Neuronal Apoptotic Signaling Pathways Probed and Intervened by Synthetically and Modularly Modified (SMM) Chemokines*

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As the main coreceptors for human immunodeficiency virus type 1 (HIV-1) entry, CXCR4 and CCR5 play important roles in HIV-associated dementia (HAD). HIV-1 glycoprotein gp120 contributes to HAD by causing neuronal damage and death, either directly by triggering apoptotic pathways or indirectly by stimulating glial cells to release neurotoxins. Here, to understand the mechanism of CXCR4 or CCR5 signaling in neuronal apoptosis associated with HAD, we have applied synthetically and modularly modified (SMM)-chemokine analogs derived from natural stromal cell-derived factor-1α or viral macrophage inflammatory protein-II as chemical probes of the mechanism(s) whereby these SMM-chemokines prevent or promote neuronal apoptosis. We show that inherently neurotoxic natural ligands of CXCR4, such as stromal cell-derived factor-1α or viral macrophage inflammatory protein-II, can be modified to protect neurons from apoptosis induced by CXCR4-prefering gp120H9251 and that the inhibition of CCR5 by antagonist SMM-chemokines, unlike neuroprotective CCR5 natural ligands, leads to neurotoxicity by activating a p38 mitogen-activated protein kinase (MAPK)-dependent pathway. Furthermore, we discover distinct signaling pathways activated by different chemokine ligands that are either natural agonists or synthetic antagonists, thus demonstrating a chemical biology strategy of using chemically engineered inhibitors of chemokine receptors to study the signaling mechanism of neuronal apoptosis and survival.

HAD2 is manifested by cognitive and motor dysfunction observed after infection with HIV-1 (1). Although about half of children and a quarter of adults infected with HIV-1 eventually develop some form of dementia (2, 3), there is no specific treatment for HAD. In recent years, chemokine receptors have been found to play important roles in the pathogenesis of HIV-1 infection, including HAD. In a plausible model, HIV-1 enters target cells through a direct fusion process in which HIV/gp120 binds to CD4, the main receptor on the target cell surface, plus one of two chemokine receptors, usually CXCR4 or CCR5 (4, 5). Natural chemokines that bind CXCR4 and CCR5 can inhibit HIV-1 infection (6, 7), probably by blocking common binding sites on the chemokine receptors that are also required for gp120 interaction (8, 9) or by inducing receptor internalization (10, 11). Although CXCR4 is expressed in virtually all tissues, including neurons, microglia, and astrocytes in the brain (12, 13), CCR5 is the primary receptor by which the cells in the nervous system get infected (14, 15).

There are two major mechanisms for gp120-induced neuronal apoptosis. The predominant pathway is an indirect process that results in the release of toxic factors from immune-stimulated or HIV-infected cells such as macrophages and microglia. HIV-1 can stimulate or infect microglia and macrophages by interacting with chemokine receptors in conjunction with CD4. Macrophages and microglia can also be activated by factors such as shed gp120 released from infected cells (1, 16). The second mechanism is direct interaction of gp120 with neurons and possibly astrocytes via their chemokine receptors (17). In fact, picomolar concentrations of CXCR4 (or X4)-preferring or CXCR4/CCR5 (or dual)-tropic gp120 are known to induce neurotoxicity through CXCR4 despite the fact that neurons do not express CD4 on their cell surface (18–20). Also the finding that gp120-induced neuronal damage can be prevented by anti-gp120 antibodies but not by anti-CD4 antibodies not only proves the specificity of the effect of gp120 but also implies that CD4 is not necessary for neurotoxicity (21, 22). Whether or not neuronal damage and apoptosis occur via indirect or direct pathways or both, chemokine receptors are involved in the development of HAD, which suggests that therapeutic interventions at the level of chemokine receptors may reverse or attenuate the symptoms associated with HAD.

Because of the profound activities of chemokine receptors in HAD, developing selective, potent inhibitors of chemokine receptors and understanding the physiological or pathological processes of HAD are crucial for devising novel strategies of...
clinal interventions. As such, we have been working toward the development of a family of unnatural chemokines, termed SMM-chemokines, which have higher receptor selectivity, binding affinity, and/or antiviral activities than natural chemokines. We previously demonstrated how this SMM-chemokine approach could be applied to convert the nonselective vMIP-II into highly selective ligands of CXCR4 or CCR5 in terms of their binding, signaling, and/or antiviral activity (23). We also used a similar strategy to modify the biological and pharmacological properties of SDF-1α (24). This is of particular importance because SDF-1α, the only known naturally occurring ligand for CXCR4, is neurotoxic in the presence or absence of HIV/gp120 (19).

Although inhibitors of chemokine receptors may be beneficial for HAD, the true benefit of these compounds remains to be shown. Interestingly, certain natural chemokines have been shown to protect neurons against X4-prefering gp120, but they do so by activating a neuroprotective pathway via non-CXCR4 receptors, including CCR5 (19) and CX3CR1 (25). It is still not known whether the same or different neuroprotective pathway can be initiated via CXCR4, or the direct blockade of CXCR4 by its inhibitors alone can sufficiently reverse gp120-induced neuronal apoptosis. Also the effect of CCR5 inhibition by agents other than its natural agonists has never been examined before. To address these questions, we used rodent cerebrocortical cultures (RCCs) that not only contained the type and proportion of cells found in human brain, i.e. neurons, astrocytes, and macrophages/microglia, but also expressed CXCR4, CCR5, and other chemokine receptor homologues (14, 26–28) that are capable of mediating HIV-1 infection via gp120 binding similar to their human homologues (29, 30). We examined the potential neuroprotective or neurotoxic effects of CXCR4- and CCR5-selective SMM-chemokines and their mechanism(s) of action in preventing or inducing neuronal apoptosis, which would provide new insights into the distinct signaling pathways of HAD-associated neuronal apoptosis activated by different chemokine receptor ligands that were either agonists or antagonists.

**EXPERIMENTAL PROCEDURES**

**Total Chemical Synthesis of SMM-Chemokines**—The automated stepwise incorporation of protected amino acids was performed using an Applied Biosystems 433A peptide synthesizer (Foster City, CA) with a CLEAR amide resin (Peptides International, Louisville, KY) as the solid support. N-(9-fluorenyl)methoxycarbonyl chemistry was employed for the synthesis (23, 24). 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole (HOBT) were used as coupling reagents in the presence of disopropylethylamine (DIEA). In certain coupling steps with potentially slow reaction rates, double coupling followed by capping of the unreacted amino functional groups was performed. After incorporation of the 50th residue, 2% v/v of Me3SO was introduced to the solution to enhance the coupling reaction. After removing N-terminal N-(9-fluorenyl)methoxycarbonyl protection, the protein was cleaved from the resin support by adding a cleavage mixture comprised of phenol (4% wt/v), thioanisole (5% v/v), water (5% v/v), ethanedithiol (2.5% v/v), triisopropylsilane (1.5% v/v), and trifluoroacetic acid (TFA, 82% v/v). The protein was precipitated by adding ice-cold tert-butyl methyl ether and washed repeatedly in cold ether. The crude protein was dissolved in 25% CH3CN in water containing 0.1% TFA before being lyophilized, and it was dissolved in water and purified using semi-preparative reverse phase-high performance liquid chromatography (RP-HPLC). Folding of the purified protein was performed in 1 m guanidinium hydrochloride and 0.1 m trisoma base at pH 8.5 (1 mg protein/ml folding buffer) and was monitored by analytical RP-HPLC using a Vyde C-18 column (0.46 × 15 cm, 5 μm) with a flow rate of 1 ml/min; solvent A, water with 0.1% TFA; solvent B, 20% water in CH3CN with 0.1% TFA; and a linear gradient 30–70% B over 30 min. Protein desalination and purification were then performed. The purified protein was characterized by matrix-assisted laser desorption ionization time-of-flight-mass spectroscopy (MALDI-TOF-MS).

**Preparation and Treatment of RCCs**—RCCs were prepared from Sprague-Dawley rat embryos at days 15–17 of gestation (19, 31, 32) and used for experiments after 17–24 days in culture. D10C extra growth media containing 160 ml of Dulbecco’s modified Eagle’s medium with glucose (Sigma, D5671), 20 ml of F-12 growth serum (Sigma, N4888), 20 ml of heat-inactivated bovine serum (Hyclone, Logan, UT, SH30072.03), 0.48 ml of penicillin-streptomycin (Sigma), 2 ml of 200 mm glutamine (Sigma), and 5 ml of HEPES at pH 7.0 (Omega, Tarzana, CA; HB-20) were used to grow and maintain the RCCs.

**Treatment with gp120, SMM-Chemokines, and Various Inhibitors of Intracellular Signaling Cascades**—Mixed neuronal/glial cells cultured on coverslips were transferred into 24-well plates containing Earle’s balanced salt solution (Invitrogen) supplemented with 1.8 mm CaCl2 and 5 μM glycine. By following a modified procedure published by others, including our own laboratory (1, 9, 19, 23, 25, 33), the cultures were exposed to gp120 (400 pm), SMM-chemokines (20 nm), the Ser/Thr kinase Akt inhibitor (LV294002, 50 μM), extracellular regulated kinase (ERK) inhibitor (PD98059, 2 μM), c-JUN N-terminal kinase (JNK) inhibitor (SP600125, 10 μM), p38 mitogen-activated protein kinase (MAPK) inhibitor (SB203580, 10 μM), or combinations thereof for 24 h. SMM-chemokines were applied for 5 min and intracellular signaling cascade inhibitors for 15 min prior to gp120 exposure. HIV-1/gp120 from X4-tropic strain IIIB was purchased from ImmunoDiagnostics (Woburn, MA); gp120 from the CCR5-prefering strain BAL was obtained through the AIDS Research and Reference Reagent program (Division of AIDS, NIAID, National Institutes of Health, Bethesda, MD). All of the inhibitors of intracellular signaling cascades were purchased from the BioMol (Plymouth Meeting, PA).

**Assessment of Neuronal Survival**—Following a modified procedure published by others, including our own laboratory (9, 19, 25, 33), neuronal survival was determined by staining permeabilized cells with Hoechst 33342 (Molecular Probes, Eugene, OR) to detect changes in the nuclear morphology of apoptotic cells and with a neuron-specific antibody, anti-microtubule-associated protein-2 (MAP-2, Sigma), to identify cell type. Prior to staining, cells were fixed for 25 min with 4% (wt/v) paraformaldehyde followed by overnight incubation with 0.1%
paraformaldehyde at 4 °C. After three washes with PBS, paraformaldehyde-fixed cells were permeabilized with 0.2% Tween 20/PBS (Sigma). Nonspecific binding sites were blocked by 30-min incubation with a 10% solution of heat-inactivated goat serum (Invitrogen) in 0.2% Tween 20/PBS. To specifically stain neurons, the cells were incubated for 4 h at room temperature with 1:500 dilution of anti-microtubule-associated protein-2. After three washes with 0.2% Tween 20/PBS, the cells were incubated with secondary antibody, Alexa Fluor 594 antirat IgG (Molecular Probes), for 30 min at room temperature. The cells were washed twice with 0.2% Tween 20/PBS followed by two washes in PBS. Cell nuclei were subsequently stained with Hoechst 33342 (12 μM) for 5 min in the dark. After washing twice with PBS, coverslips containing the cells were mounted onto glass slides. Ten microscopic fields were counted for each coverslip, and two to three coverslips per treatment were used for each experiment. For each coverslip, the number of cells analyzed ranged from 900–1300. At least three independent experiments were performed for each paradigm.

**Western Blot Experiments**—RCCs were incubated for 24 h with SMM-chemokines and gp120 from X4-tropic strain IIIB, washed once with ice-cold PBS and lysed with 1% lysis buffer (Cell Signaling Technology, Danvers, MA) containing one tablet of complete protease inhibitor mixture and 5 mM NaF. Equal amounts of protein were separated under reducing conditions by SDS-PAGE (NuPAGE, Invitrogen) and electrotransferred onto the polyvinylidene difluoride membranes (Millipore, Billerica, MA). After blocking with 5% bovine serum albumin in Tris-buffered saline Tween 20 (Bio-Rad), the membranes were incubated with antibodies against the indicated antigens. An extra sample lane was incubated without the primary antibody as a control. All the antibodies were purchased from the Cell Signaling Technology except anti-phospho-p38 (Promega, Madison, WI), anti-α-tubulin (Sigma), and horseradish peroxidase-conjugated secondary antibodies (anti-rabbit IgG, Pierce) and anti-murine IgG (Amersham Biosciences). After visualization by chemiluminescence (SuperSignal, Pierce) or ECL (Amersham Biosciences), the detected proteins were quantified using densitometry (n = 3; statistical comparison by unpaired Student’s t test; *, p < 0.05). The Western blot shows one representative example of three independent experiments.

**Statistical Analysis**—Statistical significance was determined using an analysis of variance followed by a Scheffé or Bonferroni/Dunn post hoc test.

**RESULTS AND DISCUSSION**

**Design and Selection of SMM-Chemokines**—The SMM-chemokines used for the present study included two CXCR4-selective inhibitors, D-(1–10)-vMIP-II and D-(1–10)-vMIP-II (9–68)-SDF-1α, and two CCR5-selective ligands, (1–10)-MIP-1β-(11–71)-vMIP-II and D-(1–10)-MIP-1β-(11–71)-vMIP-II. These SMM-chemokines contained various de novo designed sequence replacements or D-amino acid substitutions and displayed enhanced receptor selectivity, binding affinity, and anti-HIV activities compared with natural chemokines (23, 24). With the exception of (1–10)-MIP-1β-(11–71)-vMIP-II (designated here RCP188), which can trigger Ca2+ mobilization in CCR5-expressing cell lines at high concentrations (i.e. 1 μM), all other analogs were antagonist ligands of their corresponding target receptors, as they could not mobilize Ca2+ at any concentration. D-(1–10)-vMIP-II (RCP168), (1–10)-MIP-1β-(11–71)-vMIP-II (RCP188), and D-(1–10)-MIP-1β-(11–71)-vMIP-II (RCP189) were generated by modifying only a small N-terminal module of 10 residues of vMIP-II, the most nonspecific and cross-reactive chemokine ligand known to date (34). We previously demonstrated that RCP168 and RCP188 are selective for CXCR4 and CCR5, respectively, as they show similar or significantly enhanced binding affinities for their corresponding target receptors but drastically decreased or even completely abolished cross-binding activities for other receptors. RCP189 was also shown to maintain its selectivity toward CCR5, although its binding affinity was weaker than that of RCP188. In another study, we replaced the N-terminal (1–8) sequence module of SDF-1α, the only natural ligand for CXCR4, with all D-forms of (1–10) residues of vMIP-II (24). Subsequent binding and antiviral assays showed that D-(1–10)-vMIP-II-(9–68)-SDF-1α (RCP222) was not only a selective CXCR4 ligand but also an effective HIV-1 inhibitor. The natural chemokines, SDF-1α and vMIP-II, were used as positive controls and for comparisons. Their sequences and modifications are provided in Table 1.

**Neuroprotective Effects of Antagonist CXCR4-selective SMM-Chemokines**—Two CXCR4-specific SMM-chemokines, RCP168 and RCP222, were examined for their potential neuroprotective effects after exposure to the X4-preferring gp120IIIb (Fig. 1A). Stimulation of CXCR4 by gp120 led to 10–20% neuronal apoptosis, which is statistically significant and consistent with the previous reports on gp120-induced neurotoxicity in both RCCs and hippocampal neuronal cultures (1, 19, 25, 33). The same studies also demonstrated the neuroprotective effects of natural chemokine receptor ligands of CCR5 and CXCR1, such as macrophage inflammatory protein (MIP)-1β, regulated on activation normal T cell expressed and secreted (RANTES), and fractalkine, which could prevent the toxicity of 10–20% induced by HIV-1 gp120. Note that RCCs, which express CXCR4, CCR5, and other chemokine receptor homologues with good sequence similarities to the corresponding human chemokine receptors (14, 26–28, 35, 36), are a suitable model system to study the actions of gp120IIIb human natural chemokines, and their synthetic derivatives, as X4- or dual-tropic virus is known to cause HIV-1 infection and/or neuronal apoptosis in both human and RCCs (20), and its toxicity was reversed by MIP-1β, RANTES, and fractalkine (1, 19, 25, 33). In fact, in addition to having homologous chemokine receptors, rats express chemokine ligand homologues that share great sequence similarities with their human counterparts, as shown by rat SDF-1α, which is highly conserved with >99% identity to its known human, feline, and murine forms (37).

Two natural chemokines, vMIP-II and SDF-1α, were not only incapable of protecting neurons from gp120IIIb-induced neuronal apoptosis but were also neurotoxic on their own (Fig. 1A), which demonstrates the potential problems associated with the use of natural, agonist, or nonspecific chemokines in clinical applications. Therefore, we treated the RCCs with antagonist CXCR4-selective SMM-chemokines chemically engineered for higher target selectivity. As shown in Fig. 1A,
RCP168 or RCP222 was able to abrogate gp120\textsubscript{IIIB}-induced neuronal apoptosis, which demonstrates that inherently neurotoxic, natural chemokines can be synthetically modified to reverse gp120\textsubscript{IIIB} neurotoxicity. Note that we did not improve the neuroprotective effects of natural chemokines, which are inherently neurotoxic, but rather completely converted neurotoxic, natural chemokines into nontoxic, neuroprotective agents. These drugs presumably inhibit the neurotoxic effect of gp120\textsubscript{IIIB} at least in part, in a direct manner, as the CXCR4-specific SMM-chemokines and gp120\textsubscript{IIIB} all interact with CXCR4. Furthermore, none of these CXCR4-selective SMM-chemokines produced neurotoxicity on their own, which is consistent with our toxicity data on these compounds using human white blood cells (23). Taken together, our data indicate that inhibition of CXCR4 is an effective way of protecting neurons from gp120\textsubscript{IIIB}-induced neurotoxicity, providing that the inhibitors used for blocking gp120\textsubscript{IIIB}-CXCR4 interaction are not inherently neurotoxic like SDF-1\textalpha or vMIP-II.

**Neurotoxic Effects of Antagonist CCR5-selective SMM-Chemokines**—Following our finding that CXCR4 antagonists prevent gp120\textsubscript{IIIB}-induced neurotoxicity, we then examined the potential neuroprotective effect of the CCR5-selective agonist, RCP188. Based on previous studies demonstrating that natural ligands of CCR5, such as MIP-1\beta and RANTES, can protect neurons from gp120-induced apoptosis (19), we hypothesized that RCP188, in which the N-terminal (1–10) residues of vMIP-II are replaced with the (1–10) residues of MIP-1\beta (Table 1), might also be able to provide neuroprotection from gp120\textsubscript{IIIB}-induced neurotoxicity. However, as shown in Fig. 1B, RCP188 was not only incapable of protecting neurons from gp120\textsubscript{IIIB}-induced neuronal apoptosis but also was inherently neurotoxic itself. We hypothesized that the neurotoxic effect of RCP188 in the presence of gp120\textsubscript{IIIB} might be because of its inability to activate CCR5 and subsequent neuroprotective pathways at low concentrations (20 nM). In support of this premise, our previous Ca\textsuperscript{2+} mobilization assays showed that although RCP188 can activate Ca\textsuperscript{2+} release at concentrations greater than 1 \textmu M, its signaling intensity at these concentrations is still 3-fold less than that of wild-type MIP-1\beta at 100 nM (23). In fact, RCP188 cannot trigger Ca\textsuperscript{2+} release at concentrations less than 1 \textmu M, suggesting that RCP188 may act as an antagonist ligand at lower concentrations. In other words, the failure to activate CCR5 and subsequent neuroprotective pathways via CCR5 by RCP188 may lead to neurotoxicity in the presence of gp120\textsubscript{IIIB}.

To test the notion that antagonists of CCR5 would not prevent neurotoxicity engendered by gp120\textsubscript{IIIB}, we generated RCP189 by replacing the N-terminal (1–10) residues of RCP188 with all D-forms and tested its activity in neuronal survival experiments. Our binding and Ca\textsuperscript{2+} mobilization assays showed that RCP189 is an antagonist CCR5 ligand, although its binding activity is diminished compared with that of RCP188 (23). As expected, RCP189 was not able to rescue neurons from gp120\textsubscript{IIIB}-induced neuronal apoptosis (Fig. 1B). Taken together, the data for RCP188 and RCP189 demonstrate that CCR5 antagonists do not protect neurons from gp120\textsubscript{IIIB}-induced neurotoxicity, as they fail to activate CCR5 and/or subsequent neuroprotective pathways via CCR5.

### TABLE 1

| Analog Designation | Modification Diagrams | Amino Acid Sequences$^{\alpha}$ |
|--------------------|-----------------------|---------------------------------|
| SDF-1\textalpha    | 1-8 9-68              | KPVSLSYRPCRFFESHVA              |
|                    | NT SDF-1\textalpha Core | RANYVKHKLKNLTPNCAIQI            |
|                    |                      | VARLKNNRQVCIDPPLK              |
|                    |                      | WIQEYLEKALNK                   |
| vMIP-II            | 1-10 11-71            | LGASWHRPDKDCGLGTYQK             |
|                    | NT vMIP-II Core       | RPLPQLVLLSSWYPSQLCS             |
|                    |                      | KPGVIFLTKRGRQVCADKS             |
|                    |                      | KDWWKLMQQLPVTAR                |
| RCP168             | 1-10 11-71            | LGASWHRPDKDCGLGTYQK             |
|                    | NT vMIP-II Core       | RPLPQLVLLSSWYPSQLCS             |
|                    |                      | KPGVIFLTKRGRQVCADKS             |
|                    |                      | KDWWKLMQQLPVTAR                |
| RCP222             | 1-10 9-68             | D-Amino Acids                   |
| RCP188             | 1-10 11-71            | APMGSDDPPTACGLGTYQK             |
|                    | NT vMIP-II Core       | RPLPQLVLLSSWYPSQLCS             |
|                    |                      | KPGVIFLTKRGRQVCADKS             |
|                    |                      | KDWWKLMQQLPVTAR                |
| RCP189             | 1-10 11-71            | APMGSDDPPTACGLGTYQK             |
|                    | NT vMIP-II Core       | RPLPQLVLLSSWYPSQLCS             |
|                    |                      | KPGVIFLTKRGRQVCADKS             |
|                    |                      | KDWWKLMQQLPVTAR                |

$^{\alpha}$ D-amino acids are shown in italic, whereas substituted residues (from vMIP-II, SDF-1\textalpha, or MIP-1\beta) are shown in bold.
Mechanism(s) by Which Antagonist CCR5-selective SMM-chemokines Induce Neurotoxicity in the Absence of gp120 IIIB

An even more intriguing issue regarding the neurotoxic effects of antagonist CCR5-selective SMM-chemokines is that they can induce neurotoxicity even in the absence of gp120 IIIB. To understand the mechanism(s) by which CCR5 antagonists...
FIGURE 3. Neuroprotective effects of CXCR4-selective SMM-chemokines independent of Akt, ERK, JNK, or p38 MAPK pathway. Inhibition of Akt, ERK, JNK, or p38 MAPK by LY294002 (A), PD98059 (B), SP600125 (C), and SB203580 (D), respectively, did not affect RCP168- or RCP222-mediated neuroprotection from gp120IIIB. *, p < 0.001 compared with value for gp120, vMIP-II, SDF-1α, or combinations; **, p < 0.001 for LY294002-, PD98059-, SP600125-, and SB203580-treated cells.
induce gp120\textsubscript{IIIB}-independent neurotoxicity, a variety of inhibitors of intracellular signaling cascades were tested for their ability to prevent neuronal apoptosis associated with antagonist CCR5-specific SMM-chemokines. These inhibitors included PD98059 to inhibit ERK, SP600125 to prevent JNK activation, and SB203580 to inactivate p38 MAPK. As an added control, the effect of Akt inhibitor, LY294002, on the neurotoxicity of CCR5 antagonists was also examined. As protein kinase Akt is a major component of prosurvival and antiapoptotic mechanisms in neurons and other cell types (25, 38–41) and is involved in the neuroprotective pathway activated by natural CCR5 ligands (42), we hypothesized that neurotoxic CCR5 inhibitors would not activate Akt. This finding would further support our hypothesis that CCR5 antagonists lead to neurotoxicity because they fail to activate CCR5 and subsequent neuroprotective pathways.

Among the inhibitors examined, only SB203580 substantially reversed neuronal apoptosis induced by neurotoxic CCR5-specific SMM-chemokines (Fig. 2A). Thus, the neuroprotective CXCR4 ligands RCP168 and RCP222 were not able to rescue neurons from neurotoxicity mediated by R5-preferring RCP188 (A), RCP189 (B), or gp120\textsubscript{BAL} (C). * \( p < 0.0001 \) compared with value for gp120, RCP188, RCP189, or combinations.

**FIGURE 4.** Lack of neuroprotection by CXCR4-selective SMM-chemokines from CCR5-mediated neurotoxicity. The neuroprotective CXCR4 ligands RCP168 and RCP222 were not able to rescue neurons from neurotoxicity mediated by R5-preferring RCP188 (A), RCP189 (B), or gp120\textsubscript{BAL} (C). *, \( p < 0.0001 \) compared with value for gp120, RCP188, RCP189, or combinations.
by LY294002, ERK by PD98059, or JNK by SP600125 did not reverse the neurotoxicity induced by CCR5 antagonists or gp120

*Inhibitors of intracellular signaling cascades for their ability to reverse the neuroprotective effects of CXCR4 antagonists. We found that neither PD98059 (to inhibit ERK) nor SP600125 (to inhibit JNK) attenuated the neuroprotective action of CXCR4-selective SMM-chemokines (Fig. 3, B and C). Additionally, inhibition by SB203580 of p38 MAPK, an important contributor to gp120*Inhibitors of intracellular signaling cascades for their ability to reverse the neuroprotective effects of CXCR4 antagonists. We found that neither PD98059 (to inhibit ERK) nor SP600125 (to inhibit JNK) attenuated the neuroprotective action of CXCR4-selective SMM-chemokines (Fig. 3, B and C). Additionally, inhibition by SB203580 of p38 MAPK, an important contributor to gp120*Inhibitors of intracellular signaling cascades for their ability to reverse the neuroprotective effects of CXCR4 antagonists. 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SMM-Chemokines as Probes and Inhibitors of HAD

![Diagram](image)

**FIGURE 6. A model for the molecular mechanisms of action of neuroprotective CXCR4-selective SMM-chemokines versus neurotoxic CCR5-specific SMM-chemokines.** Antagonistic CXCR4-selective SMM-chemokines prevent gp120IIIB-induced neuronal apoptosis most likely via steric hindrance without activating Akt, ERK, or JNK, whereas natural CXCR4 agonist SDF-1α (and HIV-1 gp120) activates the pathway involving p38 MAPK. Antagonistic CCR5-selective SMM-chemokines lead to neuronal apoptosis via the p38 MAPK pathway, whereas natural CCR5 agonist MIP-1β activates a different pathway involving Akt.

The lack of activation of these antiapoptotic pathways by neuroprotective CXCR4 inhibitors suggested that an alternative action, such as steric hindrance (8, 9) or internalization (10, 11), may play a role in the neuroprotective mechanism. Because these D-amino acid-containing SMM-chemokines have been shown not to induce CXCR4 internalization (23), thus demonstrating that CXCR4-mediated neuroprotection does not require CXCR4 internalization, we decided to focus our attention on steric hindrance as a possible mechanism of action. Thus, RCCs were incubated simultaneously with both a neuroprotective CXCR4 ligand (i.e. RCP168 or RCP222) and a neurotoxic CCR5-selective SMM-chemokine (i.e. RCP188 or RCP189) to examine whether the neuroprotective CXCR4-selective ligand could still provide neuroprotection from CCR5-mediated neurotoxicity. As a control, the neuroprotective CXCR4 ligands were also incubated with the neurotoxic CCR5 ligands RCP188, RCP189, and gp120BAL, which cause neuronal death via a p38 MAPK-dependent pathway.

**Western Blot Experiments**—To verify the finding that antagonist CCR5-selective SMM-chemokines induce gp120IIIB-independent neurotoxicity via p38 MAPK activation, we performed Western blot experiments (Fig. 5A) and quantified the relative density of phospho-p38 using densitometry. As shown in Fig. 5B, only RCP189 and gp120 were able to induce a statistically significant expression of phospho-p38, which provides, in addition to our pharmacological data, direct biochemical evidence showing the activation of the p38 pathway downstream of CCR5 after CCR5 antagonist binding. This finding again demonstrates that CCR5-selective inhibitors and gp120IIIB use the common MAPK signaling pathway involving p38 to initiate their neurotoxic processes. As previously demonstrated by the pharmacological data, p38 MAPK expression was not induced by neuroprotective CXCR4-selective SMM-chemokines because the p38 MAPK pathway is an important contributor to gp120IIIB- and CCR5 inhibitor-induced neuronal apoptosis, not a neuroprotective mechanism. As an added control, the relative density of phospho-Akt was also measured. No SMM-chemokine was able to induce phospho-Akt expression (Fig. 5C), which is consistent with the pharmacological data.

**Significance**—We examined the neuroprotective and neurodestructive actions of novel CXCR4- or CCR5-specific SMM-chemokines and studied the mechanism(s) whereby these SMM-chemokines prevent or promote neuronal apoptosis. As shown in Fig. 6, the present study demonstrates that unlike natural agonist SDF-1α, which causes neuronal death via a p38 MAPK-dependent pathway (19), antagonistic CXCR4-selective SMM-chemokines can effectively prevent gp120IIIB-induced neuronal apoptosis. These novel CXCR4-selective SMM chemokines manifest a neuroprotective mechanism neither involving activation of Akt, ERK, JNK, or Ca²⁺ mobilization pathway nor inducing CXCR4 internalization. Also compared with natural CCR5 agonist ligands that are known to promote neuronal survival by activating Akt (42), our data on unnatural CCR5 agonist ligands that show overlap in the CXCR4 binding residues of these SMM-chemokines with X4-preferring gp120 but not with SDF-1α (48, 49). Another mechanism whereby CCR5-selective ligands could suppress the neuroprotective action of these new CXCR4 antagonists would be heterogeneous desensitization (50, 51), but such an effect need not be invoked here because the CCR5 ligands RCP188, RCP189, and gp120BAL are neurotoxic on their own.

M. Kaul, K. E. Medders, M. K. Desai, and S. A. Lipton, unpublished data.
SMM-chemokine or R5-prefering gp120<sub>HAL</sub> leads to neurotoxicity. This finding is consistent with the notion that steric hindrance of the CXCR4 binding site is important in the neuroprotective mechanism of antagonist CXCR4-specific SMM-chemokines. Taken together, using novel synthetic SMM-chemokines as mechanistic probes and/or potential inhibitors, we have obtained new insights into the distinct signaling pathways of neuronal apoptosis associated with HAD activated by different chemokine receptor ligands that are either agonists or antagonists. This study also demonstrates a strategy of using chemically engineered inhibitors of chemokine receptors to study the signaling mechanism and intervention strategy of neuronal cell death and survival. A similar strategy may find its application in the study of other neurodegenerative diseases as chemokine pathways to neuronal protection or damage may, at least in part, be common to other central nervous system disorders including stroke, spinal cord injury, and Alzheimer disease.

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