The chemokine receptor CCR5 provides a portal of entry for human immunodeficiency virus type 1 (HIV-1) into susceptible CD4+ cells. Both monoclonal antibody (MAb) and small-molecule CCR5 inhibitors have entered human clinical testing, but little is known regarding their potential interactions. We evaluated the interactions between CCR5 MAbs, small-molecule CCR5 antagonists, and inhibitors of HIV-1 gp120, gp41, and reverse transcriptase in vitro. Inhibition data were analyzed for cooperative effects using the combination index (CI) method and stringent statistical criteria. Potent, statistically significant antiviral synergy was observed between the CCR5 MAb PRO 140 and the small-molecule CCR5 antagonists maraviroc (UK-427,857), vicriviroc (SCH-D), and TAK-779. High-level synergy was observed consistently across various assay systems, HIV-1 envelopes, CCR5 target cells, and inhibition levels. CI values ranged from 0.18 to 0.64 and translated into in vitro dose reductions of up to 14-fold. Competition binding studies revealed nonreciprocal patterns of CCR5 binding by MAb and small-molecule CCR5 inhibitors, suggesting that synergy occurs at the level of receptor binding. In addition, both PRO 140 and maraviroc synergized with the chemokine RANTES, a natural ligand for CCR5; however, additive effects were observed for both small-molecule CCR5 antagonists and PRO 140 in combination with other classes of HIV-1 inhibitors. The findings provide a rationale for clinical exploration of MAb and small-molecule CCR5 inhibitors in novel dual-CCR5 regimens for HIV-1 therapy.

The armamentarium for human immunodeficiency virus type 1 (HIV-1) infection currently includes 22 antiretroviral agents drawn from four mechanistic treatment classes: nucleoside reverse transcriptase inhibitors (NRTI), nonnucleoside reverse transcriptase inhibitors (NNRTI), protease inhibitors, and fusion inhibitors. The standard of care for HIV-1 infection involves combination use of three or more antiretroviral agents. Where available, such therapies have markedly reduced HIV-1 morbidity and mortality (34). However, current therapies are limited by the emergence of multidrug-resistant virus, by treatment-related toxicities, by unfavorable drug-drug interactions, and by often-complex dosing regimens that can reduce adherence to therapy. Consequently, many patients eventually exhaust their treatment options, and there is an urgent need for new agents that can be deployed in novel combination regimens.

In 1996, we and others demonstrated that the chemokine receptor CCR5 serves as an entry coreceptor for HIV-1 (1, 10, 12). HIV-1 entry proceeds through a cascade of events mediated by the HIV-1 envelope glycoproteins gp120 and gp41: gp120 sequentially binds CD4 and then CCR5 or another coreceptor molecule, thereby triggering gp41-mediated fusion of the viral and cellular membranes. CCR5 has emerged as an important target for novel HIV-1 therapies (reviewed in reference 35). Both small-molecule and monoclonal antibody (MAb) inhibitors of CCR5 have entered human testing, and the first of these has demonstrated potent antiviral effects in HIV-infected individuals (14, 21).

PRO 140 is a humanized CCR5 MAb that has entered phase Ib testing for HIV-1 therapy. PRO 140 and the parent mouse MAb (PA14) broadly and potently block CCR5-mediated HIV-1 entry in vitro (32, 33, 45). Although PRO 140 and small-molecule CCR5 antagonists target the same protein, their properties are complementary in a number of important respects. Whereas the available small-molecule CCR5 inhibitors potently block the natural activity of CCR5 (11, 39, 40, 48), antiviral concentrations of PRO 140 do not block CCR5 function in vitro (33). In addition, preliminary studies indicate that PRO 140 is highly active against viruses that are resistant to small-molecule CCR5 antagonists (20, 27). These functional differences are likely related to the distinct differences in CCR5 binding. Small-molecule CCR5 antagonists bind a hydrophobic pocket formed by the transmembrane helices of CCR5 and inhibit HIV-1 via allosteric mechanisms (13, 30, 47, 48), while PRO 140 binds an extracellular epitope on CCR5 and appears to act as a competitive inhibitor (33).

Given the mechanistic differences between PRO 140 and small-molecule CCR5 antagonists in clinical development and the need for novel combination regimens, we examined the interactions between these agents in vitro. PRO 140, structurally diverse small-molecule CCR5 antagonists, and other classes of HIV-1 inhibitors were tested alone and in combination for the ability to inhibit HIV-1 membrane fusion and viral entry. Surprisingly, we observed potent antiviral synergy for PRO 140 in combination with each of several small-molecule CCR5 antagonists but not for PRO 140 in combination with...
agents that target different stages of HIV-1 entry. Both PRO 140 and small-molecular CCR5 antagonists synergized with RANTES (CCL5), a natural ligand for CCR5, but purely additive effects were observed when different small-molecule CCR5 antagonists were combined. Competition binding experiments were conducted and offer a mechanism for the cooperative effects observed. Coupled with the available viral resistance data, these findings indicate that PRO 140 and small-molecule CCR5 drugs may represent distinct subclasses of CCR5 inhibitors.

MATERIALS AND METHODS

Inhibitors. PRO 140 was expressed in mammalian cells and purified by protein A, ion exchange, and hydroxyapatite chromatographies. Maraviroc (UK-427,857; Pfizer) (11), vicriviroc (SCH-D; Schering-Plough Corporation) (39), TAK-779 (Takeda Pharmaceuticals) (5), enfuvirtide (T-20; Trimeris/Roche) (49), BMS-184874 (Bristol-Myers Squibb) (23), and PRO 542 (CD4-IgG2; Progenics) (2) were prepared according to published methods. Zidovudine (azidothymidine), RANTES, the CCR5 MAb 2D7, and the CD4 MAb Leu-3A were purchased from Sigma Chemicals (St. Louis, MO), R&D Systems (Minneapolis, MN), Pharmingen (San Diego, CA), and Becton Dickinson (Franklin Lakes, NJ), respectively. Maraviroc and vicriviroc were radiolabeled with tritium by GE Healthcare (Piscataway, NJ), and PRO 140 was conjugated to phycoerythrin (PE) by Southern Biotech, Inc. (Birmingham, AL).

HIV-1 membrane fusion assay. HIV-1 envelope-mediated membrane fusion was examined using a fluorescence resonance energy transfer (RET) assay (24) with modifications. Briefly, HeLa cells that stably express HIV-1la.r.t., gp120/ gp41 (24) and CEM.NKRR-CCR5 cells (NIH AIDS Research and Reference Reagent Program) (38, 46) were labeled separately overnight with fluorescein octadecyl ester (F18; Molecular Probes, Eugene, OR) and rhodamine octadecyl ester (R18; Molecular Probes, respectively). Cells were washed in phosphate-buffered saline containing 1% fetal bovine serum and resuspended at 15,000 cells/well into a 384-well plate. Inhibitors were added, and the plates were incubated in phosphate-buffered saline containing 1% fetal bovine serum plus 0.5% dimethyl sulfoxide for 4 h at 37°C prior to measurement of RET using a Victor2 plate reader (PerkinElmer, Boston, MA) as previously described (24). The CD4 MAb Leu3a was used as a control inhibitor, and percent inhibition was calculated as follows: RET in the presence of inhibitor/RET in the absence of inhibitor – RET in the presence of Leu3a) × 100.

HIV-1 pseudovirus assay. A self-inactivating vector was derived from the pNL4-3ΔEnv-luciferase vector (12) by deleting 507 base pairs in the U3 region of HIV-1 membrane fusion assay.

RESULTS

Inhibition of HIV-1 membrane fusion. PRO 140 and maraviroc were used individually and together to inhibit HIV-1JR-FL, envelope-mediated membrane fusion in the RET cell-cell fusion assay, and representative dose-response curves for the individual agents and combination are illustrated in Fig. 1A. Although both PRO 140 and maraviroc individually blocked HIV-1 fusion at low nanomolar potency, the combination was markedly more potent. In this assay, 50% inhibition was obtained using 2.9 nM PRO 140 alone, 5.0 nM maraviroc alone, or 2.1 nM of the combination (1.05 nM PRO 140 plus 1.05 nM maraviroc). This supra-additive effect is indicative of antiviral synergy between the two agents.

In contrast, the combination of vicriviroc and maraviroc was no more potent than individual agents (Fig. 1B). In this example, the dose-response curves for the individual inhibitors and the combination were overlapping, with 50% inhibition requiring 9.7 nM maraviroc, 5.5 nM vicriviroc, and 6.1 nM of the combination. The data suggest purely additive effects for these inhibitors.
The CI50 and CI90 values were significantly different from 1. As times for each condition. CI50 and CI90 values were calculated gp41 (enfuvirtide) inhibitors and were repeated four or more

RANTES, and 2D7), gp120 (BMS-378806 and PRO 542), and

140 combination were 0.97 and 0.96, respectively (Table 1); 9.7 nM and 21 nM for maraviroc, and 2.1 nM and

4.6 nM for the combination, respectively. CI50 and CI 90 values were 0.87 and 0.73, respectively. (B) Vicriviroc (SCH-D) and maraviroc
demonstrated synergy that met our criteria for statistical sig-
rangefrom0.36to0.61, andthesesequencestranslatedinto
dose reductions ranging from three- to eightfold across the
different conditions. Synergies were greater at 90% inhibition
than at 50% inhibition. Synergy between PRO 140 and small-
moleculeCCR5 antagonists was robust in that it was observed
at both the 50% and 90% inhibition levels in every instance.
The exception was TAK-779, which did not mediate 90% inhibi-
tion when used individually, and therefore, a CI90 was not
determined. Similarly, potent synergy was observed when
RANTES was used in combination with either PRO 140 or
maraviroc (Table 1).

Additional tests examined combinations of two small-mol-
cule CCR5 antagonists (vicriviroc/maraviroc and vicriviroc/
TAK-779) or two CCR5 MAbs (PRO 140/2D7). No significant
synergy was observed for these combinations, although the
vicriviroc/maraviroc CI90 values trended towards significance.
The findings are consistent with prior observations of over-
lapping binding sites for PRO 140 and 2D7 (33) and for vicriviroc
and TAK-779 (37).

PRO 140 was also tested in combination with the gp41
fusion inhibitor enfuvirtide and with the gp120 attachment
inhibitors PRO 542 and BMS-378806 (Table 1). CI values
ranged from 0.84 to 1.28, and none of these combinations
demonstrated synergy that met our criteria for statistical sig-
ificance. For the PRO 140/BMS-378806 combination, modest
antagonism was observed at 50% but not 90% inhibition. The
biological significance of this result is unclear.

Inhibition of HIV-1 pseudoviruses. Next, we used single-
cycle HIV-1 reporter viruses to examine whether the synergis-
tic effects were limited to cell-cell fusion or whether they ex-
tended to other modes of HIV-1 entry. Signals in this assay
require both viral entry and reverse transcription, enabling us
to include NRTI and NNRTI in the analyses. Each combina-
tion was tested against reporter viruses pseudotyped with en-
velopes from HIV-1JR-FL and HIV-1SF162 in at least four
independent assays per virus. A PRO 140/PRO 140 mock
combination was again included as an assay control and
demonstrated additive effects against both HIV-1JR-FL and
HIV-1SF162 pseudoviruses as expected (Table 2).

PRO 140 potently synergized with both maraviroc and vicri-
viroc in blocking virus-cell fusion, and the results met our
criteria for statistical significance. Comparable levels of syn-
ergy were observed against both HIV-1JR-FL and HIV-1SF162
pseudoviruses at 50% and 90% inhibition (Table 2), with CI
values ranging from 0.18 to 0.64. These synergies translated
into dose reductions of up to 14-fold. These results are in good
agreement with those obtained with the cell-cell fusion assay
(Table 1). Neither TAK-779 nor RANTES mediated consis-
tent, high-level inhibition of HIV-1 pseudovirus entry, and
therefore, these compounds were not included in this analysis
(data not shown).

Additive effects were observed for both the maraviroc/vicri-
viroc and PRO 140/2D7 combinations (Table 2). Similarly,
additivity was observed for PRO 140 used in combination with the gp120 inhibitors PRO 542 and BMS-378806. No antagonism was observed for the PRO 140/BMS-378806 combination against either virus. Overall, these findings are consistent with those seen for cell-cell fusion. Lastly, additive effects were observed for PRO 140 in combination with either zidovudine (NRTI) or nevirapine (NNRTI).

**Competition binding studies.** As described above, additive antiviral effects were observed for inhibitors known (PRO 140 and 2D7) or inferred (maraviroc and vicriviroc) to compete for CCR5 binding; however, little is known regarding the competitive binding of synergistic compounds (e.g., PRO 140/maraviroc and PRO 140/vicriviroc). Since noncompetitive binding provides a possible mechanism for synergy between CCR5 inhibitors, we explored this issue by using labeled forms of maraviroc and PRO 140.

Flow cytometry was used to examine inhibition of PRO 140-PE binding to CEM.NRK-CCR5 cells by unlabeled PRO 140, maraviroc, and vicriviroc. PRO 140-PE binding was efficiently inhibited by unlabeled PRO 140, as expected. Complete inhibition was observed in terms of both MFI values (Fig. 2A) and the percentages of cells gated for positive binding (Fig. 2B). The EC₅₀ based on MFI data was 2.5 nM (Fig. 2A), and this value compares favorably with the antiviral IC₅₀ of PRO 140 (Tables 1 and 2). Since the percentage of cells gated is a readout for essentially complete inhibition of binding, the EC₅₀ value was calculated to be 17 nM, and this value is similar to the antiviral IC₅₀ values observed for PRO 140 (Tables 1 and 2). 2D7 also completely inhibited PRO 140-PE binding to CEM.NKR-CCR5 cells (data not shown). The CCR5 specificity of PRO 140-PE was also demonstrated by its inability to bind parental CEM.NKR-CCR5 cells (data not shown).

In sharp contrast, modest levels of inhibition were observed for maraviroc and vicriviroc (Fig. 2). Micromolar concentrations of maraviroc and vicriviroc reduced PRO 140-PE MFI values by 50% or less (Fig. 2A). More dramatically, maraviroc and vicriviroc had little impact on the percentage of cells gated is a readout for essentially complete inhibition of binding; the EC₅₀ value was calculated to be 17 nM, and this value is similar to the antiviral IC₅₀ values observed for PRO 140 (Tables 1 and 2). 2D7 also completely inhibited binding of PRO 140-PE to CEM.NKR-CCR5 cells (data not shown).

The findings suggest that maraviroc and vicriviroc partially reduce the number of binding, the EC₉₀ value was calculated to be 17 nM, and this value is similar to the antiviral IC₅₀ values observed for PRO 140 (Tables 1 and 2).

**TABLE 1. CI values for inhibition of HIV-1 envelope-mediated membrane fusion**

<table>
<thead>
<tr>
<th>First inhibitor</th>
<th>Target</th>
<th>HIV-1 envelope</th>
<th>IC₅₀ (nM)</th>
<th>IC₉₀ (nM)</th>
<th>Second inhibitor</th>
<th>CI₅₀</th>
<th>P value</th>
<th>CI₉₀</th>
<th>P value</th>
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<tbody>
<tr>
<td>PRO 140</td>
<td>CCR5</td>
<td>JR-FL</td>
<td>2.2</td>
<td>28</td>
<td>PRO 140</td>
<td>1.2</td>
<td>0.32</td>
<td>1.0</td>
<td>0.16</td>
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<td></td>
<td></td>
<td>SF162</td>
<td>1.3</td>
<td>20</td>
<td>PRO 140</td>
<td>1.0</td>
<td>0.27</td>
<td>1.0</td>
<td>0.86 ± 0.33</td>
</tr>
<tr>
<td>Maraviroc</td>
<td>CCR5</td>
<td>JR-FL</td>
<td>2.4</td>
<td>44</td>
<td>PRO 140</td>
<td>0.47</td>
<td>0.15</td>
<td>&lt;0.001</td>
<td>0.18 ± 0.04</td>
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<tr>
<td></td>
<td></td>
<td>SF162</td>
<td>0.34</td>
<td>14</td>
<td>PRO 140</td>
<td>0.60</td>
<td>0.17</td>
<td>&lt;0.001</td>
<td>0.28 ± 0.11</td>
</tr>
<tr>
<td>Vicriviroc</td>
<td>CCR5</td>
<td>JR-FL</td>
<td>7.4</td>
<td>46</td>
<td>PRO 140</td>
<td>0.44</td>
<td>0.06</td>
<td>&lt;0.001</td>
<td>0.24 ± 0.11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SF162</td>
<td>0.87</td>
<td>13</td>
<td>Vicriviroc</td>
<td>0.64</td>
<td>0.07</td>
<td>&lt;0.001</td>
<td>0.31 ± 0.11</td>
</tr>
<tr>
<td>Maraviroc</td>
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<td>JR-FL</td>
<td>7.4</td>
<td>46</td>
<td>Vicriviroc</td>
<td>0.71</td>
<td>0.11</td>
<td>0.16</td>
<td>1.2 ± 0.15</td>
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<tr>
<td></td>
<td></td>
<td>SF162</td>
<td>0.87</td>
<td>13</td>
<td>Vicriviroc</td>
<td>0.87</td>
<td>0.06</td>
<td>0.19</td>
<td>0.86 ± 0.28</td>
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<tr>
<td>2D7</td>
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<td>JR-FL</td>
<td>8.8</td>
<td>&gt;200</td>
<td>PRO 140</td>
<td>1.5</td>
<td>0.25</td>
<td>0.024</td>
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<tr>
<td></td>
<td></td>
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<td>74</td>
<td>PRO 140</td>
<td>1.1</td>
<td>0.47</td>
<td>0.61</td>
<td>1.0 ± 0.16</td>
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<tr>
<td>PRO 542</td>
<td>gp120</td>
<td>JR-FL</td>
<td>0.19</td>
<td>2.9</td>
<td>PRO 140</td>
<td>1.2</td>
<td>0.32</td>
<td>0.22</td>
<td>1.0 ± 0.18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SF162</td>
<td>0.36</td>
<td>7.1</td>
<td>PRO 140</td>
<td>0.98</td>
<td>0.28</td>
<td>0.84</td>
<td>0.64 ± 0.26</td>
</tr>
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<td>BMS-378806</td>
<td>gp120</td>
<td>JR-FL</td>
<td>1.2</td>
<td>11</td>
<td>PRO 140</td>
<td>1.2</td>
<td>0.38</td>
<td>0.43</td>
<td>0.74 ± 0.23</td>
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<td>Nevirapine</td>
<td>RT</td>
<td>JR-FL</td>
<td>0.03</td>
<td>0.42</td>
<td>PRO 140</td>
<td>1.1</td>
<td>0.28</td>
<td>0.36</td>
<td>0.82 ± 0.21</td>
</tr>
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<td></td>
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<td>42</td>
<td>280</td>
<td>PRO 140</td>
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<td>0.34</td>
<td>0.30</td>
<td>0.63 ± 0.19</td>
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<tr>
<td>Zidovudine</td>
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<td>JR-FL</td>
<td>140</td>
<td>1900</td>
<td>PRO 140</td>
<td>1.1</td>
<td>0.38</td>
<td>0.37</td>
<td>0.85 ± 0.26</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>86</td>
<td>2100</td>
<td>PRO 140</td>
<td>0.99</td>
<td>0.27</td>
<td>0.91</td>
<td>1.0 ± 0.38</td>
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</table>

*Statistically significant results (P < 0.0016 after application of the Bonferroni correction for multiple comparisons) are indicated in italicized bold text. IC₅₀ and IC₉₀ refer to values for the first inhibitor. RT, reverse transcriptase; NA, not applicable. 2D7 did not consistently achieve 90% inhibition in the assay. CI values represent the means and standard deviations of four or more independent assays.
umber of PRO 140-PE molecules bound per cell; however, these compounds do not reduce the number of cells that bind measurable amounts of PRO 140-PE. Therefore, maraviroc and vicriviroc represent partial antagonists of PRO 140 binding, and this finding provides a mechanism for the antiviral synergy observed between PRO 140 and these small-molecule CCR5 antagonists.

Next, we examined inhibition of 3H-maraviroc binding by unlabeled maraviroc, vicriviroc, and PRO 140. Binding of 3H-maraviroc to CEM.NKR-CCR5 cells was efficiently inhibited by unlabeled maraviroc (Fig. 3A). The EC50 for binding was 4.3 nM and is similar to the antiviral IC50 values observed for maraviroc (Tables 1 and 2).

Vicriviroc also blocked 3H-maraviroc binding to background levels (Fig. 3A). However, there was no correlation between the compounds’ antiviral potency and their potency in blocking 3H-maraviroc binding. For example, whereas vicriviroc demonstrated equal or slightly greater antiviral potency than maraviroc (Tables 1 and 2), vicriviroc was less potent in blocking 3H-maraviroc binding (EC50 = 17 nM) (Fig. 3A). This result is consistent with minor differences in the CCR5 binding sites of these compounds.

Surprisingly, PRO 140 also blocked 3H-maraviroc binding to background levels (Fig. 3A), and this result contrasts with the modest inhibition of PRO 140-PE binding by maraviroc (Fig. 2). PRO 140 inhibited 3H-maraviroc binding with an EC50 of 14 nM, which is 5- to 10-fold higher than the antiviral IC50 of PRO 140 (Tables 1 and 2).

A final experiment examined the stability of maraviroc binding to CEM.NKR-CCR5 cells under the conditions of the competition assay. For this, we preincubated cells with 3H-maraviroc prior to washing, the addition of unlabeled compounds for 30 min, and processing as described above.

FIG. 2. Inhibition of PRO 140-PE binding to CEM.NKR-CCR5 cells by unlabeled PRO 140, maraviroc, and vicriviroc. CEM.NKR-CCR5 cells were incubated with various concentrations of unlabeled PRO 140, maraviroc, or vicriviroc for 30 min at room temperature in PBSA buffer prior to the addition of 5 nM PRO 140-PE for an additional 30 min. Cells were washed and then analyzed by flow cytometry for both the MFI of binding and the percentage of cells gated for positive binding of PRO 140-PE. Inhibition was assessed on the basis of both MFI (A) and the percentage of cells gated (B).

FIG. 3. Inhibition of 3H-maraviroc binding by unlabeled maraviroc, vicriviroc, and PRO 140. (A) CEM.NKR-CCR5 cells were preincubated with various concentrations of unlabeled maraviroc, vicriviroc, or PRO 140 for 30 min in PBSA buffer at ambient temperature prior to the addition of 2 nM 3H-maraviroc for an additional 30 min. Cells were washed and then analyzed for radioactivity by scintillation counting. (B) The stability of maraviroc binding under the assay conditions was examined by preincubating CEM.NKR-CCR5 cells with 2 nM 3H-maraviroc prior to washing, the addition of unlabeled compounds for 30 min, and processing as described above.
DISCUSSION

This study is the first to examine combinations of CCR5 drugs that are currently in development for HIV-1 therapy. Surprisingly, we observed potent antiviral synergy between the CCR5 MAb PRO 140 and each of three structurally different small-molecule CCR5 antagonists. Consistent, high-level synergy was observed across various assay systems, viral isolates, target cells, and inhibition levels. PRO 140 and small-molecule CCR5 antagonists were more potently synergistic when used together rather than in combination with inhibitors that block other stages of HIV-1 entry. In contrast, additive effects were observed for combinations of two small-molecule CCR5 antagonists. Competition binding studies revealed complex and nonreciprocal patterns of CCR5 binding by MAb and small-molecule CCR5 inhibitors and suggest that the synergistic interactions occur at the level of receptor binding. Our findings have implications for the potential use of novel dual-CCR5 regimens for HIV-1 therapy.

Robust synergy between MAb and small-molecule CCR5 inhibitors was observed in this study, and this finding is consistent with that of a recent report (36). Potent synergy was observed for both cell-cell and virus-cell fusion, and there was a good concordance of findings in these two well-established assay systems. Comparable levels of synergy were observed for PRO 140 in combination with each of three small-molecule CCR5 antagonists from unrelated chemical series. In addition, consistent synergy was observed for each of two CCR5 target cells and two well-characterized HIV-1 envelopes. HIV-1JR-FL was isolated from the brain of an AIDS patient at autopsy (31). Like other late-stage neurovirulent viruses (17), HIV-1JR-FL encodes an envelope with high binding affinity for CCR5 (36). Lastly, similar levels of synergy were observed when PRO 140 and maraviroc were tested against HIV-1BaL pseudoviruses (15) in preliminary studies (data not shown).

Synergy increased with increasing levels of viral inhibition and translated into in vitro dose reductions of up to 14-fold. Viewed alternatively, this degree of synergy provides a corresponding increase in antiviral pressure at a given concentration of drugs, thereby improving viral suppression and potentially delaying the emergence of drug-resistant virus.

We also observed potent synergy for RANTES used in combination with either maraviroc or PRO 140. Endogenous levels of RANTES may afford some protection against HIV-1 disease progression during natural infection (16, 25), and therefore, our finding of synergy has important and positive implications for CCR5-targeted therapies of HIV-1. Antiviral synergy between RANTES and PRO 140 is not surprising based on our prior observation that RANTES signaling is not blocked by antiviral concentrations of murine PRO 140 (PA14) (33). Synergy between RANTES and maraviroc is less easily explained given that maraviroc is a potent CCR5 antagonist. However, our findings are consistent with prior observations of synergy between the small-molecule CCR5 antagonist SCH-C and aminoxypentane-RANTES (44), a RANTES derivative that has been evaluated as a potential topical microbicide (19).

In contrast to the robust synergy observed between MAb and small-molecule CCR5 antagonists, additive effects were observed for combinations of small-molecule CCR5 antagonists. A lack of cooperativity is consistent with the view that these molecules compete for binding to a common pocket on CCR5 (13, 30, 47, 48). However, synergy is not required to derive clinical benefit from combination therapy. No interference was observed between small-molecule CCR5 antagonists, and this finding has relevance when considering combining such agents in the clinic.

Similarly, we did not observe potent synergy between PRO 140 and inhibitors of HIV-1 attachment (PRO 542 and BMS-378806), fusion (enfuvirtide), or reverse transcriptase (zidovudine and nevirapine), and these findings underscore the significance of the synergy observed for PRO 140 and small-molecule CCR5 antagonists. A number of prior studies have examined interactions between various small-molecule CCR5 antagonists (maraviroc, SCH-C, TAK-220, TAK-652, and E913) and drugs from each of the existing HIV-1 treatment classes. Most (42–44) but not all (11, 26) studies have reported broad synergy between CCR5 inhibitors and the other HIV-1 treatment classes, and the divergent results may reflect differences in the compounds and methods used for antiviral testing as well as differences in the methods used for data analysis. When maraviroc was tested against 20 licensed antiretroviral agents, additive effects were observed in all but three cases, in which modest synergy was reported (11). This result is consistent with our findings for combinations of PRO 140 and HIV-1 inhibitors that do not target CCR5.

Synergy between anti-HIV-1 drugs may stem from a variety of mechanisms. In mixed virus cultures, one compound may inhibit virus resistant to a second compound (18), and NRTI/NNRTI combinations may overcome specific reverse transcriptase-mediated resistance mechanisms (4, 5). Metabolic interactions between inhibitors may increase their effective intracellular drug concentrations (28), and synergistic entry inhibitors may disrupt interdependent steps in the entry cascade (29, 41). The present study examined clonal viral envelopes rather than mixed populations, and the extracellular nature of the target argues against metabolic interactions. Multiple domains of gp120 contribute to CCR5 binding (8), but it is unclear at present whether these interactions represent separate or discrete events during infection.

Our findings indicate that antiviral synergy between MAb and small-molecule CCR5 inhibitors may occur at the level of the receptor, although the mechanism remains poorly defined. As discussed above, MAbs and small molecules bind distinct loci on CCR5 (13, 30, 33, 47, 48). When preincubated with CCR5 cells in the present study, PRO 140 completely blocked subsequent binding of maraviroc to the receptor, although the PRO 140 concentrations were higher than those needed to block HIV-1 entry into the same cells. In contrast, preincubation of CCR5 cells with supersaturating concentrations of maraviroc or vicriviroc reduced PRO 140 binding by 50% or less. As one possible explanation, PRO 140 could recognize CCR5 conformers that are not bound by maraviroc or vicriviroc. Although cell surface CCR5 exists in multiple conformations (22), it seems unlikely that the small-molecule antagonists could demonstrate potent antiviral activity while failing to bind a significant fraction of cell surface CCR5. In this regard, it is important to note that a common cellular background (CEM.NKR-CCR5 cells) was used for competition binding and antiviral studies, and therefore, the findings are not related to cell-specific differences in CCR5 expression.
In our view, a more plausible explanation for our findings is that PRO 140 is capable of forming a ternary complex with maraviroc-bound CCR5, and this ternary complex provides an increased barrier to HIV-1 entry. Within the context of this model, PRO 140 may bind maraviroc-bound CCR5 somewhat less efficiently than free CCR5, as evidenced by the modest reduction in PRO 140 binding in the presence of maraviroc. However, we note that our studies do not demonstrate the existence of a ternary complex, and additional studies will be required to test this model and to further define the mechanisms of synergy between MAb and small-molecule CCR5 inhibitors. To this end, time-of-addition studies have indicated that murine PRO 140 (PA14) acts at a later stage of the fusion cascade than does the small-molecule CCR5 antagonist SCH-C (36). This report proposed that SCH-C blocks gp120 binding to CCR5, while PA14 prevents ill-defined postbinding events.

The combination index method is widely used to assess drug-drug interactions. In this method, cooperativity is often defined on the basis of empirical CI values (e.g., <0.9 for synergy and >1.1 for antagonism) irrespective of interassay variability. Statistical analyses are performed infrequently, and even more rarely are adjustments made for multiple comparisons. In the absence of such analyses, there is increased potential to overestimate the number of synergistic combinations.

We adopted a rigorous approach for identifying synergistic effects. CI values were tested for statistical significance against the null hypothesis of additivity (CI = 1). In addition, our studies determined 20 to 30 different CI values per experiment (Tables 1 and 2), as is common in synergy studies. In order to reduce the potential for spurious positive results, we reduced the significance level using the Bonferroni correction. We also evaluated a mock combination as a control. We conclude that numerous apparent synergies (CI < 0.9) could not be distinguished from interassay variation based on the available data. Nevertheless, PRO 140 and small-molecule inhibitors demonstrated significant synergy under every test condition, lending credence to this finding. Combinations with CI values that trend toward significance in the present survey, such as the PRO 140/enfuvirtide combination, could be explored in future studies.

A growing body of data indicates that MAb and small-molecule CCR5 antagonists represent distinct subclasses of CCR5 inhibitors, and a number of important parallels can be drawn between NRTI and NNRTI inhibitors on the one hand and between MAb and small-molecule CCR5 antagonists on the other. In each instance, there are distinct binding loci for the inhibitors on the target protein (reverse transcriptase or CCR5). One set of inhibitors (NNRTI or small-molecule CCR5 antagonists) acts via allosteric mechanisms, while the other set (NRTI or CCR5 MAb) acts as a competitive inhibitor. Like NRTI and NNRTI, MAb and small-molecule CCR5 inhibitors are synergetic and possess complementary patterns of viral resistance in vitro in preliminary testing (20, 27). NRTI and NNRTI represent important and distinct treatment classes even though they target the same protein, and MAb and small-molecule CCR5 inhibitors similarly may offer distinct HIV-1 treatment modalities.

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