Expedited Articles

Inhibition of in Vitro and in Vivo HIV Replication by a Distamycin Analogue That Interferes with Chemokine Receptor Function: A Candidate for Chemotherapeutic and Microbicidal Application

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Select chemokine receptors act as coreceptors for HIV-1 entry into human cells and represent targets for antiviral therapy. In this report we describe a distamycin analogue, 2,2′-[4,4′-[[aminocarbonyl]amino]bis[*N*,4′-di[pyrrole-2-carboxamide-1,1′-dimethyl]]-6,8-naphthalenedisulfonic acid]hexasodium salt (NSC 651016), that selectively inhibited chemokine binding to CCR5, CCR3, CCR1, and CXCR4, but not to CXCR2 or CCR2b, and blocked chemokine-induced calcium flux. Inhibition was not due to nonspecific charge interactions at the cell surface, but was based on a specific competition for the ligand receptor interaction sites since the inhibitory effect was specific for some but not all chemoattractant receptors. NSC 651016 inhibited in vitro replication of a wide range of HIV-1 isolates, as well as HIV-2 and SIV, and exhibited in vivo anti-HIV-1 activity in a murine model. In contrast, a distamycin analogue with similar structure and charge and the monomeric form of NSC 651016 demonstrated no inhibitory effects. These data demonstrate that molecules which interfere with HIV-1 entry into cells by targeting specific chemokine coreceptors can provide a viable approach to anti-HIV-1 therapy. NSC 651016 represents an attractive candidate for the chemotherapeutic treatment of HIV-1 infection and as a microbicide to prevent the sexual transmisssion of HIV-1. Moreover, NSC 651016 can serve as a template for medicinal chemical modifications leading to more effective antivirals.

Introduction

Specific members of the seven transmembrane G-protein-coupled chemokine receptor family have recently been identified as coreceptors for entry of the human immunodeficiency virus, type 1 (HIV-1) into human cells.[1] Examples of such chemokine receptors used as HIV-1 coreceptors include CCR2b, CCR3, CCR5, STRL33, and CXCR4.[2−8] Additionally, the CMV-derived US28 chemokine receptor also allowed for HIV-1 entry into human cells.[9] In this report we have focused primarily on the CXCR4 and CCR5 receptors. CXCR4 (fusin) is an α-chemokine receptor that binds stromal-derived factor-1α (SDF-1α), while CCR5, a *β*-chemokine receptor, binds MIP-1α, MIP-1*β*, and RANTES.[8] Naturally occurring mutant forms of CCR5 can confer some level of protection from infection by HIV-1, indicating a strong correlation between CCR5 and HIV-1 entry.[10,11] The entry of HIV-1 into host cells can be inhibited by ligands for CCR5 (including RANTES, MIP-1α, MIP-1*β*, and MCP-2)[12−14] and by several chemokine derivatives with antagonist activity.[15] Thus, it is plausible that synthetic small molecules could also prevent HIV-1 infection by targeting the cell surface chemokine receptors.

In the search for such inhibitors of chemokine receptors, we surveyed the chemical repository of the National Cancer Institute for compounds that might inhibit HIV-1 replication by blocking the entry of virus into host target cells. The search parameters were narrowed to include only molecules that did not possess undesirable side effects, that would not yield potentially carcinogenic metabolites, that were relatively simple to synthesize and purify, and that demonstrated an effective in vitro anti-HIV therapeutic index. This led us to focus on a series of distamycin analogues that inhibit in vitro anti-HIV-1 replication in the concentration range 1−10 *μ*M.[16]

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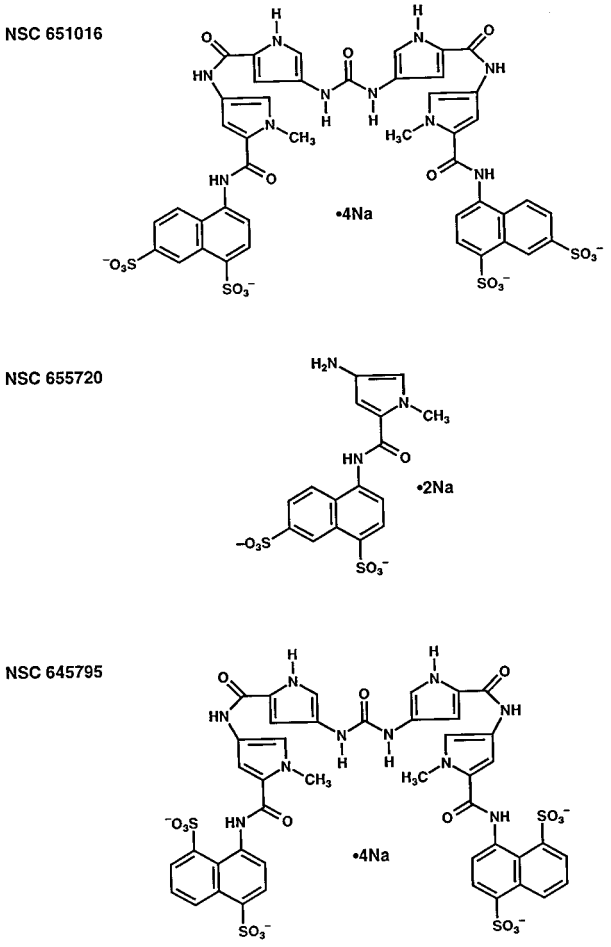

Figure 1. Structures of specific distamycin analogues. Structure of NSC 651016 (active dimer), NSC 655720 (inactive monomer), NSC 645795 (inactive dimer).

One particularly promising agent, 2,2′-[4,4′-[[aminocarbonyl]amino]bis[*N*,4′-di[pyrrole-2-carboxamide-1,1′-dimethyl]]-6,8-naphthalenedisulfonic acid]hexasodium salt, termed NSC 651016 (Figure 1), was selected for detailed investigation. To probe structural specificity, we also investigated the actions of a monomeric form of 651016 (NSC 655720, see Figure 1) and a structurally similar dimeric analogue (NSC 645795, see Figure 1).

In this report we demonstrate that the mechanism of anti-HIV-1 action of NSC 651016 results from specific interaction with HIV-1 chemokine coreceptors. Using both cellular and molecular target-based assays, we found that NSC 651016 inhibits fusion of HIV-1 with host cells. NSC 651016 demonstrated serum stability and inhibited HIV-1 replication in an in vivo model. Assessment of the effect of NSC 651016 on the interactions of chemokines with chemokine receptors and the resulting calcium flux revealed specific inhibition of only select receptor and ligand interactions with an accompanying block of calcium flux. Thus, NSC 651016 was identified as a distamycin analogue with in vivo anti-HIV-1 activity that specifically blocks chemokine receptor function, and this agent may have application for the systemic treatment of HIV-1 infection and as a preventive agent to block the sexual transmission of the virus.

Table 1. Range of Antiviral Action of NSC 651016

		EC_{50} (μM)[c]		
virus strain[a]	cell type[b]	mean	±SD	*n*
Laboratory HIV-1				
RF	CEM-SS	1.5	0.2	4
IIIB	MT-4	3.3	3.9	3
Clinical HIV-1 Isolate				
WEJO	PBLs	5.3	1.1	3
Drug-Resistant HIV-1				
6R(AZT)	MT-4	4.5	0.1	2
6S(AZT)	MT-4	7.2	1.2	2
A17 (Pyr^r)	MT-4	4.5	1.4	4
DPS^r-181	MT-4	4.8	0.6	2
N119 (Nev^r)	MT-4	1.2	1.0	2
Other Retroviruses				
HIV-2 (ROD)	CEM-SS	13.9	1.7	6
SIV (MAC251)	MT-4	26	0.2	2

[a] 6R and 6S refer to AZT-resistant and AZT-sensitive isolates from the same individual; Pyr^r, DPS^r, and Nev^r strains of HIV-1 resistant to pyridinone, diphenyl sulfone, and nevirapine, respectively. [b] PBLs, peripheral blood lymphocytes. [c] Anti-HIV studies with lymphocyte-derived cell lines were performed by the XTT cytopathicity assay, while antiviral assays with peripheral blood lymphocytes were performed by measuring cell-free p24 levels. The XTT cytoprotection studies with HIV-1 were confirmed by measurement of RT, p24, and infectious virus titers in the supernatant. The mean ± SD are derived from the number of EC_{50} determinations (*n*) from separate 96-well plates.

Results and Discussion

Inhibition of in Vitro and in Vivo HIV-1 Infection. As reported previously, NSC 651016 displayed anti-HIV-1 activity in an XTT-based cytoprotection assay in CEM-SS cells against $HIV\text{-}1_{RF}$ with an EC_{50} (concentration providing 50% reduction in virus replication) in the 1−10 μM range and an $CC_{50} \geq 316$ μM (concentration resulting in killing of 50% of the cells).[16] In the current study, NSC 651016 was found to be active against laboratory, clinical, and drug resistant isolates (resistant to RT inhibitors) of HIV-1 and representative isolates of simian immunodeficiency virus (SIV) and HIV, type 2 (HIV-2) (see Table 1). Although polyanionic compounds such as NSC 651016 often experience serum instability, NSC 651016 retained anti-HIV-1 activity in the presence of increasing concentrations of serum, demonstrating EC_{50} values ≤ 10 μM even in 50% serum (Figure 2A).

The broad antiviral activity and serum stability of NSC 651016 led us to determine if it would also demonstrate in vivo properties that would merit further investigation. NSC 651016 was active in a SCID mouse hollow fiber model[17] that measures the ability of a compound to inhibit acute replication of HIV-1 in CEM-SS cells in an in vivo environment. As illustrated in Figure 2B, NSC 651016 caused a concentration-dependent decrease in HIV-1 replication following intraperitoneal (ip) administration with three daily injections of ≥ 75 mg/kg of body weight, resulting in inhibition of viral p24 levels in plasma and peritoneal washes. NSC 651016 was additive to synergistic with AZT in this in vivo model (Figure 2C), and neither organ nor cellular toxicity was observed at antiviral doses. Consistent with these results, Ussery et al. found that NSC 651016 also inhibited HIV-1 replication in the HuPBMC-SCID model.[18]

Pharmacokinetic studies of NSC 651016 in mice[19] demonstrated that a single intravenous (iv) bolus of 50

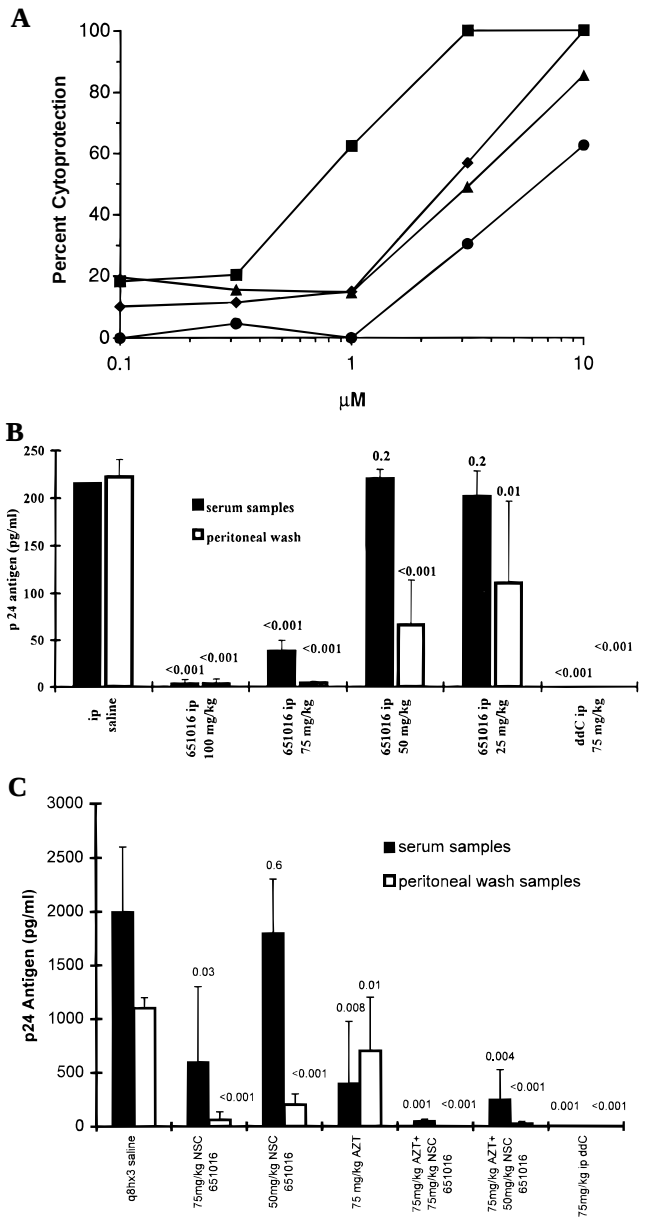

Figure 2. In vitro and in vivo anti-HIV-1 activity of NSC 651016. (A) Maintenance of anti-HIV-1 activity in serum. XTT cytoprotection studies measured the concentration of NSC 651016 required to protect CEM-SS cells from the cytopathic effect of HIV-1_{RF} by 50% (EC_{50}) in the presence of 5% (■), 10% (◆), 25% (▲), or 50% (●) serum. Each point represents the mean of triplicate samples. (B) In vivo inhibition of HIV-1 replication in murine hollow fiber model.[17] In vivo anti-HIV-1 activity was obtained with thee times daily (q8h) dosing of 75 mg/kg by the intraperitoneal route. Compound treatments were initiated on the morning of day 0 with the first dose given 1−3 h prior to fiber implant. The p24 antigen concentrations in serum (filled bars) and peritoneal washes (open bars) after 7 days of treatment showed a statistically significant (p values are shown above the SD error bars) reduction in viral antigen following treatment with >25 mg/kg NSC 651016 or with ddC. Decreased p24 antigen expression was not the result of cytotoxicity to the CEM-SS cells, as the viable cell mass in the hollow fiber samples exceeded the day 0 viable cell mass for all compound treated groups. (C) Additivity of AZT and NSC 651016. In vivo combination studies with AZT were conducted wherein three ip doses of NSC 651016 were combined with three subcutaneous doses of AZT in a checkerboard fashion.

mg/kg produced a fitted peak plasma concentration (C_0) of 699 μM and a prolonged terminal elimination half-life of 51 h. Plasma levels remained above the effective in vitro antiviral concentrations (~10 μM) for more than 24 h after iv dosing. Intraperitoneal or subcutaneous (sc) bolus administration of 50 mg/kg resulted in a peak plasma concentration of 65−114 μM, but substantial levels persisted in the plasma for several days after dosing by either route (measured plasma concentration = 16.7 μM at 96 h after sc administration). The ip and sc bioavailabilities of NSC 651016 were 60% and 100%, respectively; however, the compound was not orally bioavailable. Thus, NSC 651016 possessed favorable iv, sc, and ip pharmacokinetics and demonstrated sufficient in vivo activity to warrant a closer examination of its mechanism of action.

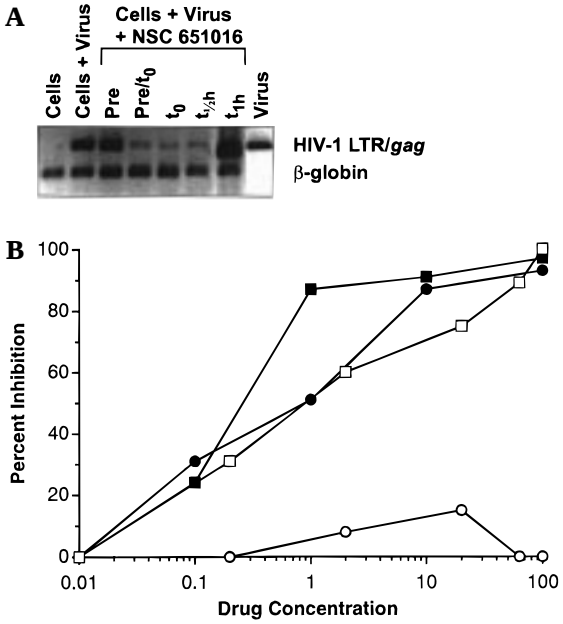

Figure 3. Antiviral Mechanism of Action Studies. (A) Time-of-addition effect on NSC 651016 on anti-HIV-1 activity. CEM-SS cells were preincubated with HIV-1_{IIIB} for 1 h at 0−4 °C, washed to remove unbound virus, and warmed to 37 °C to allow infection to proceed. NSC 651016 (100 μM) was included during the preincubation step only (Pre), included during the preincubation and then added back after warming to 37 °C at the designated time zero point (Pre/t_0), or added only at indicated times after shifting to 37 °C (t_0, $t_{1/2h}$, or t_{1h}). Cells were collected at 24 h, lysed, and then PCR amplified for the HIV-1 LTR/*gag*, a 200 bp product. Control lanes represent uninfected cells (Cells), infected cells with no addition of NSC 651016 (Cells + Virus), and an HIV-1 lysate (Virus). (B) Effects of NSC 651016 on virus binding and fusion. Binding of HIV-1_{RF} to CEM-SS cells was quantitated by a p24-based assay in the presence of increasing concentration of NSC 651016 (○) or dextran sulfate (●). Effects of NSC 651016 (□) or dextran sulfate (■) on fusion events were quantitated by measurement of blue cell formation after mixture of HeLa-CD4-LTR-β-gal cells with HL2/3 cells expressing HIV-1 Env and Tat proteins. Values represent the mean of triplicate samples for each point. The concentration of NSC 651016 is in μM units while dextran sulfate concentration is given in μg/mL.

NSC 651016 Inhibits HIV-1 Replication by Blockage of Virus Fusion to Host Cells. Time course studies[20] were performed to determine the stage of viral replication at which the NSC 651016 acted (Figure 3A). NSC 651016 added immediately after binding (t_0) of HIV-1 to cells or as much as 30 min after binding ($t_{1/2h}$) blocked infection (measured by PCR-based proviral DNA synthesis), but the antiviral effect was lost if the

Table 2. Inhibition of Chemokine Binding by Distamycin Analogues

HEK 293 cells transfected with	chemokine	% inhibn by NSC 651016	% inhibn by NSC 655720	% inhibn by NSC 645795
CCR1	RANTES	67 ± 1.0[a]	N/A	N/A
	MCP-3	53.1 ± 0.6		
CCR2b	MCP-1	12.8 ± 7	N/A	N/A
	MCP-3	10 ± 5		
CCR3	eotaxin	71.7 ± 0.3[a]	N/A	N/A
CCR5	RANTES	64.4 ± 2[a]	−1 ± 2.6	−3.1 ± 10
	MIP-1α	37.3 ± 0.2[a]	9.5 ± 2.5	6.5 ± 2.9
	MIP-1β	20 ± 1.3[a]	6.6 ± 3.2	−10.5 ± 1.2
CXCR2	IL-8	2.8 ± 5.9	0.3 ± 13	5.4 ± 9.8
CXCR4	SDF-1α	88.8 ± 0.5[a]	N/A	N/A
human monocytes expressing at least CXCR4 CCR1, 2b, and 5	SDF-1α	88 ± 6[a]	N/A	N/A
	RANTES	68 ± 2[a]		
	MIP-1α	75 ± 6[a]		
	MIP-1β	30.7 ± 10		

[a] A student *t*-test value of $p \leq 0.0002$ was determined for the drug vs no inhibitor control. All assays are at 200 μM NSC 651016. N/A, not assayed. $N \geq 2$.

compound was added more than 1 h after binding (t_{1h}) of virus to cells. When the compound was preincubated with virus and cells during the attachment phase, followed by removal of the compound by washing, NSC 651016 failed to inhibit infection (Pre condition, see Figure 3A). This indicated that the compound was not an effective inhibitor of virus binding to the high-affinity CD4 receptor molecule. Inhibitors of virus binding characteristically block infection during the Pre condition but not thereafter, while inhibitors of reverse transcriptase exert antiviral effects through the first 4−6 h of infection.[20] Since the antiviral action of NSC 651016 occurred temporally between the inhibition profiles of binding or reverse transcriptase inhibitors, this suggested that NSC 651016 was acting as an inhibitor of virus fusion.

Further mechanistic studies were performed to discriminate between the actions of NSC 651016 on virus binding versus fusion. As shown in Figure 3B, the compound did not inhibit direct binding of HIV-1 to host cells, as measured by a p24-based assay to quantitate cell-associated virions (open circles) but effectively inhibited the fusion event with an $IC_{50} = 2$ μM, as measured by the blockage of fusion of HeLa-CD4-LTR-β-gal cells with gp120-expressing and Tat-containing HL2/3 cells (open squares). Dextran sulfate (closed symbols), used as a positive control inhibitor of HIV-1 binding, prevented virus binding with an $IC_{50} = 1$ μg/mL, and this translated into prevention of subsequent infection in the fusion assay. In contrast, NSC 651016 (open symbols) only blocked the fusion event. Thus, the failure of NSC 651016 to block virus attachment (gp120/CD4 interaction), while inhibiting fusion of MAGI and HL2/3 cells supports the hypothesis that NSC 651016 is inhibiting the fusion of virions to cells without altering virus binding to cells.

Association of Antifusion Activity with Chemokine Receptors. The recently elucidated role of chemokine receptors, particularly CXCR4 and CCR5, as determinants for HIV-1 fusion with host cells led us to evaluate a potential role for the chemokine receptors in the antiviral action of NSC 651016. For this purpose, HEK-293 cells (a human embryonic kidney cell line that does not normally express CD4 or chemokine receptors) were stably transfected with either CXCR4 or CCR5 and subsequently transiently cotransfected with a CD4 expression vector. When analyzed by fluorescence-activated cell scanning (FACS) for CD4 expression, the transiently transfected cells were all strongly positive for CD4 for up to 10 days following transfection (data not shown). As predicted, HEK-293 cells lacking CD4 and chemokine receptors, or cells having either CD4 or either chemokine receptor alone, were not infectable by monocytotropic (HIV-1_{Ba-L}) or lymphocytotropic HIV-1 (HIV-1_{RF}) isolates when evaluated for proviral DNA expression by PCR analysis. Cells expressing CD4 and CXCR4 were infectable by HIV-1_{RF}, but not by HIV-1_{Ba-L}, and the infection was blocked by NSC 651016 (Figure 4). Likewise, cells expressing CD4 and CCR5 were infected by HIV-1_{Ba-L} (but not HIV-1_{RF}), and infection was blocked by NSC 651016. These data suggested the mechanism of action of NSC 651016 might be due to interference with the chemokine receptors essential to HIV-1 fusion.

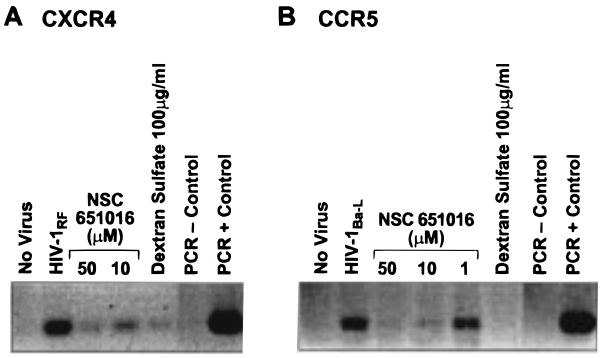

Figure 4. NSC 651016 inhibits HIV-1 infection of CD4+ CXCR4/HEK and CD4+ CCR5/HEK cells. (A) CD4+ CXCR4/HEK cells were infected with HIV-1_{RF} in the presence or absence of NSC 651016. Proviral DNA expression was determined by PCR at 24 h. (B) CD4+ CCR5/HEK cells were infected with HIV-1_{Ba-L} in the presence or absence of NSC 651016. Proviral DNA expression was determined by PCR amplication of the *gag* proviral DNA at 24 h. The PCR negative control was performed with DNA isolated from uninfected CEM-SS cells. The PCR positive control was performed with DNA isolated from HIV-1_{RF}-infected CEM-SS cells. The samples are correspondingly labeled.

Inhibition of Chemokine Binding. To further characterize the action of NSC 651016 on chemokine receptors, we tested the ability of it and related compounds to inhibit binding of radiolabeled chemokines to HEK-293 cells transfected with various chemokine receptors or to human leukocytes.[21,22] Concentration-dependent studies were performed to evaluate the effects of compounds on the various interactions; how-

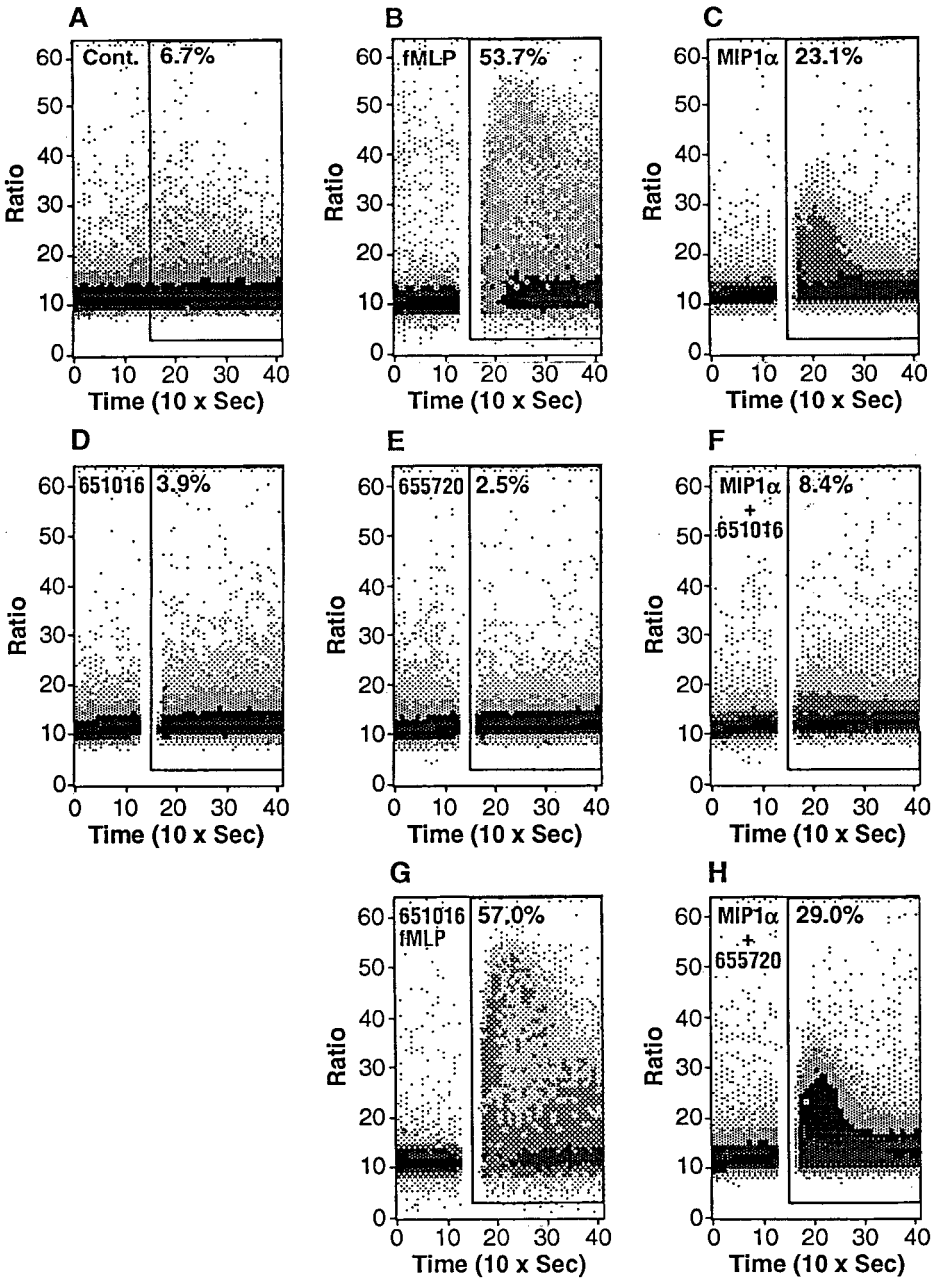

Figure 5. Effect of distamycin compounds on intracellular calcium flux in human monocytes in response to chemoattractants. Chemoattractant was added to 0.5 mL of indo-1-loaded monocytes (1×10^6 cell/mL), and the bound vs free calcium was calculated based on emission ratios.[23] A baseline was established for 150 s before the stimulant was added. The flux ratio was quantitated from 170 to 420 s (shown in boxed area); the resulting bound vs free percentage is shown in the inset. The final concentration of the chemokine was 100 ng/mL (12.5 nM). Calcium flux data were collected using monocytes from five different volunteers; the reported data are representative of all assays: (A) unstimulated control, (B) 10^{-8} M fMLP, (C) 100 ng/mL MIP-1α, (D) 200 μM NSC 651016, (E) 200 μM NSC 655720, (F) 200 μM NSC 651016 and 100 ng/mL MIP-1α, (G) indo-1-loaded monocytes pretreated with 200 μM 651016 for 150 s were subsequently treated with 10^{-8} M fMLP, (H) 200 μM 655720 and 100 ng/mL MIP-1α.

ever, because we were interested in determining qualitative differences in the ability of compounds to block specific receptor−ligand pairs, only the data comparing a single high test concentration of each compound is reported (see Table 2). Addition of NSC 651016 to HEK-293 cells transfected to express only CCR5 (CCR5/HEK-293) resulted in inhibition of RANTES, MIP-1α, and MIP-1β binding. The compound also inhibited SDF-1α binding to HEK-293 cells stably expressing only CXCR4 (CXCR4/HEK-293). In contrast, NSC 651016 was unable to inhibit the binding of IL-8 to CXCR2 or the binding of MCP-1 or MCP-3 to CCR2b. These findings revealed selectivity of the inhibitory effect of NSC 651016 for specific chemokine receptors and that CD4 was not required for inhibition of ligand binding. Furthermore, the monomeric form of NSC 651016 (NSC 655720, see Figure 1) and a structurally similar dimeric distamycin analogue (NSC 645795, see Figure 1), which did not inhibit HIV-1 infection,[16] did not inhibit chemokine binding (Table 2).

The inhibitory effects of NSC 651016 on chemokine binding were confirmed with normal human monocytes, which express a variety of chemokine receptors (summarized in Table 2). A Student *t*-test value of $p \leq$

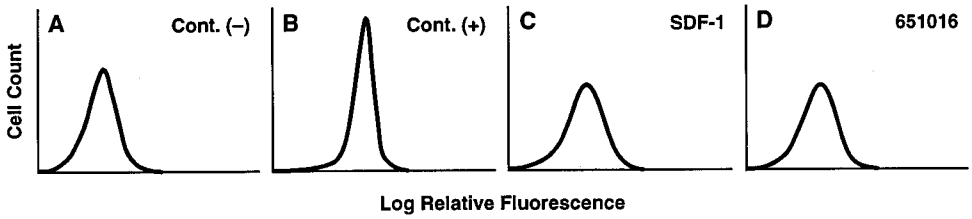

Figure 6. Inhibition of CXCR4-Specific Monoclonal Antibody Binding to Human Monocytes by SDF-1 or NSC 651016 as Determined by FACS Analysis. (A) Human monocytes incubated with nonspecific mouse IgG and then with FITC-conjugated goat antibodies to mouse IgG. (B) Human monocytes incubated with anti-CXCR4 (44716.111) and then with FITC-conjugated goat antibodies to mouse IgG. (C) Human monocytes treated with 200 ng/mL SDF-1 and then sequentially incubated with anti-CXCR4 and with FITC-conjugated goat antibodies to mouse IgG. (D) Human monocytes treated with 200 μM NSC 651016 and then sequentially incubated with anti-CXCR4 and with FITC-conjugated goat antibodies to mouse IgG.

0.0002 was determined for drug-treated vs the drug-free control. As with the HEK-293 transfectants, NSC 651016 was a potent inhibitor of chemokine binding. Thus, not only was NSC 651016 able to block chemokine binding under the highly artificial conditions found in transfected cells but it was equally effective on monocytes where the chemokine receptors are expressed endogenously without the caveats associated withinappropriate and overexpression of receptors in transfection systems.

Inhibition of Chemokine-Induced Signals. To further support our hypothesis that NSC 651016 was interacting with chemokine receptors, we determined if this compound could block the calcium flux initiated by binding of chemokines to their appropriate receptors.[23] Two-dimensional dot plots from flow cytometric analyses were utilized to evaluate the effects of NSC 651016 on chemoattractant-induced intracellular calcium flux in human monocytes. As shown in Figure 5, intracellular calcium flux was induced by both the monocyte chemoattractant fMLP (Figure 5B) and MIP-1α (Figure 5C), but the distamycin analogues do not themselves induce intracellular calcium flux, despite inhibiting chemokine binding (Figure 5D,E). More importantly, NSC 651016 completely blocked intracellular calcium flux in response to MIP-1α (Figure 5F), while the inactive NSC 655720 did not (Figure 5H). In addition to MIP-1α, the RANTES, MIP-1β, and SDF-1α chemokines induced intracellular calcium flux, and NSC 651016 blocked the signaling induced by each of these chemokines (data not shown).

To determine if human monocytes could still respond to chemoattractants by calcium flux in the presence of NSC 651016, monocytes were pretreated for 150 s with NSC 651016 followed by the addition of fMLP. There was no decrease in calcium flux in response to fMLP (Figure 5G). Thus, the cells are still functional and mediate a calcium flux in the presence of NSC 651016. While it is difficult to determine a half-maximum response using this methodology, we observed reductions in the calcium flux with 5 μM NSC 651016. These data illustrate that NSC 651016 only inhibited specific receptor signal transduction.

Inhibition of CXCR4 Specific Antibody Binding. Further proof of the targeted blockage of the chemokine receptors by NSC 651016 can be seen in Figure 6. The interaction of chemokines with the seven transmembrane domain chemokine receptors may require interaction with multiple areas of the receptor surface. To determine if NSC 651016 blocks the interaction of chemokine ligand for CXCR4 receptor via a steric hindrance mechanism, competition studies were performed with the 44716.111 monoclonal antibody that binds the extracellular domain of the CXCR4 chemokine receptor. Preincubation of SDF-1α (CXCR4 ligand) with human monocytes at 4 °C prevents binding of 44716.111 (Figure 6C). NSC 651016 mixed with human monocytes at 4 °C followed by addition of 44716.111 resulted in the same decrease in fluorescence as seen with the SDF-1α incubation (Figure 6D). These data suggest that NSC 651016 is acting by specifically interacting with at least domain 2 of the CXCR4 receptor, blocking subsequent chemokine binding.

Conclusions

The recent identification of the seven transmembrane G-protein-coupled chemokine receptors as coreceptors for HIV-1 infection has enhanced our understanding of virus interaction with cells.[1−15] This breakthrough provided new targets for antiviral therapy. To take advantage of these new targets, we investigated a series of compounds, represented by NSC 651016, which were previously reported to have antiviral activity and to target events very early in the HIV-1 replication cycle.[16] Mechanistic studies showed that NSC 651016 did not interfere with viral attachment but blocked fusion of virus to host cells. To determine if inhibition of the fusion event was due to interference with chemokine receptors, a combination of transient transfection assays using specific coreceptors, chemokine-induced calcium flux, and chemokine-directed chemotaxis assays were performed. These assays all identified NSC 651016 as an inhibitor of specific chemokine receptor functions. We found that endogenously relevant HIV-1 coreceptors (CXCR4, CCR5 and CCR3) and also CCR1 were inhibited by NSC 651016, while the CCR2b (MCP-1 and MCP-3), CXCR2 (IL-8 receptor), and fMLP receptors were unaffected. Besides its selective inhibition of chemokine receptor function, we also report that NSC 651016 inhibits a wide range of retrovirus isolates, possesses favorable sc, ip, and iv pharmacokinetics, and mediates an anti-HIV-1 effect in an in vivo model. In summary, NSC 651016 is a chemically defined nonpeptidyl compound with broad spectrum in vitro and in vivo anti-HIV activity and favorable pharmacokinetics.

Evidence for NSC 651016 as an inhibitor of virus and target cell fusion was derived from cell-based assays used to identify specific events in the HIV-1 life cycle. Time course studies indicated that this compound acted after binding of virus to cells but prior to initiation of reverse transcription. The inability of NSC 651016 to block virus attachment in the cell-based p24 virion

attachment assay further strengthened our hypothesis that NSC 651016 was acting as a fusion inhibitor. Using the MAGI HL2/3 fusion assay,[24] we could distinguish a known attachment inhibitor (dextran sulfate) from NSC 651016. Thus, mechanistically NSC 651016 is a fusion inhibitor, and in light of recent descriptions of the seven transmembrane chemokine receptors as the HIV-1 fusion coreceptor we investigated the possibility that NSC 651016 was mediating its antiviral effects by interrupting coreceptor dependent fusion.

NSC 651016 blocked interaction of chemokines with CXCR4, CCR1, CCR3 and CCR5 but did not affect binding of IL-8 to the IL-8 receptor (CXCR2) nor did it block binding of MCP-1 and MCP-3 with CCR2b. Selected chemokine-induced intracellular calcium flux was also inhibited by NSC 651016, whereas fMLP-induced flux was not. Furthermore, structurally similar analogues of NSC 651016 did not affect chemokine binding or calcium flux. These findings demonstrated that the specificity of inhibition by NSC 651016 was not merely due to interference with the low affinity and nonspecific binding of chemokines (such as IL-8 and RANTES) to glycosaminoglycans (GAGs).[25,26] Moreover, the nonspecific binding of chemokines to GAG molecules applies to all chemokines and is related to the overall charge of the chemokine C-terminal helix. While several ureido analogues of distamycin inhibit HIV replication in vitro, not all compounds that have similar structure and charge inhibit HIV replication or chemokine binding (ref 16 and data summarized in Table 1). These findings distinguish NSC 651016 from typical polyanionic compounds, such as dextran sulfate, that promote nonspecific effects and do not discriminate between virus binding and fusion.[20] In vivo studies have also shown that certain surface active agents such as dextran sulfate, heparan sulfate, and suramin can induce hematological disorders in treated animals.[27] These trends are qualitatively different from NSC 651016, for which analogues with similar structure and charge did not inhibit HIV-1 replication or chemokine binding, and it did not induce in vivo toxicities in the mouse model. Thus, the interaction of NSC 651016 with chemokine receptors appears to represent a structurally selective event that is not merely charge related.

It is unlikely that NSC 651016 acts by blocking G-protein function because the fMLP-induced calcium flux and MCP-1-induced chemotaxis were not inhibited. Furthermore, inhibition of G-proteins associated with chemokine receptors does not inhibit HIV-1 infection.[28] Extensive work has also shown that internalization of the receptor is not essential for HIV-1 infection or inhibition of HIV-1 infection.[28−31] This would suggest that the inhibition of HIV-1 infection by NSC 651016 observed in the CD4/CCR5 transfectants was not solely due to receptor internalization (Figure 6).

The discrepancy in the high doses of NSC 651016 needed to inhibit chemokine binding compared with the lower concentrations needed to inhibit HIV-1 infection and chemokine-induced chemotaxis[32] suggests that NSC 651016 is not binding to the high-affinity chemokine binding site on the receptor but to another site with lower affinity. Analysis of the NSC 651016 binding affinity for various chemokine receptors awaits development of radiolabeled NSC 651016 molecules. Thus, NSC 651016 may prove useful in the mapping of chemokine interaction with the specific receptors. While these data do not identify the precise chemokine−chemokine receptor interactions blocked by NSC 651016, they imply that the broadly distributed regions of the HIV coreceptors, recently identified as essential for infectivity, are likely blocked functional domains.[33−35] On the other hand, these data do not rule out that NSC 651016 might bind to the selected chemokines molecules themselves, thereby inhibiting their binding to specific receptors.

Currently, only inhibitors of reverse transcriptase and protease are sanctioned for clinical usage for the treatment of HIV-1 infection and AIDS. Due to the potential cross-resistance among many forms of compounds within each of these classes of agents, it is imperative that new agents targeting other sites be identified. The HIV-1 coreceptors provide a new target for such agents, yet the recently identified inhibitors of chemokine receptor functions are limited in their target specificity to a single coreceptor.[15,36−39] In contrast, NSC 651016 targets several coreceptors, and the broad antiviral range of NSC 651016 provides a potential salvage therapy for patients that are unable to tolerate current anti-HIV agents or patients that have developed resistance to various inhibitors of reverse transcriptase or protease. Additionally, NSC 651016 is being evaluated as a candidate for usage as an intravaginal microbicide to block the sexual transmission of HIV-1. Finally, the central role of chemokines in inflammation and its resolution raise the intriguing possibility that these compounds, beyond their potent antiviral activity, may also show efficacy in chemokine-mediated inflammatory diseases such as atherosclerosis and asthma. Hopefully, NSC 651016 can serve as a tool for study and a template for future generations of chemokine−chemokine receptor inhibitors (CCRIs).

Experimental Procedures

Detection of in Vitro and in Vivo Antiviral Activity. The 2,2′-[4,4′-[[aminocarbonyl]amino]bis[*N*,4′-di[pryrrole-2-carboxamide-1,1′-dimethyl]]-6,8-naphthalenedisulfonic acid]hexasodium salt (NSC 651016), as well as the two other distamycin analogues (NSC 655720 and NSC 645795) and 3′-azido-2′,3′-dideoxythymidine (AZT, NSC 602670), were derived from the Chemical Repository of the National Cancer Institute. NSC 651016 was synthesized as previously described[40] by Pharmacia & Upjohn, and its analytical purity was determined at >98% by HPLC analysis. Satisfactory elemental analyses were also obtained.

Measurement of the in vitro anti-HIV-1 activity of the experimental compounds was performed with the HIV-1_{RF} cytopathic strain and CEM-SS cells using the XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide) cytoprotection assay as previously described.[20] The in vivo assay was described previously by Hollingshead et al.[17] CEM-SS cells (NIH AIDS Research and Reference Reagent Program, Bethesda, MD) were infected at a low multiplicity of infection (MOI) 0.001 with HIV-1_{IIIB} (NIH AIDS Research and Reference Reagent Program, Bethesda, MD). Infected cells were immediately loaded into polyvinylidene fluoride hollow fibers (1 mm i.d.) (Spectrum Medical Corp., Houston, TX) with a molecular weight exclusion of ≥500 000 Da. Three fiber cultures were implanted subcutaneously (sc) and three fiber cultures were implanted intraperitoneally (ip) into each SCID mouse (NCI Animal Production Facility, NCI-FCRDC, Frederick, MD). For each experiment, groups of mice received treatment with the compound vehicle (phosphate buffered saline), the test compound, or the positive

control compound, ddC. The treatments were administered on days 0 (day of fiber implant) through 6 with samples collection on day 7. The fibers were taken for cell viability determination and reverse transcriptase (RT) quantitation. Serum and peritoneal wash samples were harvested for p24 antigen quantitation. The p24 antigen concentrations were determined with a commercially available ELISA kit (Coulter Diagnostics, Hialeah, FL). Cell viability was determined with a stable endpoint MTT dye conversion assay. The treatment route and schedule were varied among several experiments. NSC 651016 and ddC were solubilized in physiologic saline for administration by ip or sc route. Animal experiments were done in accordance with current NIH guidelines on the humane care and use of laboratory animals in research.

Pharmacokinetic Studies. NSC 651016 was dissolved in water for injection and administered to $CD2F_1$ mice by iv, sc, or ip bolus injection or by oral gavage. Mice were bled by retro-orbital puncture, and plasma was obtained by immediate centrifugation. Plasma samples (50 μL) were processed by adding an equal volume of 0.5 M tetrabutylammonium phosphate (TBAP), 300 μL of acetonitrile:methanol (1:1), and 50 μL of 2 M ammonium acetate. Following centrifugation, NSC 651016 was determined in the supernatant by HPLC. The method utilized a ODS stationary phase (Alltech Econosphere or Water Novapak) and isocratic elution (1 mL/min) with a mobile phase containing methanol:acetonitrile:phosphate buffer, pH 6.6 (42:11:47) and 5 mM TBAP. Detection was by UV absorbance at 320 nm.

Standard pharmacokinetic parameters were obtained by fitting plasma concentration−time data to appropriate poly-exponential equations using a nonlinear least squares minimization algorithm (RSTRIP-MicroMath Scientific Software). Bioavailabilites were estimated by $F = AUC/AUC_{iv}$, where AUC is the area under the fitted plasma concentration−time curve from t_0 to infinity for a given nonparenteral route and AUC_{iv} is the corresponding value obtained after iv administration.

In Vitro Mechanistic Assays. Binding of HIV-1_{RF} to CEM-SS cells was measured by a p24-based assay as previously described.[20] The ability of NSC 651016 to block HIV-1 fusion was measured using a surrogate fusion assay as previously described.[20] This assay employs the HeLa-CD4-LTR-β-gal (MAGI) cells and the HIV-1 Tat and Env expressing HL2/3 cells. The HL2/3 cells, which express HIV-1 Env protein on the cell surface and the Tat protein in the cytoplasm, can fuse with the MAGI cells and activate the β-galactosidase expression in the syncytium. Cell monolayers were fixed for 5 min with 2% formaldehyde−2% glutaraldehyde, washed twice with cold phosphate-buffered saline, and then stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) substrate for 50 min at 37 °C. The blue-stained cells in each well were then counted as an indicator of the relative levels of fusion events.

To identify the stage(s) of HIV infection affected by NSC 651016, the compound was evaluated in a high-MOI acute phase time-of-addition assay.[20] CEM-SS cells (10^5) were preincubated with HIV-1_{IIIB} (MOI = 1.0) at 4 °C for 1 h to allow binding of virus to cells but not fusion or reverse transcription. Samples were then washed three times with ice cold media to remove unbound virus, after which, the samples were rapidly warmed to 37 °C (at time t_0), allowing the infection cycle to proceed. NSC 651016 (100 μM) was included during the preincubation step only (Pre), included during the preincubation step and then added back at t_0 (Pre/t_0) following removal of residual virus, or added to samples only at t_0 or at various times after warming to 37 °C ($t = {}^1/_2$ h, 1 h). Dextran sulfate (100 μg/mL, NSC 620255) and ddC (10 μM NSC 606170) served as controls for inhibitors of virus attachment and reverse transcriptase, respectively. After a 24 h incubation, the cells were collected by centrifugation, lysed in QuickLyse buffer (10 mM Tris, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.1 mg/mL of gelatin, 0.45% Nonidet P-40, 0.45% Tween-20) containing 100 μg/mL of proteinase K, incubated at 56 °C for 2 h, and boiled for 10 min. Products of viral reverse transcription were amplified by PCR using LTR/gag primer pairs (M667/M661) and the β-globin primer pairs.[41] Amplified products were analyzed by electrophoresis in 2% agarose gels and visualized by ethidium bromide staining. The specific products were verified by restriction enzyme cleavage and Southern hybridization.

Inhibition of HIV-1 Infection of HEK Transfectants. Stably transfected HEK cells expressing either CXCR4 or CCR5 were cotransfected using electroporation[21] with a CD4 expression vector fragment excised from pTB4 (NIH AIDS Research and Reference Reagent Program, Bethesda, MD) and cloned into pSVZeo (Invitrogen, Carlsbad, CA). At 24 h posttransfection the cells were plated at a density of 10^5/well in a 24-well plate for 24 h. Media was removed, and the cultures were preincubated with either NSC 651016 or dextran sulfate (100 μg/mL) for 30 min. HIV-1_{RF} or HIV-1_{Ba-L} (MOI of 1 to 0.1) were added, and cultures continued for 24 h. The cells were lysed and genomic DNA isolated by the proteinase K/phenol:chloroform method. Polymerase chain reactions were performed on 1 μg of DNA using the M661/M667 primer pair for identification of late reverse transcription products.[41] DNA from uninfected CEM-SS cells was used for the negative control while DNA from HIV-1_{RF}-infected CEM-SS cells was used for the positive control. PCR products were identified on 2% agarose gel after ethidium bromide staining and documented photographically.

Chemokine Receptor Function Analyses. Peripheral blood monocytes were obtained by centrifugal elutriation from buffy coats isolated during routine leukapheresis of normal HIV-1 negative donors. The monocytes (>90% by nonspecific esterase staining) were cultured at 37 °C, 5% CO_2, for 16−18 h in AIMV media (Gibco BRL Life Technologies, Gaithersburg, MD) supplemented with 1% pooled human AB+ serum (Sigma, St. Louis, MO). Monocytes were collected and used directly for binding or calcium flux determination. Peripheral blood was obtained from normal hepatitis and HIV-1 negative volunteers following the NIH guidelines.

Binding assays were performed in triplicate by adding increasing amounts of competitor and constant radiolabeled chemokine, 0.2 ng/assay (New England Nuclear, RANTES-NEX 292, MIP-1α-NEX 298, MIP-1β-NEX 299; SDF-1-NEX 346 was a kind gift from Dr. Garth Brown of NEN, Boston, MA), to individual 1.5 mL microfuge tubes.[22] Then, 200 μL/sample of cells (4×10^6 cells/ml monocytes, or 1×10^6 cells/mL of HEK-293 transfectants) suspended in binding media (RPMI 1640, 1% BSA, 5 mM HEPES pH 8.0) were added to the tubes and mixed by continuous rotation at room temperature for 45 min. After incubation the cells were centrifuged through a 10% sucrose/PBS cushion, and the cell-associated radioactivity was measured by 1272 Wallac gamma counter. All assays were performed in triplicate, $n \geq 2$.

Flow cytometric measurement of intracellular calcium was performed as described by Badolato et al.[23] with an Epics 753 cytometer (Coulter Corp, Hialeah, FL). Changes in intracellular calcium concentration were determined by gated analysis of the ratio of fluorescence of indo-1 bound to calcium (emission at 485 nm) to fluorescence of free indo-1 (emission at 395 nm). The bound/free ratio was plotted against time. To assist in determining the number of fluxing cells, the baseline of the bound/free ratio was used to set the cut off. Cells with bound/free ratios higher than baseline were considered to be fluxing, and their percentage was calculated vs the total number of gated cells.

Measurement of the surface expression of CXCR4 was performed by fluorescence-activated cell screening (FACS) as previously described[15] using the monoclonal 44716.111 antibody (R &D Systems, Minneapolis, MN). Human monocytes were washed with 4 °C FACS media (PBS containing 1% FCS and 0.01% sodium azide), resuspended in FACS media (10^6 cells/200 μL), and treated with either 125 nM SDF-1 or 200 μM NSC 651016. All samples were placed on ice for 15 min before adding anti-CXCR4. Antibody was incubated for an additional 30 min followed by addition of a 20-fold volume of 4 °C FACS media wash. The presence of the primary antibody

was detected with FITC conjugated antimurine antibody (Sigma, St. Louis, MO). Although these data were collected using 200 μM NSC 651016 for comparison with other studies, we have also observed partial inhibition of antibody binding with nM concentrations of NSC 651016 (data not shown).

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