavir, 0.021 to 0.211 μM; ZDV, 0.300 μM; ddC, 0.758 μM; d4T, 1.68 μM; ddI, 9.74 μM; 3TC, 0.306 μM; saquinavir, 0.016 μM; ritonavir, 0.111 μM; indinavir, 0.028 μM. In the three-way drug regimen of nelfinavir with ZDV and 3TC, ZDV and 3TC were combined at a fixed ratio, with the highest concentrations used being 0.019 μM for ZDV and 0.079 μM for 3TC.

RESULTS

Antiviral effects of two-way drug combinations of nelfinavir and RT inhibitors. The antiviral efficacy and cytotoxicity of nelfinavir alone and in combination with the RT inhibitors ZDV, 3TC, ddC, d4T, and ddI were evaluated against an acute HIV-1 infection of CEM-SS cells in vitro. In these and subsequent experiments, five successive twofold dilutions of nelfinavir were evaluated alone and in all possible combinations with five successive twofold dilutions of a second drug. In all experiments only minimal cellular cytotoxicity was observed with any drug alone and in combination (data not shown).

Data from the drug combination experiments were initially analyzed by the URSA method specified by Greco et al. (11). Parameter estimates and standard errors from SYNFIT are summarized in Table 1. The EC50s of nelfinavir in all experiments ranged from 0.027 to 0.355 μM. Data from these analyses indicate that the two-drug combinations of nelfinavir with ZDV, 3TC, and ddC resulted in statistically significant synergy. This was demonstrated by a positive value for the interaction parameter, α. The statistical significance of this synergy was demonstrated since the calculated 95% confidence intervals contained only positive (synergetic) values. Results from two-drug combinations of nelfinavir with d4T and ddI were inter-

TABLE 1. Parameter estimates for the interaction of nelfinavir mesylate and other antiretroviral agents against acute HIV-1 RF infection of CEM-SS cells

<table>
<thead>
<tr>
<th>Second drug</th>
<th>Expt. no.</th>
<th>EC50 (μM)</th>
<th>α</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nelfinavir</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZDV</td>
<td>1</td>
<td>0.063 ± 0.004</td>
<td>0.034 ± 0.001</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.117 ± 0.013</td>
<td>0.064 ± 0.010</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.301 ± 0.036</td>
<td>0.217 ± 0.010</td>
</tr>
<tr>
<td>ddI</td>
<td>1</td>
<td>0.125 ± 0.005</td>
<td>6.966 ± 0.092</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.355 ± 0.036</td>
<td>10.974 ± 0.167</td>
</tr>
<tr>
<td>ddC</td>
<td>1</td>
<td>0.182 ± 0.012</td>
<td>0.171 ± 0.002</td>
</tr>
<tr>
<td>ddT</td>
<td>1</td>
<td>0.125 ± 0.002</td>
<td>1.672 ± 0.042</td>
</tr>
<tr>
<td>3TC</td>
<td>1</td>
<td>0.081 ± 0.006</td>
<td>0.15 ± 0.003</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZDV + 3TC</td>
<td>1</td>
<td>0.048 ± 0.001</td>
<td>ND</td>
</tr>
<tr>
<td>Saquinavir</td>
<td>1</td>
<td>0.047 ± 0.009</td>
<td>0.008 ± 0.001</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.032 ± 0.002</td>
<td>0.005 ± 0.010</td>
</tr>
<tr>
<td>Ritonavir</td>
<td>1</td>
<td>0.036 ± 0.001</td>
<td>0.047 ± 0.006</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.029 ± 0.001</td>
<td>0.042 ± 0.002</td>
</tr>
<tr>
<td>Indinavir</td>
<td>1</td>
<td>0.039 ± 0.001</td>
<td>0.023 ± 0.012</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.027 ± 0.001</td>
<td>0.015 ± 0.002</td>
</tr>
</tbody>
</table>

*Mean ± standard error parameter estimates were calculated by using a mathematical model (11) of synergism-antagonism in which the EC50 is the median effective concentration and α is the synergism-antagonism parameter. ND, not determined.

†Five successive twofold dilutions of nelfinavir were evaluated alone and in all possible combinations with five successive twofold dilutions of a second drug. The highest concentrations of each drug used in each experiment were as follows: nelfinavir, 0.021 to 0.211 μM; ZDV, 0.300 μM; ddC, 0.758 μM; d4T, 1.68 μM; ddI, 9.74 μM; 3TC, 0.306 μM; saquinavir, 0.016 μM; ritonavir, 0.111 μM; indinavir, 0.028 μM. In the three-way drug regimen of nelfinavir with ZDV and 3TC, ZDV and 3TC were combined at a fixed ratio, with the highest concentrations used being 0.019 μM for ZDV and 0.079 μM for 3TC.

‡Data are from one to three separate experiments, each of which was done in duplicate.

§The 95% confidence intervals contained only positive (synergetic) or negative (antagonistic) values.
interpreted to be additive with a trend toward synergy; although \( \alpha \) was positive, the 95% confidence intervals contained both positive (synergistic) and negative (antagonistic) values. Analyses from the experiments that were performed multiple times were consistent. Statistically significant synergy (ZDV) and additivity with a trend toward synergy (ddI) were observed in repeat experiments performed by combining nelfinavir with ZDV \((n = 3)\) and combining nelfinavir with ddI \((n = 2)\) (Table 1).

Similar results were obtained when the data were analyzed by the technique of Prichard and Shipman (32). In the two-drug combinations of nelfinavir with ZDV (Fig. 1A), 3TC (Fig. 1B), and ddC (data not shown), total synergistic volumes above the plane of independence calculated at the 95% confidence interval exceeded 100 \( \mu M^2 \cdot \% \), indicating strong synergy. As indicated in Fig. 1A and B, peak regions of synergy were observed at specific combinations of individual drug concentrations. In the two-drug combinations of nelfinavir with d4T or ddI, total synergistic volumes above the plane of independence calculated at the 95% confidence interval were \(<25 \mu M^2 \cdot \% \) (d4T; Fig. 1C) and \(>50 \mu M^2 \cdot \% \) (ddI; data not shown), indicating slight and moderate synergies for d4T and ddI, respectively.

Antiviral effects of the three-way drug combinations of nelfinavir and ZDV and 3TC. The antiviral efficacy and cytotoxicity of the three-way combination of nelfinavir with ZDV and 3TC were also evaluated against an acute HIV-1 RF infection. In this experiment, ZDV and 3TC were combined together in a fixed ratio at concentrations that, when tested as single agents, resulted in little or no inhibition of virus replication but, when tested in a two-way combination study, resulted in synergistic interactions (data not shown). As before, five successive twofold dilutions of nelfinavir were evaluated alone and in all possible combinations with five successive twofold dilutions of the fixed-ratio mixture of 3TC and ZDV. The data analyzed by the method of Greco et al. (11) indicated synergistic interactions since the value calculated for the synergy-antagonism parameter, \( \alpha \), was positive (Table 1). Moreover, the calculated 95% confidence intervals for \( \alpha \) contained only positive values, indicating that the synergy was statistically significant.

Synergistic interactions were also indicated when the data were analyzed by the technique of Prichard and Shipman (32). Calculated at the 95% confidence interval, synergistic volumes above the plane of independence were \(>100 \mu M^2 \cdot \% \), indicating strong synergy. As indicated in Fig. 1D, a peak of synergy was observed at 0.023 to 0.090 \( \mu M \) nelfinavir when it was combined with 0.01 to 0.04 \( \mu M \) 3TC and 0.003 to 0.010 \( \mu M \) ZDV. Little or no cytotoxicity was seen with either drug alone or in combination (data not shown).

Antiviral effects of two-way drug combinations of nelfinavir and protease inhibitors. The antiviral efficacy and cytotoxicity of nelfinavir alone and in combination with each of the other three currently approved protease inhibitors, i.e., saquinavir,
ritonavir, and indinavir, were similarly evaluated. In these experiments, minimal cellular cytotoxicity was observed with nelfinavir alone and in combination with each protease inhibitor (data not shown).

In the combination of nelfinavir with saquinavir, the calculated synergy-antagonism parameter, \( \alpha \), was positive in experiment 1 and slightly negative in experiment 2 (Table 1). In both experiments, however, the 95% confidence intervals for \( \alpha \) included both positive and negative values. By this method of analysis it can be concluded that the interaction of nelfinavir and saquinavir was additive since neither the synergy of experiment 1 nor the antagonism of experiment 2 was statistically significant. Similar results were obtained when the data were analyzed by the technique of Prichard and Shipman (32). Although the calculated synergistic volume above the plane of independence ranged from 37 to 105 \( \mu M^2 \% \) \( (P < 0.05) \) in both experiments, comparable volumes indicative of antagonism, were also observed below the plane of independence, i.e., \(-43 \) to \(-66 \) \( \mu M^2 \% \) \( (P < 0.05) \) (Fig. 2A). Interestingly, regions of slight antagonism, signified by volumes below the plane of independence, were observed with combinations of higher concentrations of both drugs, whereas regions of synergy, signified by volumes above the plane of independence, were observed with combinations of lower concentrations of both drugs (Fig. 2A). These data suggest that the overall interaction of saquinavir and nelfinavir was additive, although regions of both synergy and antagonism can be detected.

The antiviral efficacy and cytotoxicity of nelfinavir alone and in combination with ritonavir were similarly evaluated. Values calculated for the synergy-antagonism parameter, \( \alpha \), were negative in both experiments, indicating antagonism (Table 1). The antagonism demonstrated by the combination of nelfinavir and ritonavir was statistically significant in experiment 2 since the 95% confidence interval for \( \alpha \) contained only negative values. However, in experiment 1, the combination of nelfinavir and ritonavir was additive since the 95% confidence interval included both negative and positive values. By the method of Prichard and Shipman (32), calculated synergistic volumes above the plane of independence were small, ranging from 0 to 29 \( \mu M^2 \% \) \( (P < 0.05) \); Fig. 2B). However, slightly larger volumes, i.e., \(-11 \) to \(-94 \) \( \mu M^2 \% \) \( (P < 0.05) \), were also observed below the plane of independence. Overall, these results indicate no interactive effects in experiment 1 and slight to moderate antagonism in experiment 2. Thus, given the experimental variability observed, the interaction of ritonavir and nelfinavir appeared to indicate additivity with a slight trend toward antagonism.

Similar experiments were performed to evaluate the combination of nelfinavir and indinavir. Values calculated for the synergy-antagonism parameter, \( \alpha \), were negative in both experiments (Table 1). The calculated 95% confidence intervals for \( \alpha \) contained only negative values, indicating that the observed antagonism was statistically significant. By the method of Prichard and Shipman (32), calculated synergistic volumes above the plane of independence were small in both experiments, ranging from 0 to 17 \( \mu M^2 \% \) \( (P < 0.05) \) (Fig. 2C). Slightly larger volumes below the plane of independence were also calculated, indicative of moderate antagonism, i.e., \(-38 \) to \(-126 \) \( \mu M^2 \% \) \( (P < 0.05) \). Overall, these data suggest that the interaction of indinavir and nelfinavir was antagonistic, although the intensity of antagonism was weak, as indicated both by the small value of \( \alpha \) (Table 1) and by the small volumes depicted below the plane of interaction (Fig. 2C).

**DISCUSSION**

In this study, we have evaluated the antiviral and cytotoxic effects of two-drug combinations of nelfinavir with other clinically approved antiretroviral therapeutic agents, ZDV, 3TC,
dDC, d4T, ddI, indinavir, saquinavir, and ritonavir, and with the three-drug combination of nelfinavir with ZDV and 3TC against an acute HIV-1 RF infection of CEM-SS cells. In all of these experiments, a range of different dose-responses was analyzed by using five successive twofold dilutions of nelfinavir alone and in all possible combinations with five successive twofold dilutions of the appropriate second drug.

Data from drug combination experiments were analyzed by two different methods. Detailed comparisons of these methods with other methods are reviewed elsewhere (11). In the first approach, data were analyzed by an URSA (11) which has been extensively used and described in the literature on cancer (2, 10, 13). All URSAs fit a surface of a particular shape to the plots of the concentration-effect drug combination data and characterize the surface to measure the combined effects of a drug combination. This characterization, usually in the form of one or more statistically significant interaction parameters, focuses on the overall combination of drugs rather than the one or more statistically significant interaction parameters, drug combination. This characterization, usually in the form of a quantitative measure of the type and intensity of the interaction of the combination of drugs. Values of $a$ larger in magnitude correspond to more intense interaction effects. When $a$ is positive, synergy is indicated; when $a$ is negative, antagonism is indicated; and when $a$ is 0, additivity is indicated. In the cases for which $a$ is equal to 0, the equation fit to the data (11) is reduced to the additivity equation used to generate isobolograms.

The data were also analyzed by the method of Prichard and Shipman (32). This method calculates the difference in antiviral activity between the observed effects of a drug combination and those expected if the drugs had no interactive effect. Line segments connect these values, and the volumes above and below the plane representing the effects of no drug interaction are calculated. The calculated volumes are used to approximate the true volumes between the concentration-effect surface for a drug combination and a concentration-effect surface that would be seen if the drugs have no interactive effect in combination. Although the method of Prichard and Shipman (32) illustrates the effects of drug combinations, e.g., regions of synergism, antagonism, or no interaction, there is no precise way to measure how well the calculated volumes approximate the volumes associated with a true concentration-effect surface. Thus, while error due to experimental variability is accounted for by statistical calculations, there is no calculation for the error of approximation. Although conclusions about drug interaction in this study are based primarily on analyses performed by URSA, the conclusions generated by both methods were fairly concordant.

In this study, results from both analytical methods indicated that the two-drug combinations of nelfinavir with the RT inhibitors ZDV, 3TC, ddI, d4T, and dDC resulted in additive to statistically significant synergistic interactions. Similar results were observed when nelfinavir was combined in a three-drug combination with ZDV and 3TC. Although we cannot exclude the possibility that the observed synergy may have resulted from the interaction of only two of the three drugs, it is relevant to note that the combination of the three drugs resulted in enhanced antiviral activity at lower drug concentrations with nonoverlapping genotypic mutations. A combination-therapy strategy may also lead to the suppression of phenotypic resistance conferred by the selection of an additional mutation following the treatment with a second regimen, as has been described for ZDV and 3TC combination protocols (21). Relevant to these findings are studies which indicate that the resistance profile of nelfinavir is unique and is mediated by a previously undescribed amino acid substitution of D30N (28, 29). Preliminary results which describe the sensitivity of nelfinavir-resistant HIV isolates to other protease inhibitors suggest that nelfinavir may be a potential candidate for inclusion in combination or sequential therapeutic regimens involving other protease inhibitors.

In this study we have analyzed one component of drug interaction, whereby the antiviral effects of drugs added in combination have resulted in inhibitory effects greater than expected on the basis of the effects observed for each drug alone. The results described here suggest that administration of combinations of the appropriate doses of nelfinavir with other clinically approved antiretroviral therapeutic agents in vivo may thus result in enhanced antiviral activity. An overall understanding of all these interactions has important implications for the rational choice of the specific compounds to be used in combination-therapy regimens.

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REFERENCES


