- The graphs of combination index vs. fraction of inhibition for combination ratios of LPV and other protease inhibitors that most realistically reflect clinically observed plasma concentration are provided in Figures 2-6. Approximate 95% confidence intervals are represented by blue lines.

**Figure 2. LPV:IDV = 2:1**

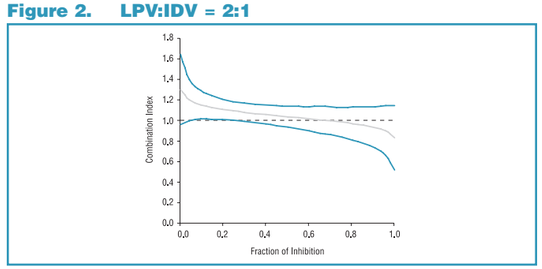



**Figure 3. LPV:APV = 2:1**

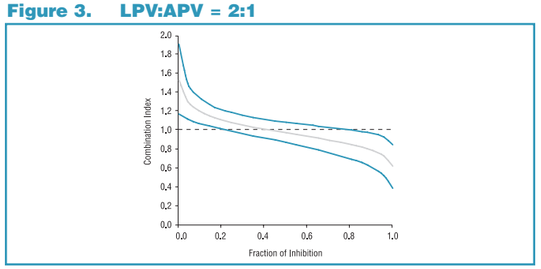



**Figure 4. LPV:NFV = 4:1**

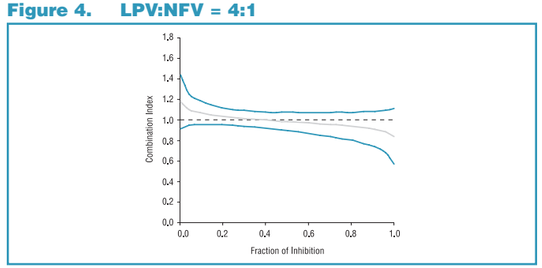



**Figure 5A. LPV:BMS-232632 = 8:1**

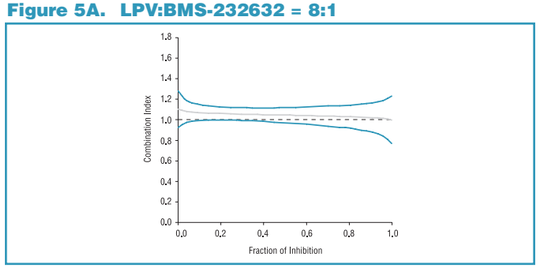



**Figure 5B. LPV:BMS-232632 = 16:1**

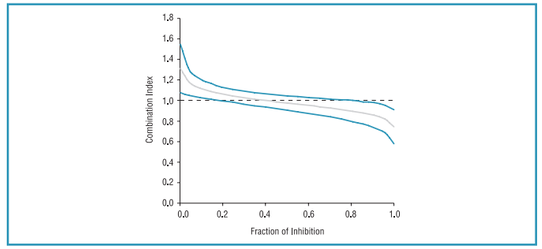
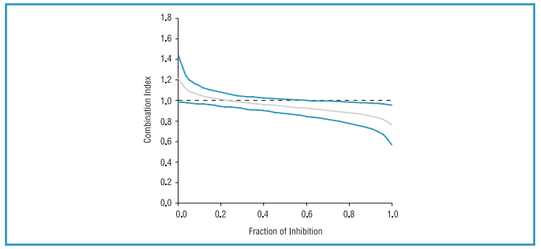



**Figure 6. LPV:TPV = 1:2**




# CONCLUSIONS

- *In vitro* (in the presence of 10% fetal calf serum), the combined action of lopinavir and saquinavir is statistically significantly synergistic.
- In contrast the combined action of lopinavir and the other protease inhibitors tested was found to be additive.
- With the exception of one combination of LPV and NFV, no antagonistic antiviral effect was observed.
- The observed *in vitro* synergy between LPV and SQV provides a basis for the clinical exploration of a novel, class-sparing regimen of Kaletra™ and Fortovase™, although the mechanism for this observation is unclear.

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# *In Vitro* Antiviral Interaction of Lopinavir with Other Protease Inhibitors


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# INTRODUCTION

Combination therapy with two protease inhibitors (PIs) such as ritonavir (RTV) and indinavir (IDV) or RTV and saquinvir (SQV) has resulted in increased viral suppression and may also delay or prevent the emergence of resistant HIV strains.[1,2] Lopinavir (LPV) is a novel peptidomimetic HIV protease inhibitor with approximately 10-fold greater *in vitro* potency than RTV in the presence of human serum.[3] Co-administration of LPV with RTV achieved plasma concentrations > 75 fold in excess of the *in vitro* $IC_{50}$ of LPV against wild type viruses. Consequently, LPV produced a significant decline in plasma HIV RNA in both treatment naïve and PI-experienced patients when combined with nucleoside and non-nucleoside reverse transcriptase inhibitors (NRTIs and NNRTIs).[4,5] To assess the potential combination of this inhibitor with other protease inhibitors, the antiviral activities of LPV alone or in combination with IDV, SQV, amprenavir (APV), nelfinavir (NFV), tipranavir (TPV), or BMS-232632 over a range of two-drug combination ratios were evaluated for evidence of synergy, additivity or antagonism.

# MATERIALS AND METHODS

**Compounds:** LPV, RTV, TPV and BMS-232632 were synthesized at Abbott Laboratories. SQV, IDV, NFV and APV were extracted from commercial formulations.

**Antiviral Assay:** The *in vitro* anti-HIV-1IIIB activity of LPV alone or in combination with APV, IDV, NFV, SQV, or BMS-232632 was determined using the MTT assay in MT4 cells in the presence of 10% fetal calf serum. MT4 cells were infected with 0.003 m.o.i. of HIV-1 at 1 x $10^6$ cells/ml for 1 hr, washed twice, seeded in a 96 well plate at 100 µl/well and treated with an equal volume of a 1% dimethylsulfoxide (DMSO) solution of inhibitor in a series of half log dilutions in RPMI containing 10% fetal bovine serum, in triplicate. The un-infected cell control was incubated in the absence of inhibitor or virus. Plates were incubated for 5 days in a $CO_2$ incubator at 37°C. On day 5, stock solution (4 mg/ml in PBS, Sigma) of 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) was added to all wells at 25 µl per well. Plates were further incubated for 4 hrs, then treated with 20% sodium dodecyl sulfate (SDS) plus 0.2 N HCl at 50 µl per well to lyse the cells. After an overnight incubation the amount of formazan produced was quantified by measuring the absorbance of light at 570/650-nm wave lengths on a Bio-Tek microtiter plate reader. Percent cytopathic effect reduction (fa), or percent of inhibition, was calculated from the formula below:

$$\frac{\text{O.D. test well} - \text{E.D. infected control well}}{\text{O.D. uninfected control well} - \text{O.D. infected control well}} \times 100\%$$

Assays were replicated in three batches with three plates per batch. $IC_{50}$ value for each PI alone was determined using the statistical method described below and presented in Table 1.

**Statistical Analysis:** To assess synergy, additivity or antagonism between LPV and other PIs, combination indices (CIs) as defined by Chou and Talalay (1984)[6] were calculated for each fixed ratio of LPV and another PI. Batch effect was ignored in statistical analysis as there did not appear to be differences associated with batches. For the modeling of dose response for each PI alone and for each fixed ratio of two PIs in combinations, the following sigmoid $E_{max}$ model was used: $f_a = C^\gamma/(C^\gamma+IC_{50}^\gamma)$, where $f_a$ is as defined earlier, C is total drug concentration, $IC_{50}$ is the median-effect drug concentration and h is the shape factor of the sigmoidal curve. It was chosen over the median-effect equation $[\log[f_a/(1-f_a)]$ as a linear function of log(C)] to accommodate observed $f_a$ values greater than 1. To achieve homogeneity in variances of the response variable across different drug concentrations, square-root transformed $f_a$ values were fit to the square root of the sigmoid $E_{max}$ model. The procedure NLIN of SAS version 8.0[8] was used to perform the model fitting. Once $IC_{50}$ and $\gamma$ were estimated for a fixed ratio of two PIs in combinations and for each of the two PIs alone, CI was calculated for the ratio of the two PIs in combinations for a selected $f_a$ value assuming mutual exclusivity of the two PIs. Confidence intervals of CIs were calculated by simulation of parameter estimates as suggested by Belen'kii and Schinazi (1994).[7] At each fa value, for each PI alone and for each fixed ratio of two PIs in combinations, $IC_{50}$ and $\gamma$ estimates were simulated 2000 times from a bivariate normal distribution with means equal to the actual estimates and variance-covariance matrix equal to the asymptotic variance-covariance matrix estimate. This led to 2000 simulated CI estimates at each $f_a$ value for each fixed ratio of two PIs in combinations. An approximate 95% two-sided confidence interval for CI was calculated as: actual CI estimate ± 1.96xSD, where SD is the standard deviation of the simulated CI estimates. Two additional methods for constructing confidence intervals (not presented here) gave similar results as the above method. When the upper bound of a CI confidence interval was below 1, synergism was inferred.

# RESULTS

- Protease inhibitor concentration ranges were selected based on the relative $IC_{50}$ values for each protease inhibitor alone in the presence of 10% FCS but absence of human serum (Table 1).

**Table 1. Antiviral Activity of the Protease Inhibitors Against Wild Type Determined in 10% FCS**

| Protease Inhibitor | $IC_{50}$ ± SE (µM) |
|---|---|
| ABT-378 | 0.033 ± 0.0006 |
| APV | 0.120 ± 0.0054 |
| IDV | 0.046 ± 0.0016 |
| NFV | 0.054 ± 0.0026 |
| SQV | 0.023 ± 0.0011 |
| TPV | 0.376 ± 0.0134 |
| BMS-232632 | 0.008 ± 0.0003 |

SE = Standard error.

- Combination indices, with 95% two-sided confidence intervals between LPV and the other PIs were determined using statistical methods (Table 2).
- If the 95% confidence interval overlapped a combination index of one, the combined action was judged to be additive. If the 95% confidence interval did not overlap a combination index of one, the combined action was judged to be synergistic (upper bound of confidence interval<1) or antagonistic (lower bound of confidence interval >1).
- Based on combination indices for percent of inhibition between 0.50 and 0.99, the combination of LPV with IDV, APV, TPV and BMS-232632 was additive. In the case of LPV with NFV, four combination ratios were found to be additive; however, one combination ratio (1:1) was slightly antagonistic (lower bound of 95% confidence interval 1.01-1.05).
- In contrast, at all five combination ratios tested, the combined action of LPV and SQV was statistically significantly synergistic.

## Table 2. Combination Indices for Combined Action of Lopinavir [LPV] and Another Protease Inhibitor [PI]

| PI | Concentration Ratio [LPV vs. PI] | Percent Inhibition [95% Confidence Intervals] 50 | 75 | 90 | 95 |
|---|---|---|---|---|---|
| SQV | 1:2 | **0.86 [0.76, 0.97]** | **0.79 [0.64, 0.94]** | **0.72 [0.53, 0.92]** | **0.68 [0.46, 0.91]** |
| | 1:1 | **0.88 [0.79, 0.98]** | **0.82 [0.69, 0.95]** | **0.76 [0.60, 0.93]** | **0.72 [0.53, 0.91]** |
| | 2:1 | **0.81 [0.73, 0.90]** | **0.77 [0.64, 0.90]** | **0.73 [0.57, 0.89]** | **0.70 [0.52, 0.89]** |
| | 4:1 | **0.85 [0.77, 0.94]** | **0.77 [0.66, 0.88]** | **0.70 [0.56, 0.84]** | **0.66 [0.49, 0.82]** |
| | 8:1 | **0.87 [0.79, 0.94]** | **0.79 [0.69, 0.90]** | **0.73 [0.59, 0.87]** | **0.69 [0.53, 0.84]** |
| IDV | 1:10 | 0.94 [0.85, 1.03] | 0.83 [0.71, 0.94] | 0.73 [0.58, 0.88] | 0.67 [0.50, 0.83] |
| | 1: 5 | 0.99 [0.90, 1.08] | 0.94 [0.81, 1.07] | 0.89 [0.72, 1.07] | 0.86 [0.66, 1.07] |
| | 1:3 | 1.04 [0.94, 1.14] | 1.03 [0.88, 1.17] | 1.02 [0.83, 1.21] | 1.01 [0.77, 1.25] |
| | 1:1 | 1.01 [0.92, 1.10] | 0.97 [0.84, 1.10] | 0.93 [0.75, 1.10] | 0.90 [0.70, 1.10] |
| | **2:1** | **1.04 [0.93, 1.14]** | **0.98 [0.83, 1.13]** | **0.93 [0.72, 1.14]** | **0.90 [0.64, 1.15]** |
| APV | 1:10 | 0.96 [0.86, 1.06] | 0.90 [0.76, 1.04] | 0.84 [0.66, 1.03] | 0.81 [0.59, 1.02] |
| | 1:5 | 0.98 [0.88, 1.08] | 0.94 [0.81, 1.08] | 0.91 [0.72, 1.10] | 0.88 [0.67, 1.10] |
| | 1:3 | 1.00 [0.91, 1.09] | 1.04 [0.90, 1.18] | 1.08 [0.88, 1.28] | 1.10 [0.84, 1.36] |
| | 1:1 | 1.01 [0.91, 1.12] | 0.94 [0.80, 1.08] | 0.87 [0.69, 1.05] | 0.82 [0.62, 1.03] |
| | **2:1** | **0.96 [0.86, 1.06]** | **0.86 [0.72, 0.99]** | **0.77 [0.60, 0.93]** | **0.71 [0.52, 0.90]** |
| NFV | 1:4 | 1.09 [0.97, 1.22] | 1.06 [0.88, 1.25] | 1.03 [0.77, 1.29] | 1.01 [0.71, 1.32] |
| | 1:2 | 0.95 [0.86, 1.04] | 0.92 [0.79, 1.05] | 0.90 [0.72, 1.07] | 0.87 [0.67, 1.08] |
| | 1:1 | 1.11 [1.01, 1.21] | 1.20 [1.04, 1.36] | 1.30 [1.05, 1.56] | 1.38 [1.05, 1.71] |
| | 2:1 | 0.99 [0.90, 1.09] | 0.98 [0.84, 1.11] | 0.96 [0.77, 1.15] | 0.95 [0.72, 1.18] |
| | **4:1** | **0.98 [0.88, 1.08]** | **0.94 [0.80, 1.08]** | **0.90 [0.71, 1.09]** | **0.87 [0.65, 1.10]** |
| BMS-232632 | 2:1 | 1.06 [0.97, 1.15] | 1.03 [0.90, 1.15] | 0.99 [0.82, 1.16] | 0.97 [0.76, 1.17] |
| | 4:1 | 0.93 [0.86, 1.01] | 0.86 [0.76, 0.96] | 0.79 [0.66, 0.92] | 0.75 [0.60, 0.90] |
| | **8:1** | **1.05 [0.96, 1.14]** | **1.04 [0.91, 1.16]** | **1.02 [0.85, 1.20]** | **1.01 [0.81, 1.22]** |
| | **16:1** | **0.97 [0.88, 1.06]** | **0.89 [0.77, 1.01]** | **0.82 [0.66, 0.97]** | **0.77 [0.60, 0.94]** |
| | 32:1 | 0.91 [0.83, 1.00] | 0.82 [0.70, 0.93] | 0.73 [0.59, 0.87] | 0.68 [0.52, 0.84] |
| TPV | 1:30? 32 | 0.85 [0.79, 0.92] | 0.77 [0.69, 0.86] | 0.70 [0.59, 0.81] | 0.66 [0.54, 0.78] |
| | 1:15 ? 16 | 0.92 [0.84, 0.99] | 0.86 [0.77, 0.96] | 0.82 [0.69, 0.95] | 0.79 [0.63, 0.94] |
| | 1:8 | 0.99 [0.91, 1.07] | 0.99 [0.88, 1.10] | 0.99 [0.84, 1.15] | 1.00 [0.81, 1.19] |
| | 1:4 | 0.99 [0.90, 1.07] | 0.95 [0.84, 1.07] | 0.93 [0.77, 1.08] | 0.91 [0.72, 1.09] |
| | **1:2** | **0.93 [0.85, 1.01]** | **0.87 [0.76, 0.99]** | **0.82 [0.67, 0.97]** | **0.78 [0.61, 0.96]** |

**Bold:** of the ratios tested, the combination ratios of LPV and other protease inhibitors most realistically reflect plasma combination ratios.

**Bold and blue text:** the synergistic effect of LPV and SQV at all five combination ratios tested.



- The graphs of combination index vs. fraction of inhibition for the five combinations of LPV and SQV are provided in Figure 1. In each case, statistically significant synergy was observed at concentrations producing >50% inhibition.

**Figure 1A. LPV:SQV = 1:2**

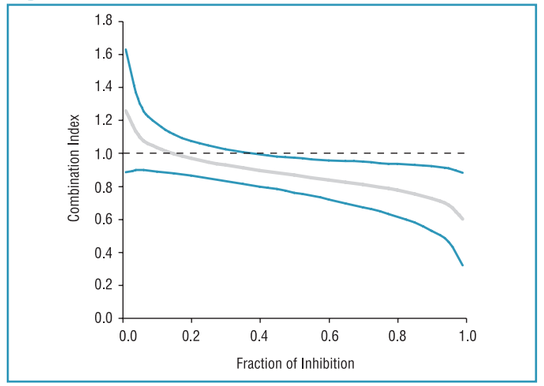


**Figure 1B. LPV:SQV = 1:1**

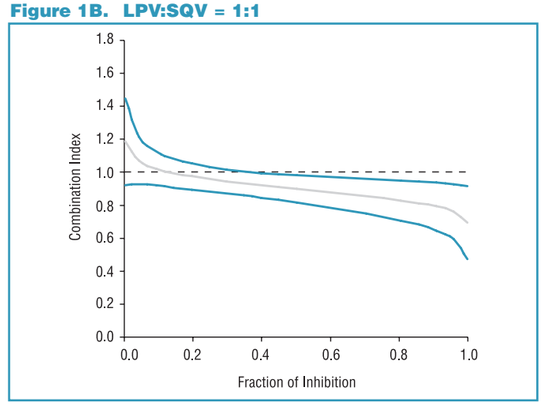


**Figure 1C. LPV:SQV = 2:1**

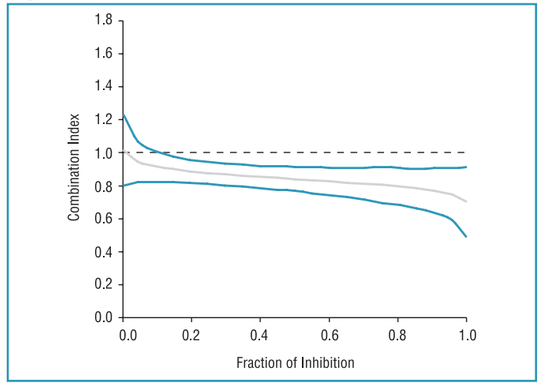


**Figure 1D. LPV:SQV = 4:1**

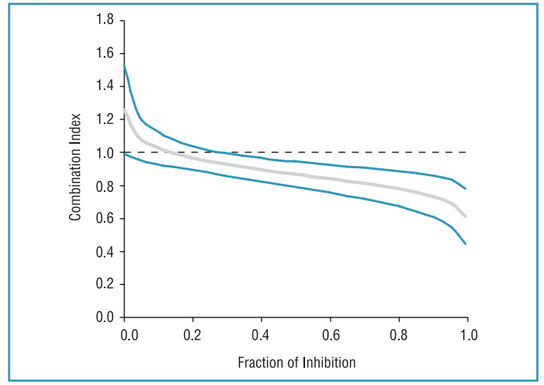


**Figure 1E. LPV:SQV = 8:1**