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**Title**

**Agonist-induced internalization of CCR5 as a mechanism to inhibit HIV replication**

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**Running title**

HIV blockade by a small-molecule agonist for CCR5

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## Abstract

The chemokine G-protein coupled receptor CCR5 is used as an entry gate by CCR5-tropic (R5) and dual- or CCR5/CXCR4-tropic (R5/X4) strains of HIV to enter the human host cells. Thus, CCR5 antagonists (i.e. maraviroc) have proven to be clinically effective by preventing the interaction between the viral gp120 and CCR5 and thus impeding viral entry into host cells. However, the emergence of HIV strains resistant to CCR5 antagonists have been reported *in vitro* and *in vivo*, where the virus has adapted to enter the cells via antagonist-bound CCR5. An alternative strategy that should obviate this mode of viral resistance would entail ablation of the CCR5 portal for HIV entry from the cell surface through agonist-induced receptor internalization. While this protective effect has been clearly demonstrated with natural CCR5 ligands, the chemoattractant properties of these chemokines have precluded them from further consideration in terms of drug development. Thus we sought to explore the possibility of developing novel small molecules and selective CCR5 agonists devoid of eliciting chemotaxis. Indeed the CCR5 agonists described herein were found to induce profound down modulation of the CCR5 (and not the CXCR4) receptor from the cell surface, and its sustained sequestration in the intracellular compartment, without inducing chemotaxis *in vitro*. The bioactivity profile of these novel CCR5 agonists is exemplified by the compound ESN-196 that potently inhibits HIV-1 infection in human PBMCs and macrophages *in vitro* with potencies comparable to that of maraviroc, and moreover demonstrates full activity against a maraviroc-resistant HIV-1 RU570 strain.

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**Introduction**

CCR5 is a G-protein coupled receptor (GPCR) that is expressed on various cells implicated in the immune response including T-lymphocytes, monocytes, macrophages, dendritic cells and microglia. Moreover, CCR5 is activated by several chemokines such as RANTES and the macrophage inflammatory proteins MIP-1α and MIP-1β. As initially reported by Cocchi and coworkers in 1995, the implication of CCR5 as a potential target for development of AIDS therapeutics relates to the role of such chemokines in inhibiting HIV infection in humans. Furthermore, CCR5 has been identified as a key co-receptor utilized by the so-called CCR5-tropic or R5 strains of HIV to gain entry into cells (Berger et al., 1999). The successful launch of maraviroc (Dorr et al., 2005), Pfizer's CCR5 antagonist, has validated this GPCR as a target for treatment of HIV-1 infections. In addition to maraviroc, several other CCR5 antagonists or monoclonal antibody inhibitors have also been optimized, some of which entered clinical development (Westby and van der Ryst 2010; Jacobson et al., 2008).

The current generation of CCR5 entry inhibitors, including maraviroc, vicriviroc and aplaviroc, have been shown to be allosteric modulators of CCR5 (Kondru et al., 2008) that block entry of R5 virus mainly by interfering with the interaction between the gp120-V3 loop and the CCR5 receptor. However, the emergence of resistance to these allosteric inhibitors has already been reported whereupon viral strains characterized by various mutations in the V3 loop are able to bind to the drug-bound form of the CCR5 receptor and proceed with viral entry (Moore and Kuritzkes 2009; Tilton et al., 2010 a and b).

As stated above, such CCR5 agonists as RANTES are also known to block viral entry, although this activity may occur through two distinct mechanisms of action. First, such

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agonists may act similarly to the antagonists, i.e. through allosteric blockade of the binding interaction between gp120 and CCR5. Second, CCR5 agonists also induce receptor internalization (Aramori et al., 1997; Escola et al., 2010) thereby obliterating the viral entry gate from the cell surface. This latter mechanism is consistent with the observation that individuals naturally deficient for cell surface expression of CCR5, due to a homozygous mutation (CCR5Δ32), present a high level of resistance to acquiring HIV, and interestingly, they do not present any associated physiological deficiency (Samson et al., 1996). Similarly, Lederman and co-workers (2006) reported a full protective effect of PSC-RANTES against R5 SHIV infection following vaginal application in macaques. This modified chemokine acts as an agonist of CCR5 and has a particularly high capacity to induce CCR5 internalization (Sabbe et al., 2001). Several relevant precedents in this area include the work on CCR5-internalizing analogs of RANTES for topical use (Gaertner et al., 2008) as well as reports on small-molecule CCR5 agonists by a few groups (Saita et al., 2006; Kellenberger et al., 2007). In addition to the latter cases, the use of agonists to elicit receptor internalization and thereby achieve the endpoint of functional antagonism also finds precedence in other projects such as the work reported on S1P1 receptor, where the immunomodulating effect of the small molecule agonist has been reported to result from the down regulation of this receptor at the surface of T-lymphocytes (Zemann et al., 2006 ; Bolli et al., 2010).

Compounds A-B (Figure 1) served as an inception point for the work described herein. Compound A had been previously reported as a CCR5 antagonist (Burrows et al., 2005). At the outset, we observed that converting the tertiary amide functionality in Compound A to a secondary amide (i.e. Compound B) renders the previously described CCR5 antagonist into an agonist (Huck et al., 2009). Subsequent internal medicinal chemistry

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efforts to optimize Compound B culminated in the discovery of ESN-196, a highly potent and selective small-molecule CCR5 agonist with an overall improved drug-like profile versus Compound B.

In this study, we describe the unique bioactivity profile of ESN-196 and in so doing illustrate that CCR5 receptor internalization is highly correlated with inhibition of R5 HIV-1 replication in cell lines and in human peripheral blood mononuclear cells (PBMCs). In addition, we highlight the fact that the i R5 HIV-1 strains resistant to the CCR5 antagonists maraviroc and vicriviroc generated *in vitro*, remained as sensitive to ESN-196 as the wild-type R5 virus affirming an advantage of functional agonism achieved through receptor internalization. These data are discussed with respect to the potential therapeutic use of CCR5 agonists in the prevention of viral transmission and treatment of HIV/AIDS.

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**Methods**

*Intracellular Calcium assay*

The activity of the compounds was systematically monitored in an aequorin calcium assay in a heterologous system based on recombinant CHO-K1-hCCR5 cells (Detheux et al., 2000). In brief, cells were collected from adherent cultures and stored frozen in liquid nitrogen in 10% DMSO. The day before the assay, cells are thawed and incubated overnight in DMEM F-12 ($1x10^6$ cells/ml) supplemented with 5µM coelenterazine H (Promega). Cells were then washed in DMEM-F12 medium and resuspended at a concentration of $1x10^5$cells/ml. The emission of light following the addition of chemokines or compounds was recorded using FDSS 6000 luminometer (Hamamadsu). Results are expressed as Relative Light Units (RLU).

*Radioligand binding assay*

Competition binding assay was performed as described (Blanpain et al., 1999) on membrane fractions prepared from CHO-K1 cell lines expressing CCR5. In brief, the membrane extracts were incubated in binding buffer (25 mM HEPES, pH 7.4; 5 mM $MgCl_2$; 1 mM CaCl, and 0.5% protease-free BSA) containing 0.05 nM radioligand $^{125}$I–MIP-1β and various concentrations of compounds. The amount of tracer associated to membranes was determined after filtration through GF/B filters (Packard) presoaked in 1% BSA or 0.3% polyethylenimine (PEI). The radioactivity was quantified using the TopCount-NXT reader (Packard). Results were normalized for total binding in the absence of competitor (100%) and nonspecific binding (0%) in the presence of a 100-fold excess of the competitor and were analyzed by nonlinear regression using a single-site competition model (Graph-Pad Prism™ Software). For saturation experiments, the binding conditions were identical to the competition experiments described above and

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increasing concentrations of the radioligand $^{125}$I–MIP-1β were added. The non-specific binding was measured in the presence of 2µM of cold MIP-1β. The Bmax was calculated using non linear regression in the Graph-Pad Prism™ Software.

*CCR5 internalization assay – fluorescence microscopy*

A vector derived from pIRESneo (Clontech) was constructed that expresses a fusion protein between CCR5 (accession number NP_000570) and Yellow Fluorescent Protein (YFP, accession number ABV26713). The chimeric gene is a continuous open reading frame that contains a Ala-Ser link between the last amino-acid of CCR5 and the Start codon of YFP. This construct was stably transfected in HEK293 cells to generate a stable recombinant cell line. A suspension of these HEK293-CCR5-YFP (grown in DMEM/F12 medium containing 0.1% (w/v) BSA) was dispensed in poly-D-lysine 96-well plates at 20000 cells per well. The compounds were added at various concentrations (DMSO final concentration 0.3%). After 1 hour of internalization at 37°C, the cells were fixed with PAF 2% (30 min), and the nuclei were dyed with DAPI 600 nM (10 min). Fluorescence microscopy images (6 images/well) were acquired with the InCell Analyzer 1000 (GE Healthcare), using 360 nM as excitation wavelengths for detection of the DAPI stained nuclei (defined as structures ≥90 $\mu m^2$), and 475 nM for the detection of the CCR5-associated YFP fluorescence concentrated in the internalization inclusions (defined as fluorescent structures of 0.9-9µM). The emitted light (460 nm and 535 nm for DAPI and YFP detection, respectively) was recorded and processed by the INCell Analyzer 1000 3.3 software using parameters provided by the manufacturer. The analysis of the images including the quantification of internalization was performed by the INCellAnalyzer 1000 Workstation 3.4 using the membrane translocation protocol where in internalization is quantified as the total area of inclusions, per cell, relative to the cellular space.



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*cAMP-based functional recovery assay*

CHO-K1 cells expressing the human CCR5 gene were seeded in 96-well black culture plates at $5x10^3$cells/ml in HamF'12 medium without antibiotic, supplemented with 10% FBS and grown for two days. The culture medium was replaced by fresh medium supplemented with 100 nM of compound or chemokine. On the next day the cells were washed, re-suspended in fresh medium without compound and incubated overnight to allow receptor recycling. Cells were then incubated for 30 min with a serial dilution of RANTES in presence of forskoline. The cells were then lysed and the intracellular level of cAMP was measured by the HTRF technology using the cAMP dynamic 2 assay from Cisbio. The fluorescence was read using the Rubystar (BMG Labtech).

*Chemotaxis assay*

The chemotaxis assay was performed in Multiscreen-MIC 96-wells plates (Millipore) equipped with 5µM filters. CHO-K1-hCCR5 cells migration from the upper well to the lower chamber containing various concentrations of RANTES, MIP-1β or ESN-196 was measured after 4h of incubation at 37°C using the ATP-Lite assay (Perkin Elmer). The chemotaxis index corresponds to the ratio between the bioluminescence measured in the well versus the control well, where no compound is applied.

*Pseudotype antiviral assay*

The pseudotyped MMLV-HIVenv viruses supernatants were produced as described (Chan et al., 2006). MAGI-R5 cells were plated in 96-well culture plates at $1x10^4$ cells/well in DMEM F12 supplemented with 10% FBS and cultured overnight. 1 h before infection, the culture medium was replaced by fresh medium supplemented with the

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appropriate concentration of compound or chemokine. Viral supernatant was added to the cultures followed by centrifugation of culture plates at 2500 rpm for 45 min at room temperature (spinoculation). The cells were then incubated at 37 °C overnight. The next day, the culture supernatant was replaced with fresh medium prepared with the same amount of compound, and the cells were incubated for another 24 h at 37 °C. At the end of incubation, the luciferase activity was measured using the Steady-Glo® Luciferase Assay System (Promega) and the TopCount-NXT reader (Packard).

*HIV-1 replication assay in PBMC and monocytes/macrophages*

Antiviral testing of the chemokines and the small molecules in PHA-stimulated peripheral blood mononuclear cells (PBMC) from healthy donors was essentially performed as described previously (Balzarini et al., 2006). Human monocyte/ macrophage cultures were prepared as previously described (Aquaro et al., 2001)
The inhibition of HIV-1 replication was evaluated by the level of p24 antigen in culture supernatants after 12-14 days of culture by specific p-24 Ag ELISA (Perkin Elmer Lifesciences). Cell viability was evaluated in parallel by microscopic evaluation and MTS staining method.

*Viruses*

HIV-1 NL4.3 (X4) and HIV-1 BaL (R5) was provided by R. C. Gallo and M. Popovic (at that time at the National Cancer Institute, National Institutes of Health, Bethesda, MD).
The clinical Russian HIV-1 isolate clade G, called RU570, was obtained through the Centralized Facility for AIDS Reagents and drug resistance against this virus was obtained by culturing this virus in the presence of maraviroc and vicriviroc in PBMC for

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12-14 days. Then supernatant was collected, viral p-24 Ag values determined and infection restarted in PBMC with or without increasing concentrations of compound. After 22 passages (~280 days) with vicriviroc or 32 passages with maraviroc (~410 days) completely resistant viruses were obtained.

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## Results

*CCR5 Binding and Functional Assays*

Five putative CCR5 ligands (Fig 1A) were characterized in radioligand binding (Fig 1B) and aequorin functional assays (Figure 1C). The synthetic CCR5 agonist YM-370749 (Saita et al., 2006) was tested as a reference ligand and was determined to be inactive in both assays. In contrast, all other compounds competitively and reversibly displaced $[^{125}I]$-MIP-1β binding with ESN-196 ($K_i$ = 0.09 nM) demonstrating more than 50-fold higher potency than the precursor compounds A and B ($K_i$ =21.2 nM and 5.6 nM, respectively) and the reference antagonist, maraviroc ($K_i$ = 0.93 nM).. In the aequorin assay (Fig 1C), maraviroc and compound A did not elicit significant receptor activation (% $E_{max}$ < 1) consistent with their characterization as antagonists. In comparison, compound B elicited partial agonist activity (% $E_{max}$ = 71) whereas ESN-196 demonstrated full agonist activity ($EC_{50}$ = 43 nM, % $E_{max}$ =110) as defined relative to the %$E_{max}$ measured for RANTES ($EC_{50}$ = 0.9 nM, %$E_{max}$ = 100 by definition).

ESN-196 selectivity for CCR5 was determined by measuring compound cross-reactivity against a panel of 18 chemokine receptors. Thus, functional assays were carried out at a single test concentration of 10 µM in both agonist and antagonist mode on human, cloned receptors expressed in CHO-recombinant systems using either the aequorin or the GTPγ$[^{35}S]$ assays. ESN-196 demonstrated no significant cross-reactivity at any target in the chemokine receptor panel, including the HIV-1 co-receptors CXCR4 and CCR3 (data not shown).

*ESN-196 induces the internalization of CCR5 in recombinant cellular system*

The protective effect of natural chemokines in HIV-infection essentially results from co-receptor internalization. Due to the potential advantages of this mechanism of action, we



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verified by fluorescence microscopy that our small molecule induces the internalization of a CCR5-YFP chimeric receptor expressed in HEK293T human cells and exposed to ESN-196, RANTES or maraviroc. As shown in Figure 2A, both the natural chemokine and the small molecule agonist induced a strong internalization of the YFP fluorescence into the intracellular compartment. The image-based analysis and quantification of the cellular location of the fluorescence demonstrates that the internalization of CCR5 is concentration-dependent with $EC_{50}$ values of 0.3 nM and 2 nM for ESN-196 and RANTES, respectively (Figure 2B). Similarly to the untreated control, the fluorescence remained associated to the cell membrane in the presence of maraviroc and YM-370749 at concentrations up to 10 μM. Radioligand binding assay also was used wherein internalization of the CCR5 receptor was observed in recombinant CHO-CCR5 cells following prolonged (i.e. 24h) exposure. Thus, the receptor density at the cell surface, expressed as the Bmax (Table 1) is reduced as a function of ESN-196 concentration following a prolonged (24h) exposure.

*ESN-196 is a potent entry inhibitor for R5 HIV in recombinant MAGI cells, PBMCs and macrophages*

During our medicinal chemistry program, we routinely evaluated the antiviral activity of our compounds in a single-cycle infection assay using recombinant MMLV-based pseudotype R5 HIV-1 (Chan et al., 2006). In this assay, the viral entry in MAGI cells expressing both CCR5 and CD4 is inhibited by ESN-196 in a dose dependent manner, with an $IC_{50}$ of 5.0 nM (n=2), compared to 0.6 nM (n=2) for maraviroc and 11.4 nM (n=15) for RANTES (Figure 3).

The antiviral activity of ESN-196 was confirmed in freshly isolated human PBMCs using the HIV-1 R5 BaL strain and a clinical HIV-1 R5 isolate (CI#15) (Figure 4). The viral



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replication was measured by p24 Ag ELISA dosage and in these experiments, ESN-196 appeared to be a full inhibitor of HIV-1 with nanomolar potency having $IC_{50}$ values of 14 nM (n=11) and 4 nM (n=10) for BaL strain and Cl#15, respectively. This inhibitory activity is in the same range as the antagonist maraviroc, always tested in parallel with an $IC_{50}$ of 6 nM (n=40) for the BaL strain and 2 nM (n=40) for the CI#15. In order to verify that the inhibition of viral replication was not due to an effect of ESN-196 on cell growth, we demonstrated that this compound does not have any growth inhibitory and cytotoxic effect on PBMCs in the conditions of the assay ($CC_{50} > 10$ µM).

Infection of human monocytes/ macrophages with HIV-1 BaL was also potently inhibited by ESN-196 with an $IC_{50}$ of 0.9 nM (n=3) as compared to 3.6 nM (n=3) for maraviroc .

As expected, no antiviral activity was observed for the CCR5 ligands in cultures of PBMC using either the CXCR4-tropic (X4) HIV-1 strains NL4.3 or a X4 HIV-1 clinical isolate from clade D ($IC_{50}$ >20 µM, n=2), or the dual-tropic R5/X4 HIV-1 HE strain ($IC_{50}$ >20 µM, n=2).

*ESN-196 is active against HIV-1 strains resistant to the CCR5 inhibitors maraviroc and vicriviroc*

The HIV-1 clinical isolate RU570 has been described as less sensitive to the CCR5 antagonists SCH-C (Strizki et al, 2001 200 nM), vicriviroc (Marozsan et al, 2005, 1.2 nM) and maraviroc (Westby et al., 2007). This virus was thus chosen to generate virus strains presenting high resistance levels to these CCR5 inhibitors in PBMCs. Briefly, 32 and 22 passages were needed to generate a virus highly resistant to maraviroc and vicriviroc, respectively. When ESN-196 was evaluated against the RU570 wild type virus the $IC_{50}$ was 87 nM and when evaluated against the maraviroc resistant virus ($IC_{50}$ >10



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μM for maraviroc) and the vicriviroc resistant virus ($IC_{50}$ >10 μM for vicriviroc) ESN-196 was active at 3.4 and 1.9 nM, respectively. As a control the natural ligand RANTES had comparable activity against the wild-type as the two antagonist-resistant virus strains ($IC_{50}$ ~ 200 nM).

*ESN-196 delays the recovery of CCR5 function*

The timing for the recovery of CCR5 receptor after exposure to a drug is a potential indicator of the duration of antiviral efficacy. Recombinant CHO-K1-hCCR5 cells were exposed to maraviroc, RANTES or ESN-196 for 24 hours. The compound or chemokine was washed away and after a recovery period of 24 hours, the functional response of these cells to RANTES was measured in a cAMP assay. As shown in Figure 5, the cells exposed to the antagonist maraviroc totally recovered their responsiveness to RANTES following wash-out of the antagonist. Cells pre-exposed to RANTES recovered their activation potency but a reduction in receptor response at maximal effective concentrations was observed. However, for cells pre-exposed to ESN-196, a sustained reduction of both potency and activation magnitude in response to RANTES (Fig. 5) indicating that ESN-196 has the most profound effect on sustained reduction CCR5 cell-surface expression of the three ligands tested.

*ESN-196 does not induce chemotaxis in CHO-CCR5 recombinant cells*

A potential issue for the development of a small molecule agonist at CCR5 is the potential to induce pro-inflammatory pharmacology as observed for the natural chemokine agonists. Thus, it is well-established that the endogenous CCR5 chemokines elicit recruitment of cells at inflammatory sites by chemotaxis. The chemoattractive potential of ESN-196 was evaluated and compared to the effect of the chemokines

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RANTES and MIP-1β in a recombinant system using a CCR5-expressing CHO cell line. The two chemokines induce a very strong chemotactic migration of the recombinant CHO cells while ESN-196 does not induce any significant migration in the range of concentrations tested in this assay (Figure 6). Migration was observed once per hour for up to four hours. This time point is displayed in the graphic coincident with the maximal Rantes response, but internalization was not observed with ESN-196 at any time. Moreover, co-treatment with ESN-196 abolished the chemotactic response to RANTES, presumably by allosteric receptor blockade, whereas a 24h pre-treatment (followed by wash-out) of ESN-196 also abolished the chemotactic response to RANTES, in this case presumably by CCR5 receptor internalization.

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**Discussion**

CCR5 is a clinically validated target for the treatment for the development of anti-HIV drugs as exemplified by the marketed product, maraviroc. Moreover, a host of other small molecule CCR5 antagonists have been advanced into development (Westby and van der Ryst 2010; Jacobson et al, 2008). In contrast, the alternative strategy based on the use of CCR5 agonists has garnered far less attention. In this approach, the ligand-receptor interaction of the CCR5 agonist would provide an allosteric blockade of the gp120-CCR5 interaction to prevent viral entry (similarly to the CCR5 antagonists) and, in addition, agonism of the CCR5 receptor causes receptor internalization thereby eliciting a sustained ablation of the entry gate for HIV. The notion of using a small-molecule CCR5 agonist in this manner has previously been reported (Saita et al, 2006), however we were unable to reproduce these results with the reference ligand (YM-370349). Nonetheless, we report here the discovery of a novel CCR5 small molecule agonist with a high anti-HIV potency profile *in vitro*.

As mentioned above, further to the initial observation that Compound B (Figure 1) provides a starting point for the CCR5 agonist program, extensive medicinal chemistry resources were devoted to improving the drug-like properties and potency of these early lead structures. These internal medicinal chemistry efforts culminated in the synthesis and characterization of over 500 compounds, of which ESN-196 is highlighted as an optimized lead. Indeed, ESN-196 is a high-affinity, potent and selective agonist able to induce CCR5 receptor internalization into the intracellular compartment, probably in the endosome recycling compartment and the trans-Golgi network (Escola et al., 2010). Fluorescence microscopy images clearly demonstrate the intracellular migration of the

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receptor stimulated by ESN-196 in contrast to the absence of such effect with the CCR5 antagonist maraviroc.

The correlation between receptor stimulation/internalization and HIV-inhibitory potential was assessed in two distinct assays. A Moloney Murine Leukemia Virus-based pseudotype anti-HIV assay (Chan et al., 2006) was applied for regular screening to help guide structure-activity relationship (SAR) development in our internal medicinal chemistry efforts. As reported by Chan et al, we also observed a globally satisfying albeit an imperfect correlation between this non-replication assay and our follow-up assay, namely HIV entry inhibition in primary human blood cells. In particular, ESN-196 was identified as one of the most active compounds in both assays and was demonstrated to be as active as the anti-HIV drug maraviroc in blocking the proliferation of virulent strains of HIV in vitro in freshly isolated human PBMCs as well as in monocytes/macrophages. As an agonist, it was observed to be equipotent to the most active natural chemokine, LD78beta, in the monocyte/macrophage assay (Aquaro et al., 2001). These observations suggest that ESN-196 is in the appropriate range of potency for potential clinical application. Its spectrum of activity is suitable for both a systemic and prophylactic drug to be employed as a topically-administered microbicide (Lederman et al., 2006), for which the local protection of monocytes and monocytes-derived cells such as macrophages would be an essential characteristic.

An important consideration in considering a CCR5 agonist strategy for drug development is the potential risk of provoking pro-inflammatory side effects as observed for the endogenous cytokines interacting at CCR5. Specifically in terms of microbicidal applications, local administration of a pro-inflammatory drug would be

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counterproductive since inflammation of the vaginal mucosa is known to indirectly increase the risk of HIV transmission (Galvin 2004). Thus, it is an important finding that ESN-196, in sharp contrast to RANTES, does not induce chemotaxis of recombinant CHO-cells expressing CCR5. Moreover, the finding that ESN-196 competitively antagonizes the chemotactic response to RANTES is consistent with the categorization of ESN-196 as an allosteric agonist at the CCR5 receptor whereupon this allosteric interaction is insufficient to elicit the chemotactic response.

From the genetic point of view, HIV is one of the viruses with the highest mutation rates (Ribeiro 1998), which explains the rapid emergence of resistance against most classes of anti-HIV drugs. CCR5 antagonists do not escape this rule, and resistant isolates have been reported both in vivo (Moore and Kuritzkes, 2009, Tilton et al., 2010 a and b) and in vitro (Westby et al., 2007; Pugach et al., 2007; Trkola et al., 2002) that are able to enter the cells by using the drug-bound CCR5. The appearance of this phenotype can result from a simple combination of four amino acids substitutions in the GP120 gene which explains the relatively high frequency of spontaneous resistance (Kuhmann et al., 2004). Similarly HIV variants resistant to another CCR5 antagonist, TAK-652, have also been isolated in vitro after incubation of PBMCs in the presence of escalating concentrations of the compound (Baba et al., 2007). Vicriviroc (SCH-417690) is another unrelated CCR5- antagonist in advanced clinical trials, for which resistance has also been reported (Tsibris et al, 2008). Thus as a CCR5 agonist, ESN-196 could offer a key advantage compared to the CCR5 antagonists, specifically that elimination of the CCR5 receptor from the cell surface likely presents a greater obstacle to the spontaneous emergence of drug-resistant HIV-1 variants. Thus, in this case the challenge for the virus would be to switch to an alternative entrance gate to infect cells and such a spontaneous emergence of

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a completely new tropism has never been reported. To date, the only alternate tropism characterized for HIV uses the CXCR4 co-receptor, and the genetic switch from R5 to X4 needs a combination of mutations that would not easily appear in a HIV population that hardly replicates. Furthermore, a study trying to evidence the switch from X4 to R5 tropism failed to identify such events (Schols et al., 1998). The aforementioned is distinct from the observation that maraviroc seems to favor the emergence of the X4 virus sub-population in the specific example of patients hosting a mixed (dual R5/X4) viral population (Westby et al., 2006). In this case, the switch from R5 to X4 tropism is considered a consequence of the recruitment of an existing reservoir of CXCR4-tropic sub-population, rather than a genetic switch from a CCR5-tropic isolate. In keeping with the observation that ESN-196 has a CCR5-specific mechanism of action, it would be similarly expected that ESN-196 would be ultimately ineffective in patients harbouring a mixed R5/X4 viral population.

In this study, we also illustrated that ESN-196 induces a prolonged loss of CCR5 function at the cell surface when compared to RANTES consistent with agonist-induced receptor internalization. RANTES derivatives such as PSC-RANTES (Pastore et al, 2003) and AOP-RANTES (Mack et al, 1998) similarly induced a prolonged down-regulation of CCR5 at the cell surface attributed to a prolonged recycling time of CCR5 to the cell surface. Antagonist compounds, as demonstrated in this report for maraviroc, do not induce receptor internalization and therefore CCR5 receptor signalling is promptly detected after antagonist wash-out. This implies that full receptor occupancy is required for the antagonist approach to be viable necessitating a strict dosing regimen. An additional advantage of the agonist approach is that there is a sustained protective effect

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due to the receptor internalization aspect implying a greater margin of flexibility in terms of drug exposure and dose regimen.

In summary, we present an alternative approach for HIV treatment through blocking R5 HIV-1 entry via CCR5 receptor internalization using the small-molecule CCR5 agonist, ESN-196. The validity of this approach has been demonstrated *in vitro* inasmuch as ESN-196 has been found to be sufficiently potent to confer full protection in human PBMCs towards HIV infection. In addition, our observations suggest that the CCR5 agonist approach offers two potential advantages versus the traditional, antagonist approach, specifically that the agonist (i) provides a sustained protective effect, and (ii) presents a greater obstacle to the emergence of spontaneous mutants resistant to the therapy.

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**Acknowledgments**

None

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**Authorship contribution**

*Participated in research design:* Ferain, Hoveyda, Ooms, Schols, and Fraser

*Conducted experiments:* Ferain, Ooms, and Schols

*Contributed new reagents or analytic tools:* Hoveyda, Ooms, Schols, and Bernard

*Performed data analysis:* Ferain, Hoveyda, Ooms, Bernard, Schols, and Fraser

*Wrote or contributed to the writing of the manuscript:* Ferain, Hoveyda, Schols, and Fraser

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**Legends for Figures**

FIGURE 1:

A.Structures of Compound A (2-(4-chlorophenyl)-N-(1-(3,3-diphenylpropyl)piperidin-4-yl)-N-methylacetamide), Compound B (2-(4-chlorophenyl)-N-(1-(3,3-diphenylpropyl)piperidin-4-yl)acetamide) , ESN-196 ((R)-2-(4-cyanophenyl)-N-(1-(1-(N,1-diphenylmethylsulfonamido)propan-2-yl)piperidin-4-yl)acetamide), YM-370749 and maraviroc

B. Representative curves of binding competition between the MIP-1b radioligand and the compounds. Results are presented as the percentage of $^{125}$I-MIP-1β binding in the presence of Compound A (Ki=21.2 nM); Compound B (Ki=5.6 nM); ESN-196 (Ki=0.09 nM); YM-370749 (inactive) and maraviroc (Ki= 0.93 nM). Experiments were performed in duplicate and the data shown are mean values ± SD

C. Representative curves of concentration dependent activation of the CCR5 receptor monitored as Calcium-induced emission of light in an aequorin CHO-CCR5 cell line. Results are presented as a percentage of RANTES maximum response (100%). EC50 and % Emax values for each compound are as follow: ES00231487 (“Compound A”): NA, <1% (10µM); ES00231486 (“Compound B”): 70 nM; 71%; ES00232155 (“ESN-196”): 43 nM; 110%; YM-370749: NA, <1% (10µM); maraviroc; NA, <1% (300 nM); RANTES: 0.9 nM; 100%. Experiments were performed in duplicate and the data shown are mean values ± SD

FIGURE 2:



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A, Cellular location of CCR5-YFP following an agonist stimulation of 1 hour in recombinant HEK-CCR5-YFP cells using fluorescence microscopy. 1, untreated control; 2, 300 nM RANTES; 3, 300 nM maraviroc; 4, 300 nM ESN-196; 5, 300 nM YM-370749 B, Representative dose-dependent internalization curves of CCR5-YFP: quantification by the membrane translocation module on the IN Cell Analyzer following 1 hour stimulation with various concentrations of compounds (ESN-196, n=2; maraviroc, n=4; YM-370749, n=4 or RANTES, n=2). Results (mean values ± SD) are expressed as the percentage of intracellular area occupied by inclusions.

FIGURE 3:

Representative curves of concentration dependent inhibition of pseudotyped viral infection of MAGI-R5 cells. Cells were incubated with serially diluted CCR5 agonists (ESN-196, RANTES, YM-370749) or antagonist (maraviroc). The inhibition of infection is measured as a ratio between the luciferase activity in cells exposed to the compound compared to the maximum infection in non-exposed but infected cells. Experiments were performed in duplicate and the data shown are mean values ± SD

FIGURE 4:

HIV-1 infection in PBMCs and treatment with ESN-196 or MVC in (i) R5 Bal strain and (ii) Clinical R5 Isolate #15. Results are plotted as single experimental points. PBMCs were isolated from the same blood donor.

FIGURE 5:

Functional recovery of CCR5 in a recombinant CHO-K1-CCR5 cell line. Cells were treated with 100 nM of compounds for 24 h. After compound washout followed by a



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recovery period of 24h, the CCR5 responsiveness to RANTES was evaluated in a cAMP assay. ESN-196-induced receptor internalization reduces both the potency (EC50 shift) and efficacy (% Emax) of RANTES. Experiments were performed in duplicate and the data shown are mean values ± SD

FIGURE 6:

ESN-196 does not elicit concentration-dependent chemotactic attraction of CHO-K1-CCR5 cells in comparison to RANTES and MIP-1β (mean values of two experiments); ESN-196 (100 nM) added 15 minutes before RANTES completely antagonizes the chemotactic response to this chemokine. Pre-treatment of cells for 24h with ESN-196 (100 nM) followed by washout noncompetitively inhibits the chemotactic response to RANTES. Experiments were performed in triplicate and the data shown are mean values ± SD

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**Table**

TABLE 1

| [ESN-196], μM | Bmax, pmole/mg protein |
|---|---|
| not exposed | 5.29 |
| 0.1 | 1.69 |
| 10 | 1.32 |

Prolonged internalization of the CCR5 receptor illustrated by the measurement of the CCR5 Bmax after a 24h exposure to ESN-196 compared to non-exposed cells

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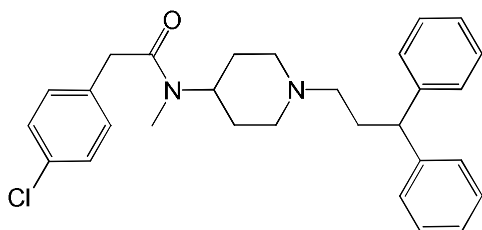
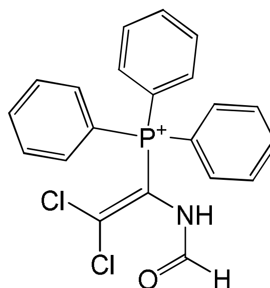
Figure 1A

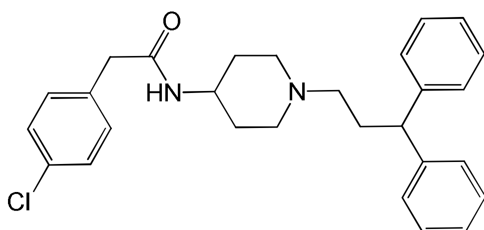
Compound A



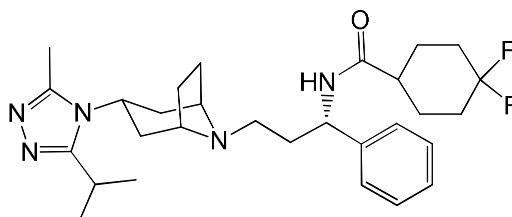

YM-370749




Compound B




Maraviroc




ESN-196

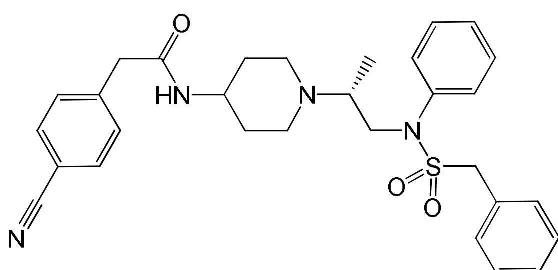



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Figure 1B

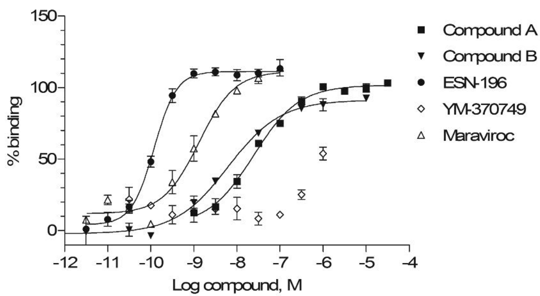
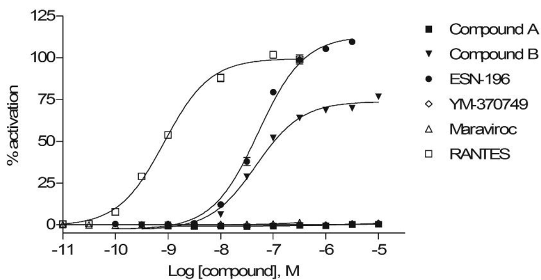


Figure 1C



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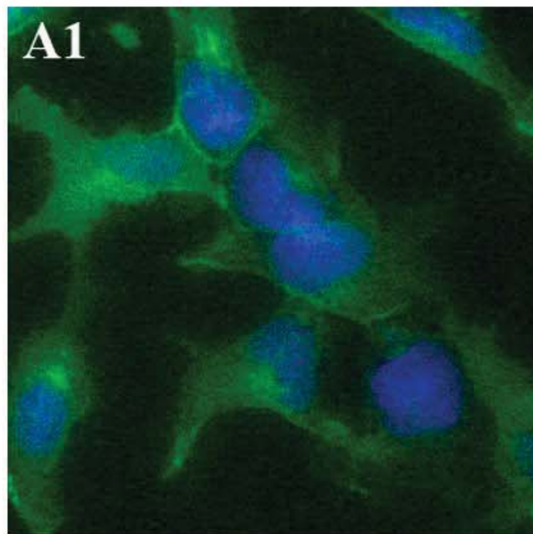


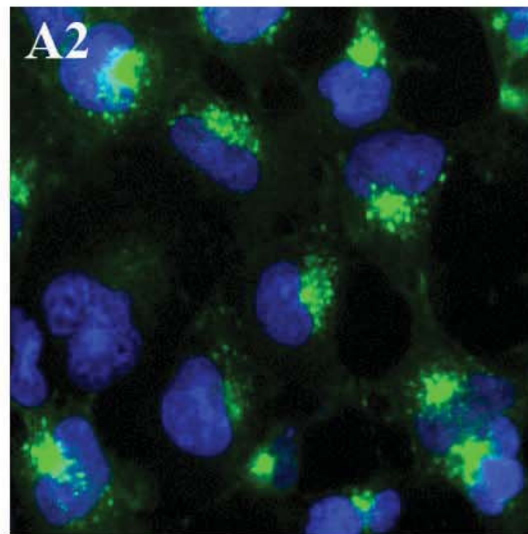


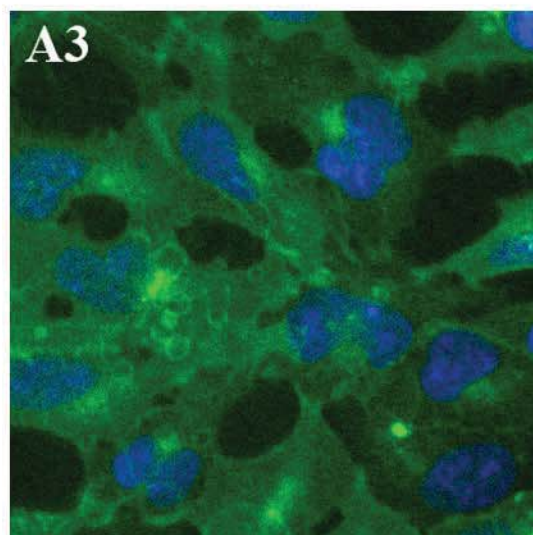


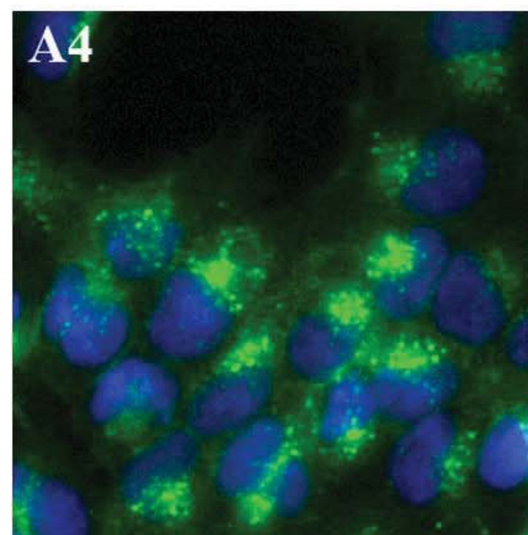


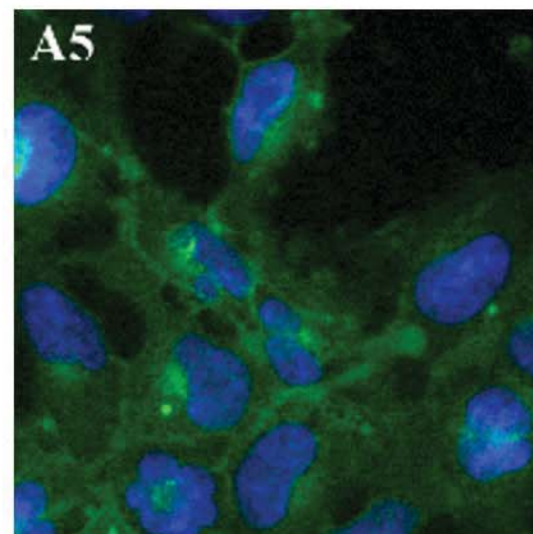


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Figure 2B

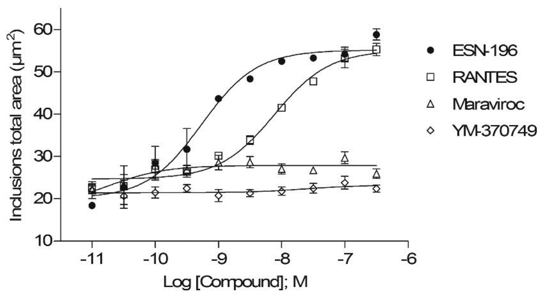


Figure 3

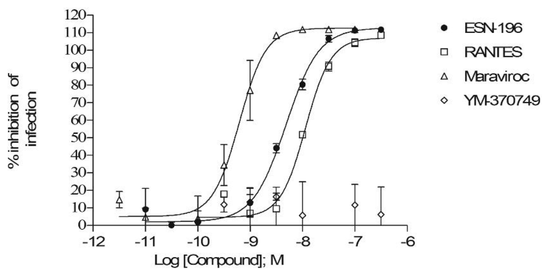
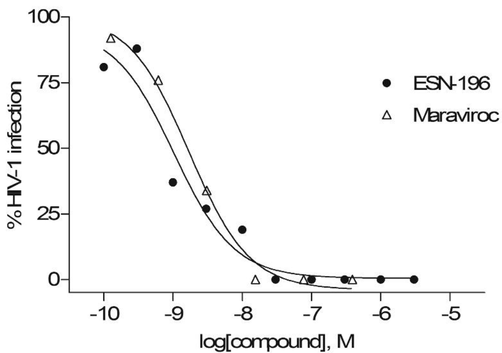


Figure 4

(i)




(ii)

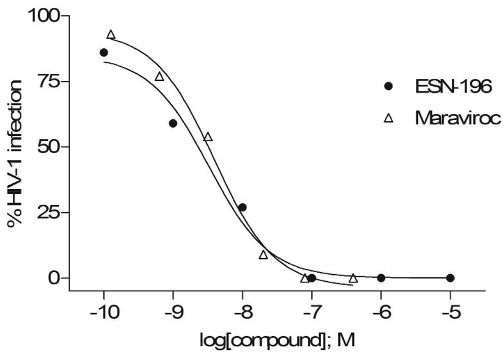
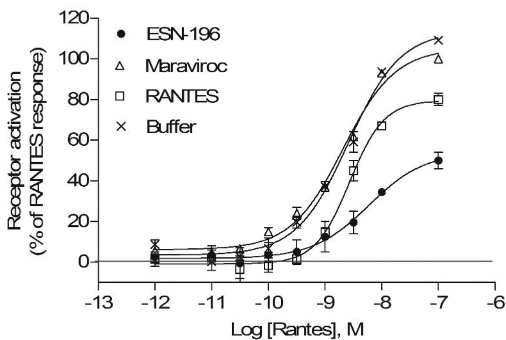


Figure 5




| | $EC_{50}$ shift (log) | $E_{max}$ (%) |
|---|---|---|
| ESN-196 | 0.36 | 48 |
| Maraviroc | -0.09 | 93 |
| RANTES | 0.02 | 70 |

Figure 6