In Vitro Antiviral Activity of the Novel, Tyrosyl-Based Human Immunodeficiency Virus (HIV) Type 1 Protease Inhibitor Brecanavir (GW640385) in Combination with Other Antiretrovirals and against a Panel of Protease Inhibitor-Resistant HIV

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Brecanavir, a novel tyrosyl-based arylsulfonamide, high-affinity, human immunodeficiency virus type 1 (HIV-1) protease inhibitor (PI), has been evaluated for anti-HIV activity in several in vitro assays. Preclinical assessment of brecanavir indicated that this compound potently inhibited HIV-1 in cell culture assays with 50% effective concentrations (EC₅₀) of 0.2 to 0.53 nM and was equally active against HIV strains utilizing either the CXCR4 or CCR5 coreceptor, as was found with other PIs. The presence of up to 40% human serum decreased the anti-HIV-1 activity of brecanavir by 5.2-fold, but under these conditions the compound retained single-digit nanomolar EC₅₀₈. When brecanavir was tested in combination with nucleoside reverse transcriptase inhibitors, the antiviral activity of brecanavir was synergistic with the effects of stavudine and additive to the effects of zidovudine, tenofovir, didanosine, abacavir, lamivudine, and emtricitabine. Brecanavir was synergistic with the nonnucleoside reverse transcriptase inhibitor nevirapine or delavirdine and was additive to the effects of efavirenz. In combination with other PIs, brecanavir was additive to the activities of indinavir, lopinavir, nefelnavir, ritonavir, amprenavir, saquinavir, and atazanavir. Clinical HIV isolates from PI-experienced patients were evaluated for sensitivity to brecanavir and other PIs in a recombinant virus assay. Brecanavir had a <5-fold increase in EC₅₀₈ against 80% of patient isolates tested and had a greater mean in vitro potency than amprenavir, indinavir, lopinavir, ritonavir, amprenavir, saquinavir, and atazanavir. Clinical HIV isolates were more sensitive to brecanavir than to other PIs and brecanavir was the most potent PI tested in vitro.

Human immunodeficiency virus (HIV) protease is an essential enzyme required for viral proliferation. HIV protease inhibitors (PIs) are among the most potent and effective antiretrovirals and are considered essential components of successful combination therapy or highly active antiretroviral therapy (HAART) to treat HIV disease. Current guidelines include an initial treatment option that specifies the use of two nucleoside reverse transcriptase inhibitors (NRTIs) with a ritonavir (RTV)-boosted PI (5). However, emergence of viral resistance to HIV PIs and cross-resistance between members of the PI class are some of several major factors linked to the failure of the clinical management of HIV disease. Additionally, treatment regimens composed of several RT inhibitors and PIs have produced complex patterns of compound interaction and cross-resistance among drug classes (2). The development of compounds that are active against PI-resistant strains of HIV appears needed to assure optimal treatment of PI-experienced patients. Moreover, recent evidence indicates the transmission of drug-resistant HIV strains to treatment-naïve patient populations (1, 6, 15), and this has implications for the treatment regimens selected for recently diagnosed patients. It is also essential that new antiretrovirals interact favorably with other components of the complex HAART regimens.

In an attempt to overcome the challenge of cross-resistance within the PI class, novel inhibitors need to be designed that target critical sites on the protease enzyme that are not targeted by currently used PIs. The goal of drug discovery should involve the identification of inhibitors of a broad spectrum of mutant HIV-1 strains, which are compatible with existing treatments and select unique protease mutations which remain susceptible to other PIs.

Our research has identified novel inhibitors of the HIV protease that possess potent activities against a variety of clinically relevant mutant HIV strains, are compatible with other anti-HIV agents, and have unique resistance profiles. Recently we described a series of novel arylsulfonamide PIs with potent anti-HIV activities against both wild-type and drug-resistant viral strains (18). Additional structural modifications that introduced a tyrosine moiety into the PI position on the series have led to exceptionally potent compounds. In this article, we...
describe the in vitro activities of brecanavir (BCV) (Fig. 1), a
pyrrolidomimetic with low nanomolar activities against
both wild-type and PI-resistant HIV, additive to synergistic
activities in combination with other antiretrovirals, and
having a unique in vitro resistance profile.

(Results of this study were presented in part at the 2nd
International AIDS Society Conference on HIV Pathology and
Treatment, Paris, France, 13 to 16 July 2003.)

MATERIALS AND METHODS

Compounds. BCV (GW40385), (3R,3aS,6aR)-hexahydrofu2,3-b]furan-3-yl
(1S,2R)-[(1,3-benzodioxol-5-yl-sulfonyl)(isobutyl)amino]-2-hydroxy-1-\{4-\{(2-
methyl-1-thiazol-4-yl)ethoxy(phenyl)propylcarbamate (Fig. 1), was synthe-
sized at GlaxoSmithKline (Research Triangle Park, NC). Zidovudine (ZDV),
abacavir (ABC), stavudine (d4T), didoxycycline (dld), didanosine (ddI), ne-
virapine (NVP), delavirdine (DLV), lamivudine (3TC), darunavir (DRV), ad-
efsevir (ADV), and amprenavir (APV) were synthesized at GlaxoSmithKline.
The nucleotide produg tenofovir disoproxil fumarate was manufactured in
the pharmacy, and the active drug substance, \(R\)-9\{(2-phenoxymethoxpropyl)-
adenine (tenofovir [TFV]), was isolated in the Medicinal Chemistry Department
at GlaxoSmithKline. The PI irapnavir (TPV) was isolated from the commercial
formulation, Aptivus. The marketed PIs indinavir (IDV), lopinavir (LPV), nelfi-
navir (NFV), RTV, atazanavir (ATV), and saquinavir (SQV) were obtained from
the Medicinal Chemistry Department at GlaxoSmithKline.

Cell lines and primary cell cultures. MT-4 cells, a human T-cell leukemia virus
type 1-transformed human T-cell line (19), were obtained from long-term cul-
tures of samples supplied by B. Larder and maintained as previously described
(3). HeLa-Cd4-LTR-β-gal cells (obtained from Michael Emerman through the
AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH)
were maintained in Dulbecco's modified Eagle's medium with 10% fetal
bovine serum (FBS) (catalog no. SH30070.03; HyClone) and under the selective
pressure of Geneticin (catalog no. 10131-035; Invitrogen) and hygromycin B
(catalog no. 15750-060; Invitrogen), 10% [vol/vol] FBS, and 10
\(\mu\)g/ml gentamicin. Approved HIV inhibitors were diluted horizontally across separate
master assay plates. Checkerboard-style dilutions were arranged by combining
aliquots from both the horizontally and vertically diluted master plates into
daughter plates, so that every concentration of BCV was tested in the presence
and absence of every concentration of the approved HIV inhibitor. Anti-HIV
activity tests were performed in a minimum of triplicate assays of each combi-
nation. Cell infection, incubation, and MTS staining were carried out by the same
methods used in the standard MT-4 cell assay.

Antiviral assay in the presence of human serum and human serum proteins. Exponentially
growing MT-4 cells were harvested and centrifuged at 400 \(g\) for
5 min at room temperature, and the cell pellet was resuspended in RPMI 1640
medium, 10% [vol/vol] FBS, and 10
\(\mu\)g/ml gentamicin. Approved HIV inhibitors were diluted horizontally across separate
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methods used in the standard MT-4 cell assay.

\[
\text{EC}_{50} = T \times F - P \times \frac{X}{K}
\]

where \(K\) is the dissociation constant for binding. In serum the concentrations of
binding proteins range from 20 \(\mu\)M (ol-acid glycoprotein) to ~500 \(\mu\)M (human
serum albumin) (21), and the inhibitors used are present in concentrations
of 1,000-fold lower. Thus, \(P\) is \(>>\)B, and in equation 1 we can substitute \(P = 0\),
which gives

\[
\text{EC}_{50} = T \times F - \frac{X}{K}
\]

Therefore, the total concentration of drug required for the maintenance of a
fixed concentration of free drug is a linear function of the protein concentra-
tion and predicts a linear increase of the \(\text{EC}_{50}\) with the serum concentration. Such a
linear relationship has been demonstrated at total drug concentrations below 100

\[
\begin{align*}
\text{EC}_{50} &= T \times F - \frac{X}{K} \\
\text{where } K &= \text{the dissociation constant for binding. In serum the concentrations of binding proteins range from 20} \mu\text{M (ol-acid glycoprotein) to } ~500 \mu\text{M (human serum albumin) (21), and the inhibitors used are present in concentrations of 1,000-fold lower. Thus, } P &= \gg B, \text{ and in equation 1 we can substitute } P = 0, \text{ which gives } \\
\text{EC}_{50} &= T \times F - \frac{X}{K} \\
\end{align*}
\]
nM (Fig. 2). Thus, since equation 2 is theoretically valid and experimentally verified over the practical range of measurement, we believe that it can be used to extrapolate the experimental data to determine the EC_{50} at 100% human serum.

**PBMC assay.** PHA-stimulated PBMCs were pelleted, washed once with PBS and resuspended to \(8 \times 10^6\) cells/mL in RPMI 1640 medium with 20% (vol/vol) FBS, 10% (vol/vol) interleukin 2, and 10% (vol/vol) gentamicin and, and 50 μL was distributed to 96-well tissue culture plates. Compounds were serially diluted in medium in fourfold increments at two times the final concentration. Fifty microliters of diluted compound was transferred to the PBMCs and placed in a humidified incubator at 37°C, 5% CO₂, for 1 h. An additional 60 μL of diluted compound was transferred to a separate 96-well plate containing 60 μL of appropriately diluted HIV-1 Ba-L and thoroughly mixed. One hundred microliters of this mixture was transferred into the PBMC-compound mixture and placed in a humidified incubator at 37°C, 5% CO₂, for 7 days. On day 7 of the assay, 50 μL of culture supernatant was transferred to a new 96-well plate. The RT levels in the supernatants were measured by the method of Schwartz et al. (23).

**Drug combinations (deviations from dose-wise additivity).** EC_{50} values were calculated by curve fitting data to the Hill equation (12), using a nonlinear least-squares curve-fitting program based on the Marquardt-Levenberg algorithm (16). The interaction of each pair of compound combinations was analyzed by the methods described by Sellest et al. (24), which provide an estimation of the strength of any interaction and of its statistical significance. Synergy and antagonism are defined as deviations from dose-wise additivity, which results when two drugs interact as if they were the same drug. Values for average deviation from additivity in the range of \(-2.0\) to \(-0.2\) indicate weak synergy and values between \(-0.2\) and \(0.5\) indicate strong synergy of interaction. Conversely, values of \(0.1\) to \(0.2\) indicate that a weak antagonism exists between the treatments.

**HeLa-CD4 MAGI antiviral assay.** Compound anti-HIV-1 activity was determined in HeLa-CD4-LTR-β-gal (14) by the method of Ferris et al. (7).

**HIV protease enzymology.** Inhibition constant (K_i) values were determined from a continuous fluorescence activity assay for HIV-1 protease or calculated from the bimolecular rate constants (k_{cat}) for association of enzyme with inhibitor and the values for the first-order rate constants (k_{-1}) for the dissociation of enzyme and inhibitor (10).

**Phenotypic susceptibilities of 94 viruses obtained from PI-experienced patients.** One hundred nine viruses obtained from PI-treated-experienced patients were studied. Ninety-four of these viruses were selected based on the presence of major protease resistance-associated mutations (RAMs) at residues 48 (n = 48), 49 (n = 49), 50 (V, n = 5; L, n = 5), 82 (n = 82), 84 (n = 84), 88 (n = 88), and 90 (n = 90) (Table 1). Viruses were selected so that single, double, triple and multiple mutations of different combinations were included. Sequence analysis and drug susceptibility testing were performed at Monogram Biosciences, Inc., South San Francisco, CA. Viral DNA sequences were determined by a thermocycling method using fluorescent dye-labeled deoxynucleotide chain terminator chemistry. Resistance-associated mutations were classified based on International AIDS Society resistance tables (13). The mean percent inhibition of each drug concentration was determined and used to calculate the EC_{50}. The n-fold change (FC) in drug susceptibility was determined by comparing the EC_{50} for the subject virus to the EC_{50} for the drug-sensitive reference virus containing the protease and RT sequences of the NL-4-3 strain of HIV-1.

**RESULTS**

**Activity of BCV against wild-type HIV.** The in vitro anti-HIV activities of BCV against wild-type laboratory strains of HIV-1 were evaluated with acutely infected MT-4 cells, human PBMCs, or HeLa-CD4 cells (Table 2). In assays with MT-4 cells acutely infected with HIV-1 that utilize the CXCR4 coreceptor, namely, strain IIIB or the molecular clone HXB2, BCV produced equivalent EC_{50}s of 0.44 or 0.53 nM, respectively. The anti-HIV-1 activity of BCV was also characterized with human PBMCs isolated from noninfected donors. BCV inhibited HIV-1 strain IIIB and the strain Ba-L, with subnanomolar activities of 0.2 and 0.42 nM, respectively. The cell culture 50% inhibitory concentration values (CCIC_{50}) for BCV were determined with a small panel of uninfected B and T cells (data not shown). For MT-4, Molt-4, IM-9, or U-937 cells, the CCIC_{50} for BCV was greater than 25 μM, the highest concentration tested. These data, in conjunction with the EC_{50} of 0.44 nM obtained with MT-4 cells with wild-type HIV-1, estimate the in vitro selectivity index (CCIC_{50}/EC_{50}) at \(>55,000\) for BCV. In assays with HeLa-CD4 cells and HIV-1 strain HXB2, BCV was inactive up to the limit of solubility (approximately 50 μM). Since this assay relies on only one round of infection, inhibitors of HIV-1 protease are uniformly inactive.

**Antiviral activity in human PBMCs.** A comparison of the anti-HIV-1 strain Ba-L activities of BCV with several PIs in human PBMCs is shown in Table 3. BCV was significantly

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**TABLE 1. Major HIV-1 protease mutations associated with PI resistance identified in clinical-isolate viruses**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>No. of isolates with mutation</th>
<th>% Viruses with mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>D30N</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>V32I</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>L33F or I</td>
<td>31</td>
<td>33</td>
</tr>
<tr>
<td>M46I or L</td>
<td>60</td>
<td>64</td>
</tr>
<tr>
<td>I47V</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>G48S or V</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>I50V or L</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>V82A or C or I or T or F or S</td>
<td>48</td>
<td>51</td>
</tr>
<tr>
<td>I84A or V</td>
<td>36</td>
<td>38</td>
</tr>
<tr>
<td>N88D or G or S or T</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>L90M</td>
<td>44</td>
<td>47</td>
</tr>
</tbody>
</table>

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* Ninety-four isolates were analyzed.

**TABLE 2. Activities of BCV against wild-type HIV**

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Cell type</th>
<th>Assay readout (nM)</th>
<th>Anti-HIV EC_{50} (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-1 IIIB</td>
<td>MT-4</td>
<td>CPE (8)</td>
<td>0.44</td>
</tr>
<tr>
<td>HIV-1 HXB2</td>
<td>MT-4</td>
<td>CPE (13)</td>
<td>0.53</td>
</tr>
<tr>
<td>HIV-1 IIIB</td>
<td>PBMC</td>
<td>RT (1)</td>
<td>0.2</td>
</tr>
<tr>
<td>HIV-1 Ba-L</td>
<td>PBMC</td>
<td>RT (8)</td>
<td>0.42</td>
</tr>
<tr>
<td>HIV-1 HXB2</td>
<td>HeLa-CD4</td>
<td>β-Gal (2)</td>
<td>&gt;90,000</td>
</tr>
</tbody>
</table>

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* CPE, HIV-induced cytopathic effect; RT, measurement of HIV RT activity in assay supernatants; β-Gal, β-galactosidase activity readout utilizing the Tropix assay system. n, number of determinations.

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Values are means ± standard deviations except in the case of HIV-1 Ba-L, where values are geometric means ± standard deviations; NA, not applicable.
more potent ($P < 0.0001$) than any of the other PIs tested (range, 6.7- to 720-fold). BCV was 720-fold more potent than the nonpeptidomimetic, TPV, 130-fold more potent than APV, 85-fold more potent than either NVP, 10-fold more potent than ATV, and 6.7-fold more potent than the structurally related PI, DRV.

**Antiviral activity in the presence of human serum and human serum proteins.** The effect of human serum on the anti-HIV activity of BCV, DRV, or TPV in MT-4 cells was estimated by determining the ratio of EC_{50s} observed in the absence or presence of various concentrations of human serum. These data, shown in Table 4, indicate that BCV maintains approximately sevenfold-greater antiviral potency than ATV, and 6.7-fold more potent than the structurally related PI, DRV.

The effect of human serum albumin (HSA) or α1-acid glycoprotein (AAG) on the potency of brecanavir or DRV was determined by methods similar to those used in experiments testing the effects of human serum (Table 5). HSA (40 mg/ml) and/or 1 mg/ml AAG resulted in a 3.7- or 7.6-fold increase in the EC_{50} of BCV, respectively. The combination of HSA and AAG resulted in an 8.2-fold increase in the EC_{50} of BCV. For DRV, there was a 1.3- or 8.8-fold increase in the EC_{50} when tested with either HSA or AAG alone, respectively. When tested in the presence of both HSA and AAG, there was an 8.7-fold increase in the EC_{50} for DRV. Since the concentrations chosen were those reported for human serum, these results are in excellent agreement with values obtained by addition of whole serum (Table 4).

**Combination antiviral activity in MT-4 cells.** Table 6 depicts the values for the deviation from additivity for combinations of BCV and currently approved anti-HIV-1 drugs. These effects are further detailed in the graphical presentation of the isobolograms for the interaction of BCV with the other agents (Fig. 3). In the current series of experiments, tests where BCV was used as both the horizontally diluted and vertically diluted agent (sham combination) show that no artifactual synergistic or antagonistic effects were seen. In combination testing with NRTIs (Table 6; Fig. 3b), the activity of BCV was additive to AAG with the FBS concentration range of 0 to 40% added human serum. These data, shown in Table 4, indicate that BCV maintains approximately sevenfold-greater antiviral potency than ATV, and 6.7-fold more potent than the structurally related PI, DRV.

**Antimicrob. Agents Chemother.**

<table>
<thead>
<tr>
<th>TABLE 3. Anti-HIV-1 Ba-L activities of BCV and approved PIs in PBMC</th>
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<tbody>
<tr>
<td><strong>Compound</strong></td>
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<tr>
<td>----------------</td>
</tr>
<tr>
<td>BCV</td>
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<tr>
<td>ATV</td>
</tr>
<tr>
<td>NVP</td>
</tr>
<tr>
<td>APV</td>
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<tr>
<td>TPV</td>
</tr>
<tr>
<td>DRV</td>
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</table>

*Values are geometric mean ± standard deviations for eight determinations; 95% confidence interval.

*Significantly different from each of the other mean EC_{50s} ($P < 0.0001$).

<table>
<thead>
<tr>
<th>TABLE 4. Effect of human serum on antiviral activity of BCV, DRV, or TPV in MT-4 cells</th>
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<tbody>
<tr>
<td><strong>% FBS</strong></td>
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<tr>
<td></td>
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<tr>
<td>10</td>
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<tr>
<td>40</td>
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<td>10</td>
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<td>10</td>
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<td>10</td>
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</tbody>
</table>

*Values are means from two determinations; FS, n-fold shift, the ratio of the EC_{50} in the presence of human serum to the EC_{50} with 10% FBS; NT, not tested. Extrapolated n-fold shifts at 100% human serum (means ± standard errors of the means) are as follows: for BCV, 11 ± 2.9; for DRV, 9.3 ± 1.9; for TPV, 39 ± 6.5.

<table>
<thead>
<tr>
<th>TABLE 5. Effect of α1-acid glycoprotein or human serum albumin on antiviral activity of BCV or DRV</th>
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<tbody>
<tr>
<td><strong>% FBS</strong></td>
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<tr>
<td>10</td>
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<td>10</td>
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<td>10</td>
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<tr>
<td>40</td>
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</table>

*Values are means from two determinations; FS, n-fold shift, the ratio of the EC_{50} in the presence of α1-acid glycoprotein or human serum albumin to the EC_{50} with 10% FBS.

<table>
<thead>
<tr>
<th>TABLE 6. Inhibition of HIV-1 strain IIIB by BCV in combination with other approved anti-HIV agents in MT-4 cells</th>
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<tbody>
<tr>
<td><strong>Compound</strong></td>
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<tr>
<td></td>
</tr>
<tr>
<td>BCV</td>
</tr>
<tr>
<td>ZDV</td>
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<tr>
<td>d4T</td>
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<tr>
<td>TFV</td>
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<td>ddC</td>
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<td>ddl</td>
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<td>ADV</td>
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<tr>
<td>ABC</td>
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<td>FTC</td>
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<td>APV</td>
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<td>SQV</td>
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<td>ATV</td>
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</table>

*Values are means ± standard errors of the means from at least three determinations. $P(t)$, probability, as determined by the test, that the deviation from additivity is actually equal to zero.
the effects of ZDV, ABC, 3TC, TFV, ddl, and ddc. BCV was synergistic with d4T. With NNRTIs (Table 6; Fig. 3c), the activity of BCV was additive to the effects of EFV but was synergistic with those of DLV and NVP. As expected, combination testing of BCV with the PIs (Table 6; Fig. 3a) APV, SQV, IDV, RTV, NFV, LPV, and ATZ resulted in additive anti-HIV effects.

Phenotypic susceptibilities of 94 viruses obtained from PI-experienced patients. The mean antiviral potency of BCV was greater than those of APV, IDV, LPV, ATV, TPV, and DRV based on testing of viruses from 94 treatment-experienced patients (Fig. 4; Table 7). The mean number of major PI RAMs per isolate was 2.8 (range, 0 to 6; median, 3), and the mean number of total protease mutations was 16 (range, 1 to 27; median, 17).

Approximately 80% (75 out of 94) of all isolates tested were fully susceptible to BCV, based on an FC in EC₅₀ of <5 compared to results with the reference wild-type virus, HIV-1 strain NL4-3. BCV was more than 200-fold more potent than APV, IDV, LPV, or TPV and more than 50-fold more potent than ATV against all of the 94 isolates. BCV was more than fivefold more potent than the structurally related PI, DRV. In addition to the absolute potency advantage for BCV, the mean FC for BCV was lower than for all other PIs except for TPV and DRV, where BCV showed a similar FC.

In general, the viruses with more protease mutations resulted in larger increases in EC₅₀ than viruses with fewer protease mutations. The 10 patient viruses with the highest FC with BCV (mean FC = 41.9; range, 8.9 to 112; median, 13) had a mean of 4.4 major PI RAMs (range, 3 to 6; median, 4) per isolate and a mean of 17.8 total protease mutations (range, 12 to 22; median, 17) per isolate. Two isolates that had an FC for BCV of >100 had genotypes containing the BCV-associated resistance mutations, M46I and A71V, that were identified by in vitro resistance selection with HIV-1 strain HXB2 (26).

Against isolates containing 0 to 3 major PI RAMs, BCV had ~3 FC compared to the reference virus and with isolates containing 4 or 5 RAMs had FCs of 10 or 48, respectively (Fig. 5). Against isolates containing 0 to 2 BCV-associated mutations identified during in vitro serial virus passage (26), there was a 1.5- to 6.2-fold change in the activity of BCV (BCV EC₅₀ range 0.1 to 10.2 nM) compared to that of the wild-type reference virus.

**DISCUSSION**

PI-based HAART regimens are demonstrably potent therapies enabling long-lasting suppression of HIV-associated disease, prolongation of life, and reduction of morbidity in an increasing patient population (22). However, the clinical effectiveness of current PI therapies has been hindered by, among other factors, difficulty in adherence to a high pill burden in dosing regimens, leading to inadequate virus suppression, which correlates with the development of viral escape mutants and disease progression (11). The development of new PIs with strong activities against drug-resistant HIV would provide a tremendous clinical benefit to patients with limited therapeutic options.

Recently we documented our efforts to optimize the arylsulfonamide scaffold of PIs (17). This work resulted in the discovery of BCV, which exhibited exceptionally high potency ($K_i = 15$ fM) against the wild-type HIV protease enzyme. In a competitive binding assay (10), BCV was found to be 2,000-fold more potent than APV ($K_i = 36$ pM) (10) and 10-fold more potent than DRV ($K_i = 147$ fM) (unpublished results). We also determined the $K_i$ value of amprenavir by inhibition of enzyme activity using a fluorescent peptide substrate (10) and found the $K_i$ value (57 pM) to be very similar to the value determined with the competitive binding assay. Another newer PI, TPV, was reported to have a $K_i$
similar to that of amprenavir (8 pM) (25). This value was also
determined with a fluorescent peptide substrate and suggests
that TPV is several orders of magnitude weaker than BCV.

In the present study, we have shown that BCV has subnano-
molar potency against several wild-type laboratory HIV strains
in both MT-4 cells and PBMCs. Compared to published anti-
HIV strain IIIB activities in MT-4 cells, BCV is more than 270-
or 850-fold more potent than the PIs APV or TPV, respectively
(20), and more than 10-fold more potent than the structurally
similar PI, DRV (4). BCV also potently suppressed the virus
strain Ba-L, with nearly equivalent activity seen with strains
IIIB and HXB2. A comparison of the activity of BCV with
those of other PIs against strain Ba-L in PBMCs showed that
BCV was significantly more potent than any of the other PIs
tested.

Like other PIs, BCV was not active against HIV in the
HeLa-CD4 cell assay. The absence of activity in the HeLa-CD4
cell assay is a common characteristic of PIs, since this assay has
only a single round of infection and has little or no production
of progeny viral particles, where PIs would be active during
maturation.

No cellular toxicity was observed in several cell lines up to
the highest concentration of BCV tested (25 μM), which indi-
cates a high selectivity index of >55,000.

The in vitro interaction of BCV with other antivirals was
determined with MT-4 cells. In combination with the marketed
anti-HIV-1 agents, the activity of BCV was found to be addi-
tive to or synergistic with the activities of the other agents. It is
not clear why results of in vitro tests of BCV in combination
with one member of a drug class would be different from those
with others in the same drug class. For example, activity of
BCV in combination with EFV was additive, whereas that in
combinations with either NVP or DLV was synergistic. The
differences seen in the interaction of BCV and various mem-
bers of the NNRTI class may be a matter of degree. By our
definition of synergy, a value of −0.1 for average deviation
from additivity would indicate a weak synergy for the combi-
nation. For combinations of EFV and BCV, the value of −0.1
achieves the minimum value that would indicate synergy,
but it fails to achieve statistical significance (P = 0.08). The
greatest value of in vitro combination studies is to identify
combinations that result in antagonistic antiviral effects and
exclude such combinations from clinical consideration.

There are many other important factors that also must be
considered in the selection of combination therapeutics,
including pharmacokinetics, interactions with drug-metabo-
lizing enzymes, and toxicities. These factors can be evalu-
ated only in clinical trials.

BCV is approximately 98% bound to serum proteins (9).
Despite this high level of protein binding, BCV retains potent
activity when antiviral assessments are performed in the pres-
ence of physiological levels of human proteins. In the absence
of added human serum, the in vitro potency of DRV was
eightfold less than the potency of BCV. In the presence of 40%
human serum, the highest concentration tested, the in vitro
potency of DRV was 5.8-fold less than the potency of BCV.

Protein binding often increases the half-life of the drug by
reducing free concentrations that are available for metabolism
or excretion. However, protein binding and the resultant
reduction of free drug also attenuate the pharmacology of the
drug (i.e., antiviral activity in this case). Therefore, these cur-
rent in vitro studies may serve as a means to estimate the

### TABLE 7. Anti-HIV-1 EC<sub>50</sub>s of several PIs against 94
clinical-isolate viruses

<table>
<thead>
<tr>
<th>Compound</th>
<th>Anti-HIV-1 EC&lt;sub&gt;50&lt;/sub&gt;, nM, mean ± SEM (range)</th>
<th>Mean FC for which FC was</th>
<th>No. of viruses</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean ± SEM (range)</td>
<td>for which FC was</td>
<td>&lt;3</td>
</tr>
<tr>
<td>BCV</td>
<td>0.88 ± 0.21 (0.1–15)</td>
<td>6.6</td>
<td>58</td>
</tr>
<tr>
<td>APV</td>
<td>190 ± 23 (1.2–1,300)</td>
<td>13.7</td>
<td>24</td>
</tr>
<tr>
<td>IDV</td>
<td>220 ± 30 (3.2–1,500)</td>
<td>26</td>
<td>23</td>
</tr>
<tr>
<td>LPV</td>
<td>230 ± 34 (0.7–1,450)</td>
<td>43</td>
<td>21</td>
</tr>
<tr>
<td>ATV</td>
<td>47 ± 7.5 (0.8–500)</td>
<td>26</td>
<td>20</td>
</tr>
<tr>
<td>TPV</td>
<td>190 ± 24 (7–1,080)</td>
<td>2.4</td>
<td>77</td>
</tr>
<tr>
<td>DRV</td>
<td>4.6 ± 1.5 (0.1–104)</td>
<td>7.2</td>
<td>60</td>
</tr>
</tbody>
</table>

*Mean fold change (FC) in EC<sub>50</sub> from that for the drug-sensitive reference
wild-type virus, HIV-1 strain NL4-3. Reference virus compound sensitivities
(EC<sub>50</sub>) were 0.18, 15.3, 9.8, 4.4, 1.7, 76.2, and 0.78 nM for BCV, APV, IDV, LPV,
ATV, TPV, and DRV, respectively.
antiviral effect of total drug concentrations determined in vivo in patient plasma.

In testing against clinical isolate viruses derived from PI-experienced patients, BCV has shown greater in vitro potency than APV, IDV, LPV, ATV, TPV, and DRV and a lower FC than all other PIs tested except for a FC similar to those of TPV and DRV. Due to the small scale of the clinical isolate data set, it was difficult to ascribe BCV resistance of the clinical isolates to specific mutational patterns.

In summary, the present study demonstrates the following in vitro preclinical properties of BCV. (i) It has potent subnanomolar in vitro antiretroviral activity against several laboratory HIV strains and is significantly more potent than other PIs tested against viruses using the CCR5 coreceptor. (ii) In the presence of added human serum or serum proteins, BCV retains a potency advantage over other PIs. (iii) It is additive or synergistic when tested in combination with other antiretrovirals. (iv) It exhibited mean subnanomolar potencies against a panel of viruses from PI-experienced patients.

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REFERENCES


