Blockade of X4-Tropic HIV-1 Cellular Entry by GSK812397, a Potent Noncompetitive CXCR4 Receptor Antagonist[†]

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GSK812397 is a potent entry inhibitor of X4-tropic strains of HIV-1, as demonstrated in multiple *in vitro* cellular assays (e.g., in peripheral blood mononuclear cells [PBMCs] and a viral human osteosarcoma [HOS] assay, mean 50% inhibitory concentrations [IC₅₀s] ± standard errors of the means were 4.60 ± 1.23 nM and 1.50 ± 0.21 nM, respectively). The primary *in vitro* potency of GSK812397 was not significantly altered by the addition of serum proteins (2.55 [±0.12]-fold shift in the presence of human serum albumin and α-acid glycoprotein in the PBMC assay). Pharmacological characterization of GSK812397 in cell-based functional assays revealed it to be a noncompetitive antagonist of the CXCR4 receptor, with GSK812397 producing a concentration-dependent decrease in both an SDF-1-mediated chemotaxis and intracellular calcium release (IC₅₀s were 0.34 ± 0.01 nM and 2.41 ± 0.50 nM, respectively). With respect to the antiviral activity of GSK812397, it was effective against a broad range of X4- and X4R5-utilizing clinical isolates. The potency and efficacy of GSK812397 were dependent on the individual isolate, with complete inhibition of infection observed with 24 of 30 isolates. GSK812397 did not show any detectable *in vitro* cytotoxicity and was highly selective for CXCR4, as determined using a wide range of receptors, enzymes, and transporters. Moreover, GSK812397 demonstrated acceptable pharmacokinetic properties and bioavailability across species. The data demonstrate that GSK812397 has antiviral activity against a broad range of X4-utilizing strains of HIV-1 via a noncompetitive antagonism of the CXCR4 receptor.

CCR5 and CXCR4 are well established as the principal coreceptors that are utilized by HIV-1 to gain entry into the host cell (23). CCR5 (R5)-utilizing HIV-1 strains are generally associated with the initial infection phase of the virus. This apparent selectivity, however, may be related to the higher expression level of CCR5 on target cells within or near the genital or rectal mucosa (10, 26). In addition, these tissues also express relatively high levels of the natural ligand for the receptor stromal cell-derived factor 1 (SDF-1), which may bind to the CXCR4 receptor blocking the interaction with X4 virus (20). As the infection progresses toward AIDS, variant forms of the virus that have the ability to either utilize both CCR5 and CXCR4 chemokine receptors (X4R5 dual-tropic viruses) or utilize solely the CXCR4 chemokine receptor (X4 viruses) to gain entry and infect the host cells (20) emerge. The infection can progress to AIDS in the absence of X4 variant viral forms; however, the appearance of these forms is strongly associated with accelerated disease progression and the decline of CD4⁺ T cells (20). Indeed, X4 variants are believed to be highly virulent since 40% of HIV-infected patients with AIDS present with X4 strains (20).

Therapeutics that target viral entry are now an established part of the armamentarium of potential antivirals available to

the patient. These include the CCR5 receptor antagonist maraviroc (Pfizer) (6) and the peptide therapeutic agent Fuzeon (Trimeris) (13), which targets the gp41 component of the viral coat protein. Several studies have highlighted a role for CXCR4 as a therapeutic target in the treatment of HIV-1. *In vitro* studies examining HIV-1 infection of primary cells have shown that addition of SDF-1, the natural ligand for CXCR4, can block the infection of these cells by X4 virus (24). Moreover, the recent development of selective tool molecules, including AMD3100 (8), AMD11070 (15), and KRH-3955 (17), that block the interaction of CXCR4 with HIV-1 gp120 has confirmed that small-molecule antagonists of CXCR4 can also block HIV-1 infection, thus opening up the opportunity for therapeutic intervention in this area. The virulent nature of the X4 strains of HIV would suggest that therapeutics that target CXCR4 and thus potentially inhibit the emergence of these strains would be useful as part of a combination therapy approach to inhibit disease progression.

In the present study we describe preclinical data highlighting the properties of a novel, orally bioavailable, and highly selective noncompetitive CXCR4 antagonist, GSK812397. The antiviral profile of GSK812397, together with its *in vivo* pharmacokinetic profile across a variety of species, is described for a number of *in vitro* viral assays.

MATERIALS AND METHODS

Compound. The synthesis of GSK812397 ([5-(4-methyl-1-piperazinyl)-2-({methyl[(8S)-5,6,7,8-tetrahydro-8-quinolinyl]amino}methyl)imidazo[1,2-*a*]pyridin-3-yl)methanol] (Fig. 1) has been described previously (2). Chemical reagents were obtained from Sigma-Aldrich (St. Louis, MO), unless otherwise speci-

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FIG. 1. Chemical structure of GSK812397.

fied. Cell culture reagents were obtained from Invitrogen (Carlsbad, CA), unless otherwise specified.

Cell preparation. Human osteosarcoma (HOS) cells expressing human CXCR4 (hCXCR4), hCCR5, and hCD4 (5) were acquired from the AIDS Reference and Reagent Program (Germantown, MD) and were engineered to express an HIV-1 long terminal repeat (LTR)-luciferase reporter (9). The cells were maintained in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal calf serum (FCS), $1 \times$ nonessential amino acids (NEAA), G418 (400 μ g/ml), puromycin (1 μ g/ml), mycophenolic acid (40 μ g/ml), xanthine (250 μ g/ml), and hypoxanthine (13.5 μ g/ml) to maintain a selection pressure for cells expressing the LTR-luciferase, hCXCR4, hCCR5, and hCD4. HEK-293 cells, stably transfected to express the human macrophage scavenging receptor (class A, type 1; GenBank accession no. D90187), were maintained in DMEM/Ham's F-12 media (1:1 mix) supplemented with 10% FCS and 1 μ g/ml puromycin. The expression of this receptor by the HEK-293 cells enhances their ability to stick to tissue culture-treated plastic ware. U937 cells were maintained in RPMI containing 10% FCS and 10 mM HEPES, $1 \times$ NEAA, 1 mM sodium pyruvate, and 3.5 μ l/liter beta-mercaptoethanol. All cell culture reagents were obtained from Invitrogen (Carlsbad, CA).

Normal donor peripheral blood mononuclear cells (PBMCs) were isolated from random buffy coats (35 to 40 ml of elutriated whole blood in anticoagulant from HIV-negative donors) received from the American Red Cross Carolina Division (Douglasville, GA). PBMCs were isolated by density gradient centrifugation over lymphocyte separation medium (Mediatech, Manassas, VA) and stimulated in 150 ml RPMI 1640 containing 20% FCS, 10% interleukin 2 (IL-2), 10 μ g/ml gentamicin, and 5 μ g/ml phytohemagglutinin (PHA) for 24 to 48 h.

BacMam baculovirus generation. Recombinant BacMam baculoviruses for CXCR4, gp160, rev, tat, and the chimeric G protein Gqi5 (4) were constructed from pFASTBacMam shuttle plasmids using the bacterial cell-based Bac-to-Bac system (Invitrogen, Carlsbad, CA) (11). Viruses were propagated in Sf9 (*Spartan frugiperda*) cells cultured in Hink's TNM-FH insect media supplemented with 10% fetal calf serum and 0.1% (vol/vol) Pluronic F-68 according to established protocols (16).

Viral HOS assay. HOS cells were suspended in DMEM supplemented with 2% FCS and $1 \times$ NEAA. Cells were dispensed into 96-well tissue culture-treated plates (6,000 per well; 50 μ l) and placed in a humidified incubator (37°C, 5% CO₂/95% air) overnight. The following day, 50 μ l of compounds in the above media were added to each well, and the incubation was continued for a further 1 h. An additional 60 μ l of compound in medium was transferred to a 96-well plate, and 60 μ l of HIV-1 (X4- or R5-tropic virus) was added to each well and thoroughly mixed. An aliquot (100 μ l) of the HIV/compound mixture was subsequently transferred to the wells containing cells/compound. The plates were placed in a humidified incubator at 37°C (5% CO₂/95% air) for 72 h. Following this incubation period, 150 μ l of supernatant was removed, 50 μ l of reconstituted LucLite Plus reagent (Promega, Madison, WI) was added to each well, and the luciferase activity of each sample was determined by measuring luminescence (TopCount; Perkin Elmer, Waltham, MA).

PBMC HIV replication cell assay. HIV-1 IIIB replication in PBMCs was quantified by measuring reverse transcriptase (RT) activity present in the supernatant as previously described (7). PHA-stimulated PBMCs were pelleted at 260 $\times g$ for 15 min, washed once with sterile phosphate-buffered saline, pelleted as described above, and resuspended to a density of 4×10^6 cells/ml in RPMI 1640 medium containing 20% FCS, 10% IL-2, and 50 μ g/ml gentamicin. Cells were dispensed into 96-well tissue culture plates (100 μ l per well). An equal volume of test compound was added, and the plates were placed in a humidified incubator at 37°C, 5% CO₂ for 1 h. A separate aliquot of diluted compound was added to HIV-1 IIIB, and 100 μ l of this mixture was then added to the PBMC-compound mixture. Infection proceeded in a humidified incubator at 37°C,

5% CO₂ for 7 days. A total of 50 μ l of cell-free culture supernatant was transferred to a new 96-well plate. A total of 10 μ l of RT extraction buffer (500 mM KCl, 50 mM dithiothreitol, 0.5% NP-40) was added and mixed, and then 40 μ l of RT assay buffer was added {1.25 mM EGTA, 125 mM Tris-HCl, 12.5 mM MgCl₂, 68 Ci/mmol methyl-[³H]deoxythymidine-5'-triphosphate, 0.62 optical density units/ml poly(rA) · poly(dT)₁₂₋₁₈}. After mixing, the RT reaction proceeded in a humidified incubator at 37°C, 5% CO₂ for 2 h. Radioactivity was captured on Unifilter DE-81 96-well plates (GE Healthcare, Waukesha, WI) using a Univac vacuum manifold (GE Healthcare, Waukesha, WI). Wells were washed a total of three times: the first wash with 5% Na₂HPO₄, followed by distilled water, and finally 95% ethanol. The radioactivity per well was then determined (TopCount; Perkin Elmer, Waltham, MA).

Cell-cell fusion assay. The fusion assay was essentially a modification of the viral HOS assay in which the virus was replaced with a human embryonic kidney (HEK) cell line transduced with BacMam baculovirus to express the CXCR4-utilizing HIV strain HXB2 envelope proteins gp120 and gp41, together with the viral proteins tat and rev. The transduction of HEK-293 cells was performed by direct addition of BacMam baculovirus-containing insect cell media to cells. The cells were simultaneously transduced with BacMam baculovirus expressing tat (1.3×10^8 PFU/ml), rev (1.5×10^8 PFU/ml), and gp160 (1.0×10^8 PFU/ml). Incubations were performed in the presence of butyric acid (2 mM), which has been shown to increase protein expression. The cells were incubated at 37°C, 5% CO₂, 95% humidity for 24 h to allow for protein expression. Transduced HEK-293 cells were subsequently added to the wells of a 96-well plate that contained 1 μ l of compound in DMSO in each well. HOS cells (50 μ l) were added at a density of 20,000 cells per well. The cells were returned to a tissue culture incubator (37°C; 5% CO₂/95% air) for an additional 24 h. Luciferase activity was determined by measuring the luminescence (TopCount; Perkin Elmer, Waltham, MA), and 100 μ l of reconstituted LucLite Plus reagent (Promega, Madison, WI) was added to each well.

Cytotoxicity assay. To measure cytotoxicity in the viral HOS assay, PBMC cell assay, and U937 chemotaxis assay, cells were treated with compound in the absence of virus. The viability of the cells was determined using CellTiter Glow reagent (Promega, Waltham, MA), which measures cellular ATP content. HOS and peripheral blood lymphocyte (PBL) cytotoxicity was determined after 96 h of incubation and for U937 cells after 2 h. In the fusion assay, compound was added to the cell mix, and the viability of the cells was determined following 24 h of incubation using CellTiter 96 MTS dye reagent (Promega, Waltham, MA).

Calcium mobilization experiments. HEK-293 cells were harvested using a nonenzymatic cell dissociation buffer (Invitrogen, Carlsbad, CA) and were resuspended in culture media supplemented with CXCR4 and Gqi5 BacMam viruses (multiplicity of infection of 50 and 12.5, respectively). The cells were plated at a density of 40,000 cells (100- μ l volume) per well in black, clear-bottomed, 96-well plates. The plates were incubated at 37°C, 5% CO₂, and 95% humidity for 24 h to allow for CXCR4 and Gqi5 protein expression. Growth media were removed from the transduced HEK-293 cells, and they were washed once with FLIPR buffer (Calcium Plus assay kit dye reagent [Molecular Devices, Sunnyvale, CA] dissolved in Dulbecco's modified Eagle's medium/Ham's F-12 medium containing 2.5 mM probenecid and 0.1% [wt/vol] bovine serum albumin). A total of 50 μ l of this dye solution was then added to each well, and the plates were incubated for 1 h at 37°C, under 5% CO₂ and 95% humidity. The effects of various ligands on intracellular calcium levels were examined using FLIPR (Fluorometric Imaging Plate Reader; Molecular Devices, Sunnyvale, CA). For antagonist profiling, the compound was added 15 min prior to the addition of the agonist ligand.

Chemotaxis assay. Chemotaxis was examined by measuring the migration of U937 cells through a porous membrane in response to the agonist ligand SDF-1 in a 96-well plate format (Neuro Probe ChemoTx; Neuro Probe Inc., Gaithersburg, MD). U937 cells were chosen as a model system due to the endogenous expression of CXCR4 in this cell line and the ability of this cell type to efficiently chemotax toward the CXCR4 ligand SDF-1. U937 cells were resuspended in chemotaxis buffer (RPMI, 2% FCS, 10 mM HEPES; 5×10^6 cells/ml) and were incubated with compound for 15 min prior to 50 μ l of the mixture being added to the top of the filter (5.0 μ m). The lower chamber of the plate contained identical concentrations of compound and a defined concentration of SDF-1 (PeptoTech Inc., Rocky Hill, NJ). The cells were allowed to migrate for 2 h, after which time the filter was removed. The media in the lower chamber were transferred to another 96-well plate, and the cell number was determined using Cell Titer Glow reagent (Promega, Waltham, MA). This reagent produces a luminescence that is dependent on the ATP content of the cells present in the well. To ensure that the decrease in signal was not due to any cytotoxicity of the compound, the effect of compound on cell viability was also determined under

TABLE 1. Potency of GSK812397 in fusion, viral HOS, and PBMC assays

Parameter	Result for assay: ^a			
	PBMC (IIB)	X4 viral HOS (IIB)	Fusion (HXB2)	R5 viral HOS (Ba-L)
Potency (nM)	4.60 ± 1.23	1.50 ± 0.21	0.56 ± 0.05	>1,000
Inhibition (%)	120 ± 9	94 ± 3	114 ± 4	ND
Cytotoxicity (nM)	>1,000	>1,000	>1,000	>1,000

^a Potency and inhibition data represent the mean IC₅₀ ± standard error of the mean. The number of experiments performed for each assay was as follows: fusion, *n* = 13; X4, *n* = 11; PBMC, *n* = 8; and R5, *n* = 11. ND, not determined.

identical conditions. Luminescence was measured using a TopCount instrument (Perkin Elmer, Waltham, MA).

Pharmacokinetic profiling Of GSK812397. The plasma concentration-time profile of GSK812397 was determined after a single oral administration at a dose of 3 mg/kg or intravenous administration at a dose of 1 mg/kg. Plasma samples were taken at successive time points over the subsequent 24-h period. GSK812397 plasma levels were measured by liquid chromatography-tandem mass spectrometry. Pharmacokinetic parameters were calculated by using WinNonlin Professional (version 3.1; Pharsight Co., Mountain View, CA).

Data analysis. Data are presented as the mean ± the standard error of the mean (SEM) unless otherwise stated. EC₅₀s and IC₅₀s are defined as the concentration of compound required to stimulate and inhibit, respectively, 50% of a maximal response. All curve fitting was performed using a four-parameter logistic equation: $y = d + \{(a - d) / [1 + (x/c)^b]\}$, where *y* is the response, *a* is the estimated response at zero concentration, *b* is the slope factor, *c* is the mid-range concentration (XC₅₀), and *d* is the estimated response at infinite concentration. All data were fitted using GraphPad Prism (GraphPad Software, La Jolla, CA).

RESULTS

Antiviral potency of GSK812397. The potency and efficacy of GSK812397 were examined in three primary assays. These included two cell-based antiviral assays that measured the ability of the compound to prevent HIV entry/replication, the viral HOS assay, and the PBMC assay. A surrogate, virus-free, cell-cell fusion assay that mimics viral fusion by replacing the virus with a cell line expressing HIV envelope proteins was also tested. The potency levels of GSK812397 were similar in all of the above CXCR4-related assays, with IC₅₀s of 4.60 ± 1.23 nM, 1.50 ± 0.21 nM, and 0.56 ± 0.05 nM for the PBMC, viral HOS, and fusion assays, respectively (Table 1). In all the assays used, the maximum inhibition produced by GSK812397 was not significantly different from the values defining 100% inhibition when analyzed using a Student *t* test. In contrast, GSK812397 did not block CCR5-mediated viral entry in the R5 viral HOS assay (IC₅₀ > 2.5 μM), thereby demonstrating selectivity for X4-utilizing virus. In addition, GSK812397 was not overtly toxic in any of the cell-based assays over the ex-

tended incubation periods examined (IC₅₀ > 1.0 μM, for all assays).

Effects of serum protein binding. It is well known that the potency of drugs can be affected by their ability to bind to human serum proteins. Although the activity of a compound *in vivo* is influenced by many parameters that cannot be adequately assessed *in vitro*, an estimate of the influence of serum protein binding on the compound's potency is useful in setting target concentrations for the clinic. Therefore, the antiviral IC₅₀s and effects of protein binding provided a means of estimating antiviral activity at physiological plasma protein levels. An assessment of the effect of serum protein binding on the activity was made by adding human serum albumin (HSA) in the absence or presence of α-acid glycoprotein (α-AGP) to the viral HOS and fusion assays (Table 2). For the baseline antiviral potency, the IC₅₀ data generated in the viral HOS assay were used. A small 2.55 (±0.12)-fold shift in GSK812397 potency was observed in the presence of a combination of 45 mg/ml HSA and 1 mg/ml α-AGP in the viral HOS assay. This resulted in a protein-adjusted IC₅₀ of 3.14 ± 0.33 nM and an IC₉₀ value of approximately 12 nM.

GSK812397 blocks entry of both X4 monotropic and X4R5 dual-tropic clinical isolates. The ability of GSK812397 to inhibit infection by X4 and X4R5 (dual- or mixed-tropic) clinical isolates was examined in a contracted study with Monogram Biosciences (South San Francisco, CA). GSK812397 was tested against 30 clinical isolates of HIV-1 (25 X4 and 5 X4R5) in Monogram's proprietary Phenosense HIV entry assay (19). This included the measurement of compound potency against each isolate and the maximal inhibition of viral infection of the host cell by GSK812397. The cells used in this study were engineered to express either CXCR4 or CCR5 but not both; hence, the entry was specific to the respective coreceptor expressed on the cell. GSK812397 was active against all X4 and X4R5 isolates tested; however, GSK812397 was not able to

TABLE 2. Effect of serum proteins on the potency of GSK812397 in fusion and viral HOS assays

Assay	Parameter	Result with protein: ^a			
		Control	HSA (45 mg/ml)	α-AGP (1 mg/ml)	HSA/α-AGP
Fusion	IC ₅₀ (nM)	2.09 ± 0.33	1.71 ± 0.22	1.99 ± 0.28	1.97 ± 0.22
	Change (fold)	1.00 ± 0.00	0.84 ± 0.05	0.98 ± 0.06	1.01 ± 0.11
HOS	IC ₅₀ (nM)	1.23 ± 0.09	2.01 ± 0.16	2.87 ± 0.92	3.14 ± 0.33
	Change (fold)	1.00 ± 0.00	1.64 ± 0.05	2.27 ± 0.56	2.55 ± 0.12

^a Data represent the geometric mean IC₅₀ ± standard error of the mean. The number of experiments performed for each assay was as follows: fusion assay, *n* = 3, and HOS assay, *n* = 6. Assays were performed in the absence or presence of HSA and α-AGP.

FIG. 2. Effect of GSK812397 on the infection of CXCR4-expressing host cells by various clinical isolates of HIV-1. This assay was performed by Monogram Biosciences (San Francisco, CA) in accordance with the methodology described in reference 18. (A) The potency of GSK812397 in inhibiting infection was compared across X4 clinical isolates (filled bars) and X4R5 dual-tropic isolates (open bars). (B) The maximal inhibition of infection for each isolate was also examined and was compared to that of the infection by each isolate in the absence of compound. Data represent the mean calculated from a single curve fitted to a concentration-response of GSK812397 in which each point was performed in triplicate.

completely inhibit (to >95%) 4 out of the 25 X4 isolates and 2 out of the 5 X4R5 isolates. In addition, the potency of GSK812397 was dependent upon the particular isolate, with IC₅₀s ranging from 1.0 to 31.6 nM. Moreover, GSK812397 did not inhibit replication of the X4R5 isolates when the R5 component was examined with host cells that expressed only CCR5. Similarly, GSK812397 had no effect on the replication of the R5 viral strain JRC5F. The data are summarized in Fig. 2A and B.

GSK812397 acts specifically through the CXCR4 receptor.

GSK812397 selectively blocks the entry into cells of X4-utilizing strains of HIV. There are several possible mechanisms for such an effect. The compound could bind to CD4, CXCR4, GP120, or GP41 and thus prevent fusion of virus with the cell. In addition, if the compound is indeed a CXCR4 antagonist, it is important to establish what the pharmacology of the compound is at the receptor in order to understand what the potential *in vivo* consequences of compound administration might be. For example, if GSK812397 blocks the function of the natural ligand at the CXCR4 receptor, this may have implications with respect to cellular chemotaxis. In order to address this question, a functional assay was established in which

HEK cells were transduced to express the human CXCR4 receptor together with a promiscuous G protein (Gqi5) that allowed the receptor to couple to intracellular calcium release. The activity of the receptor was examined by measuring the release of intracellular calcium, using FLIPR, in response to the CXCR4-selective agonist stromal cell-derived factor 1 α (SDF-1). GSK812397 alone had no effect on intracellular calcium release, demonstrating that it was not an agonist of the CXCR4 receptor. However, GSK812397 caused a concentration-dependent inhibition of SDF-1-mediated intracellular calcium release, using an EC₈₀ concentration of SDF-1, with an IC₅₀ of 2.41 ± 0.50 nM ($n = 9$) and maximal inhibition of $109.9 \pm 5.2\%$. These data demonstrate that GSK812397 is a potent inhibitor of CXCR4 receptor function, with respect to the natural ligand for CXCR4, SDF-1.

The mode of antagonism produced by GSK812397 was also studied. Concentration-response curves for SDF-1 were generated in the functional CXCR4 FLIPR assay in the presence of increasing concentrations of GSK812397. If GSK812397 was a competitive antagonist, one would expect a parallel rightward shift in the SDF-1 concentration-response curve, with no change in the maximum response to SDF-1. If GSK812397A were a noncompetitive antagonist, then no change in the EC₅₀ of the SDF-1 α curve would be expected; however, a decrease in the maximum response would be observed for the agonist. As shown in Fig. 3A, GSK812397 clearly possesses the characteristics of a noncompetitive antagonist.

GSK812397 inhibits CXCR4-mediated chemotaxis. The CXCR4 receptor is known to play a role in the chemotaxis of a number of cell types *in vivo*. The inhibition of SDF-1 function by GSK812397 at the CXCR4 receptor was examined in a cell-based chemotaxis assay first to determine if GSK812397 would indeed modulate the chemotactic function of SDF-1. Second, by examining the pharmacology of GSK812397 in this receptor-mediated system, it is possible to gain significant insight into the pharmacological profile of the compound, including determining whether it is competitive or noncompetitive. The effect of GSK812397 on SDF-1-induced chemotaxis was examined in the monocyte-derived cell line U937, a cell line which endogenously expressed the CXCR4 receptor subtype. GSK812397 produced concentration-dependent inhibition in the chemotaxis of the U937 cell line produced by an EC₈₀ concentration of SDF-1, with an IC₅₀ of 0.34 ± 0.01 nM ($n = 6$) and a maximal inhibition of $108.8 \pm 3.4\%$.

The mode of antagonism was examined in this assay to confirm the mode of antagonism observed in the calcium release assay. Concentration-response curves to SDF-1 were again generated in the presence of increasing concentrations of GSK812397 (Fig. 3B). The data generated are consistent with GSK812397 having the pharmacology of a noncompetitive antagonist, as was observed in the functional CXCR4 FLIPR assay.

Association and dissociation of compound with receptor. An estimation of how rapidly the compound reached equilibrium upon addition was made using the CXCR4 FLIPR assay described above. GSK812397 was preincubated for various periods of time prior to addition of the agonist SDF-1. GSK812397 completely blocked the response to SDF-1 following a 5-min incubation. The potency increased slightly between 5 min and 15 min (IC₅₀s of 3.36 ± 0.29 nM [$n = 4$] and 1.07 ± 0.04 nM

FIG. 3. Pharmacological profiling of GSK812397. Inhibition of SDF-1-mediated calcium release (A) and chemotaxis of U937 cells by GSK812397 (B). Calcium release assays were performed in HEK-293 cells stably transfected to express the human CXCR4 receptor and the chimeric G protein Gqi5. Cells were preincubated with increasing concentrations of GSK812397 (■, vehicle; ◇, 0.94 nM; ●, 1.88 nM; □, 3.75 nM) 15 min prior to the addition of a concentration-response curve of SDF-1. Intracellular calcium release was measured using FLIPR. Chemotaxis assays were performed using the U2OS cell line that endogenously expresses CXCR4. Cells were preincubated with increasing concentrations of GSK812397 (■, vehicle; ◇, 0.01 nM; ●, 0.06 nM; □, 0.32 nM; ▲, 1.6 nM; ▼, 8.0 nM) 15 min prior to the addition of a concentration-response curve of SDF-1. Cells were allowed to chemotax for 2 h. Data represent the mean value of the results from at least three experiments. Error bars represent the standard error of the mean.

[$n = 4$] for 5-min and 15-min incubations, respectively) before remaining constant throughout the remainder of the time course (Fig. 4A). The data demonstrate that GSK812397 reaches equilibrium with the CXCR4 receptor within 15 min from addition.

The dissociation of GSK812397 from the CXCR4 receptor was also examined to determine if the compound-receptor interaction was reversible. The functional CXCR4 FLIPR assay was again utilized. Cells were preincubated with GSK812397 for 30 min. The cells were then washed to remove excess compound, and SDF-1 was added at successive time points. The data demonstrate that the compound interaction with the CXCR4 receptor

FIG. 4. Measurement of the association and dissociation of GSK812397 from the CXCR4 receptor. (A) Association of GSK812397 from the receptor was examined by measuring the potency (IC_{50}) of the compound following increasing incubation periods (■, 5 min; ◇, 15 min; ●, 30 min; □, 45 min) using the calcium release assay. Calcium release was stimulated by addition of 30 nM SDF-1. (B) Dissociation of GSK812397 from the receptor was also examined in the calcium release assay by examining the potency (IC_{50}) of the compound following removal of the compound by washing and examining the change in potency over time (■, no wash; ◇, 15 min; ●, 30 min; □, 45 min). Data represent the mean value of the results from at least three experiments. Error bars represent the standard error of the mean.

is reversible, with the IC_{50} for GSK812397 slowly decreasing with increasing time postwash, ranging from IC_{50} s of 0.87 ± 0.04 nM ($n = 4$) in the absence of washing to 26.64 ± 17.81 nM ($n = 4$) at 45 min postwash (Fig. 4b). This format of assay gives only a crude measure of compound dissociation, but it is sufficient to show that the compound binding is indeed reversible.

Species pharmacology and pharmacokinetics. The pharmacology of GSK812397 was examined at CXCR4 receptors cloned from a number of animal species. These included human, cynomolgus macaque, rat, and mouse. The receptors were expressed in the U2-OS cell line, a human cell line that does not express the CXCR4 receptor, together with a pro-

TABLE 3. Potency and pharmacokinetic parameters for GSK812397 across species^a

Parameter	Result for species				
	Human	Mouse	Rat	Dog	Monkey
Potency (IC ₅₀ [nM])	6.6 ± 0.7	2.1 ± 0.3	4.3 ± 0.7	ND	7.8 ± 0.8
Cl (ml/min/kg)	ND	15.9	4.5	5.5	5.8
V _{ss} (liter/kg)	ND	4.2	2.1	5.3	5.9
t _{1/2} (h)	ND	4.1	8.6	>12	>12
F (% solution)	ND	10	21	21	17

^a Potency data are determined from the inhibition of SDF-1 (30 nM)-stimulated calcium release using FLIPR in HEK-293 cells expressing CXCR4 receptors from the various species examined. Data for potency represent the geometric mean ± standard error of the mean (*n* = 3). Clearance (Cl) and volume of distribution (V_{ss}) are calculated according to a 1-mg/kg intravenous dose. Half-life (t_{1/2}) and oral bioavailability (F) are calculated according to solution doses of 3 mg/kg. ND, not determined.

miscuous G protein (Gqi5). The pharmacology was examined using the same functional CXCR4 FLIPR assay described above, using changes in intracellular calcium as a readout. GSK812397 showed very similar pharmacology at the CXCR4 receptors of all the species examined, with IC₅₀ and percent maximal inhibition values being almost identical between species (Table 3). A significant attempt was made to express the dog receptor in a variety of cell systems, but with no success.

The pharmacokinetic data for GSK812397 are summarized in Table 3. Clearance and bioavailability were found to be similar among the species examined; however, the half-lives of GSK812397 for dog and monkey were found to be significantly longer (>12 h for both species) than that for mouse (4.1 h).

Compound selectivity. GSK812397 selectivity was profiled against a broad range of cellular targets in a contracted study performed by MDS PanLabs (King of Prussia, PA). The purpose of this study was to confirm that the activity of the compound was limited to CXCR4 antagonism, thus reducing the potential for adverse off-target effects when administered *in vivo*. The targets examined included 53 receptors (including adrenergic, cholinergic, dopaminergic, histaminergic, and serotonergic receptor subtypes), 16 enzymes, 8 ion channels, and 4 transporter proteins. The potential interaction of GSK812397 with these targets was examined in radiolabeled competition binding assays. In addition, a secondary selectivity panel consisting of 15 chemokine receptor subtypes was also examined in a FLIPR-based functional Ca²⁺ release assay under contract with Millipore (Billerica, MA). GSK812397 was shown to interact with only one of the above targets, namely, angiotensin-converting enzyme (ACE), with an IC₅₀ of 193 nM in an isolated enzyme assay. However, in subsequent experiments examining the ability of GSK812397 to inhibit ACE in isolated guinea pig ileum, the inhibition was less significant than that observed for the isolated enzyme, with a maximal inhibition of only 26% at 30 μM.

In subsequent studies, GSK812397 was shown not to inhibit the hERG ion channel up to a concentration of 10 μM. In addition, GSK812397 did not produce an inhibition of any of the cytochrome P450 enzyme family tested (1A2, 2C9, 2C19, 2D6, and 3A4) at concentrations up to 30 μM.

DISCUSSION

Therapies that block viral entry into the host cell have been shown to be clinically beneficial. The inhibition of HIV-1 entry by the CCR5 antagonist maraviroc produces a significant decrease in viral load in patients (6). However, potential drawbacks in targeting the HIV-1 coreceptors as an approach are the possibility of viral resistance or viral coreceptor switching. Viral resistance may arise, for example, if a mutation in the hypervariable V3 loop of the HIV-1 gp120 protein results in a version of gp120 that binds to the new drug-bound conformation of the receptor or to a distinct area of the receptor that is unaffected by bound compound. In coreceptor switching, blockade of gp120 binding to one coreceptor by a compound may result in a change in virus coreceptor preference, such that an R5 virus may utilize X4 or vice versa. This may raise concerns for CCR5 inhibitors if there is indeed a coreceptor switch to the potentially more virulent X4-utilizing HIV-1, although to date no such switch in tropism has been observed in the clinic. Given that CXCR4-utilizing virus is not the predominant form in infected individuals, it is likely that CXCR4 antagonists would be useful only in combination therapy, for example, with a CCR5 antagonist, in order to prevent the possible emergence of preexisting forms of CXCR4 virus or tropism switching. There may also be a prerequisite for tropism testing in the clinic or a limitation of the use of such a treatment to individuals in the later stages of the disease.

In the present study we describe the antiviral profile and pharmacological properties of GSK812397, a novel, potent, and bioavailable CXCR4 antagonist that is highly selective. Several other selective CXCR4 antagonists have been reported in the literature. AMD3100 was initially reported to block HIV-1 replication by preventing entry into the cell via CXCR4 (8). Although effective in this respect, this bicyclam compound was not orally bioavailable. More recent advances in the field have led to the development of orally active CXCR4 antagonists, including AMD11070 (15) and more recently KRH-3955 (17). GSK812397 is derived from a chemical class distinct from that of either AMD11070 or KRH-3955. GSK812397 is highly potent with respect to its antiviral activity, with potency in the low nanomolar range against IIIB and HXB2 strains, similar to those published for KRH-3955 (17). Moreover, the antiviral activity was not significantly reduced in the presence of human serum albumin or α-acid glycoprotein.

GSK812397 was also shown to be highly effective against a broad panel of HIV-1 clinical isolates, with respect to blocking infection via the CXCR4 receptor. GSK812397 inhibited cellular infection by X4 strains, as well as dual-tropic strains (X4R5) in host cells expressing only the CXCR4 receptor. In contrast, the compound did not inhibit CCR5-mediated viral infection, as determined by the inability to block dual-tropic viral infection of cells expressing only the CCR5 receptor. Thus, the compound is specific for the inhibition of viral infection mediated via the CXCR4 receptor. The potencies of GSK812397 for the 30 isolates examined were all within a similar range (IC₅₀s of 1 to 30 nM); however, the absolute efficacy of the compound for each isolate was markedly different. Replication of 6 out of the 30 isolates was not completely inhibited by GSK812397, in spite of the fact that the IC₅₀ of the compound for some of these isolates was in the single-digit

nanomolar range. One potential explanation for this finding could lie in the pharmacological properties of the compound. It is possible that GSK812397 is an allosteric antagonist that binds to a site on the CXCR4 receptor that is separate and distinct from the binding site of HIV-1. Once bound to this site, the conformation of the receptor changes in such a manner as to make it thermodynamically unfavorable for the virus to bind. Because of putative differences in the precise interaction of each isolate with the CXCR4 receptor, it is possible that the compound will be more efficacious against certain isolates compared with others. Indeed, it has been demonstrated that the interaction of other small-molecule antagonists that interfere with large protein-protein interactions (such as SDF-1/CXCR4 or RANTES/CCR5) is allosteric in nature (25). It is also possible that some of the isolates tested had amino acid differences that effectively rendered them partially resistant to blockade by the CXCR4 antagonist, perhaps explaining the range of IC_{50} values and lack of complete inhibition in some cases. We did not look at the sequence variation of the clinical isolates or perform resistance studies for GSK812397.

In addition to its ability to block HIV-1 interaction with CXCR4, GSK812397 has also been demonstrated to block the functional activity of SDF-1. In the present study, this ability was used to confirm that the compound was indeed interacting with the CXCR4 receptor and not with the viral proteins. In addition, it allowed for the characterization of the pharmacology of GSK812397. Two independent assays were undertaken to examine the pharmacology of GSK812397. First, the effect of the compound on CXCR4-mediated calcium release was examined. SDF-1 produced a concentration-dependent increase in calcium release in cells expressing CXCR4/Gqi5 but not in cells in which either CXCR4 or Gqi5 was absent, showing that the response generated was specific to this pathway. GSK812397 produced a concentration-dependent decrease in SDF-1-stimulated calcium release. No effect of the compound was observed when an alternative calcium-releasing stimulus was used, again demonstrating that the response was CXCR4 specific. Addition of increasing concentrations of GSK812397 produced a depression in the concentration-response curve to SDF-1 in this assay, with no significant change in the EC_{50} for SDF-1. This profile is typical of what one would observe for a noncompetitive antagonist. Similar studies performed with U937 cells in which the effect of GSK812397 on SDF-1-mediated chemotaxis was examined also produced a decrease in the maximal response of the SDF-1 concentration-response curve, with no significant change in the EC_{50} . Thus, in both assays GSK812397 was demonstrated to be a potent noncompetitive CXCR4 antagonist.

A crude kinetic analysis examining the association and dissociation of GSK812397 was performed using the FLIPR-based calcium release assay. The association of compound and receptor was rapid, as determined by the time required to maximally inhibit the SDF-1 calcium response (<15 min). The dissociation was examined by measuring the potency and efficacy of the compound following removal of the compound by washing the cells with compound-free media. The inhibition by compound decreased rapidly, with a significant 30-fold decrease in the IC_{50} being observed 45 min after the removal of compound. Previous studies with CCR5 antagonists have shown a broad array of dissociation rates with half-lives that

have been as long as several days (23). Indeed, from published data it would appear that the off-rate of KRH-3955 is markedly greater than that observed for GSK812397 (17). Such information is key in the design of clinical studies, since the pharmacokinetic properties of the compound, such as half-life of compound elimination, may be less relevant if the compound does not rapidly dissociate from the receptor.

As mentioned previously, the bicyclam CXCR4 antagonist AMD3100 (8) suffers from a lack of bioavailability, with the only route of administration being via injection. The pharmacokinetic profile of KRH-3955 is a significant improvement on AMD3100, with an oral bioavailability of 25.6% and an intravenous half-life of approximately 99 h (17). The authors suggest that this extended half-life is likely due to tissue accumulation. In comparison, the pharmacokinetic properties of GSK812397 compare favorably with those of KRH-3955. The bioavailability of GSK812397 was found to be similar across several species, including rat (21%), dog (21%), and monkey (17%). In addition the half-life was greater than 12 h in both dog and monkey. In contrast to KRH-3955, no evidence of tissue accumulation was observed with GSK812397, which could be an advantage in the development of this compound.

Although initially targeted as a compound for the treatment of HIV-1 infection, CXCR4 antagonists such as GSK812397 may also have therapeutic potential in a number of other important areas. For example, GSK812397 could be of value in the treatment of rheumatoid arthritis by blocking the homing of inflammatory cells to inflamed joints (3, 14). In addition, CXCR4 has been shown to play a role in metastatic spread as well as the direct regulation of the growth and/or survival of tumor cells (16, 22). The pharmacokinetic profile and potent CXCR4 antagonism across several species makes GSK812397 a highly valuable tool for evaluating such roles.

In summary, GSK812397 is a potent, orally bioavailable CXCR4 antagonist that inhibits CXCR4-mediated HIV-1 infection at single-digit nanomolar concentrations for a broad range of X4 and dual-tropic X4R5 isolates. GSK812397 therefore could be of value in the treatment of HIV-1 as part of a combination therapy.

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