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Molecular cloning and radioligand binding characterization of the chemokine receptor CCR5 from rhesus macaque and human

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Abbreviations:
HIV, human immunodeficiency virus
MIP, macrophage inflammatory protein
HCC-1, hemofiltrate CC chemokine 1
MCP, monocyte chemotactic protein
RANTES, regulated on activation normal T cell expressed and secreted
CTACK, cutaneous T cell-attracting chemokine
TARC, thymus and activation-regulated chemokine
LARC, liver and activation-regulated chemokine

ABSTRACT

The aim of this study was to determine if macaque represents a suitable species for the pre-clinical evaluation of novel CCR5 antagonists, such as maraviroc (UK-427,857). To do this we cloned and expressed CCR5 from rhesus macaque and compared the binding properties of [^{125}I]-MIP-1β and [^{3}H]-maraviroc with human recombinant CCR5. [^{125}I]-MIP-1β bound with similar high affinity to CCR5 from macaque ($K_d = 0.24 \pm 0.05$ nM) and human ($K_d = 0.23 \pm 0.05$ nM) and with similar kinetic properties. In competition binding studies the affinity of a range of human chemokines for macaque CCR5 was also similar to human CCR5. Maraviroc inhibited binding of [^{125}I]-MIP-1β to CCR5 from macaque and human with similar potency ($IC_{50} = 17.50 \pm 1.24$ nM and 7.18 ± 0.93 nM, respectively) and antagonised MIP-1β induced intracellular calcium release mediated through CCR5 from macaque and human with similar potency ($IC_{50} = 17.50 \pm 3.30$ nM and 12.07 ± 1.89, respectively). [^{3}H]-maraviroc bound with high affinity to CCR5 from macaque ($K_d = 1.36 \pm 0.07$ nM) and human ($K_d = 0.86 \pm 0.08$ nM), but was found to dissociate ~10-fold more quickly from macaque CCR5. However, as with the human receptor, maraviroc was shown to be a high affinity, potent functional antagonist of macaque CCR5 thereby indicating that the macaque should be a suitable species in which to evaluate the pharmacology, safety and potential mechanism-related toxicology of novel CCR5 antagonists.

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ELC, EBI, Epstein-Barr virus induced molecule 1 ligand chemokine (MIP-3β)
TECK, mature human thymus-expressed chemokine

1. Introduction

Chemokines are a family of small peptides that act as chemoattractants and activators of specific types of leukocyte to mediate a variety of immune and inflammatory responses through their binding and subsequent activation of specific receptors on the cell surface. These receptors belong to a large family of seven-transmembrane G-protein coupled receptors [1–3] that includes the C, CC, CXC and CX_3C subfamilies [4]. In addition to a role in leukocyte trafficking, chemokines and their receptors have other biological roles and are involved in haematopoiesis, angiogenesis, metastasis and HIV-1 infection [1–3]. The chemokine receptor CCR5, originally identified as a receptor for MIP-1α, MIP-1β and RANTES [5–7], has been shown to be the co-receptor for the most commonly transmitted HIV-1 strains that predominate during the early stages of infection [8–10]. HIV-1 initiates entry into cells through binding to the CD4 receptor, but CD4 binding alone is insufficient to allow viral fusion and entry and the virus requires a second receptor to reduce the energy barrier for these processes [11]. Humans that are homozygous for a 32 bp deletion in the gene encoding for CCR5 tend to have a high degree of resistance and heterozygous individuals tend to have a slower rate of HIV-1 disease progression, confirming the role of CCR5 in facilitating viral entry [12–16]. Immune function appears to be normal in such individuals and for this reason; CCR5 represents an attractive drug target for inhibition of HIV-1 entry and has led to the search for CCR5 antagonists, as new anti-retroviral treatments [17–22]. Maraviroc is a highly selective, orally bioavailable, small molecule CCR5 antagonist with potent anti-HIV-1 activity, currently undergoing clinical evaluation for the treatment of HIV-1 infection [23]. The compound has potent broad-spectrum activity against a range of HIV-1 isolates (for detailed review see [24]) and in phase II studies has been shown to reduce viral load in HIV-1 infected patients, thereby confirming the validity of this approach [25].

A key challenge faced with the development of maraviroc had been its low affinity at the CCR5 of common laboratory animals, i.e. $K_i > 10\ \mu M$ for mouse, rat and dog recombinant CCR5 (unpublished observations). This has not been an issue for the determination of the potential anti-HIV activity where human leukocytes can be used in vitro [23,24], however, for mechanism-based safety and toxicology assessments a laboratory species is required. Ideally, these assessments should be conducted in a test species that expresses the target receptor and which demonstrates the same pharmacological profile to that expected in human. Since previous studies had shown that human and macaque CCR5 genes have very close homology [26–29] and given that macaques are a relevant toxicology species [30], we sought to determine if the binding and functional properties of a range of chemokines and maraviroc at macaque CCR5 were similar to human CCR5. To do this we cloned and expressed the macaque CCR5 in HEK-293 cells. We then characterised the binding and functional properties of recombinant macaque CCR5 in comparison with the human recombinant CCR5 expressed in HEK-293 cells.

2. Methods

2.1. Materials

Due to the high degree of sequence homology between human and macaque chemokines, human recombinant chemokines were used in these studies. Chemokines and biotinylated human recombinant MIP-1β were purchased from R&D systems (London, UK). Lyophilised chemokines were dissolved in phosphate buffered saline (PBS) and stored at −20 °C in aliquots. [^{125}I]-MIP-1β (2000 Ci/mmol) was purchased from Perkin Elmer LAS, UK and [^{3}H]-maraviroc (16 Ci/mmol) was custom synthesised by Amersham Biosciences. All other chemicals were from Sigma. Cell culture reagents were obtained from Life Technologies, Merelbeke, Belgium. Maraviroc (UK-427,857) was synthesized by Pfizer Global Research and Development, Sandwich, Kent, UK.

2.2. Culture of rhesus macaque kidney cells

Primary rhesus macaque kidney (PMK) cells (ECACC 98020308, Salisbury, UK) were cultured in Eagle's minimum essential medium (EMEM, Invitrogen) containing 5% FCS, 2 mM L-glutamine, 1× non-essential amino acids (NEAA), at 37 °C in a 5% CO_2 humidified incubator.

2.3. Macaque CCR5 DNA and sequence

Macaque CCR5 gene-specific oligonucleotide primer pairs (Eurogentec, Southampton, UK) were designed using the sequence deposited in the NIH GenBank® genetic sequence database (accession number AF005660), and the Vector NTI DNA sequence analysis software (Informax Inc., Bethesda, USA). Primer sequences were: (5′) CCCTCGAGGGCCGCCAC-CATGGACTATCAAGTGTCAAGTCC and (3′) CGGGATCCCGT-CACAAGCCCACAGATATTTCC. An XhoI restriction site and a Kozak transcription initiation motif were incorporated in the 5′ primer, immediately prior to the initial methionine ATG codon, and a BamHI restriction site was incorporated in the 3′ primer. Since there are no introns in the coding region of CCR5 in human, rhesus genomic DNA isolated from PMK cells was used as a template for the PCR. Genomic DNA was isolated

from approximately 4×10^7 PMK cells using a genomic DNA kit (Promega, Southampton, UK). PCR amplification of the CCR5 coding sequence from genomic DNA isolated from PMK cells was performed using a proof-reading DNA polymerase reagent (Expand, Roche, Lewes, UK), using the proprietary protocol accompanying the polymerase. PCR products were analyzed by agarose gel electrophoresis, and the macaque CCR5 coding sequence was directionally subcloned into the pcDNA3.1(−) plasmid vector (Invitrogen, Paisley, UK) as an XhoI/BamHI insert. The pcDNA3.1(−) vector includes a CMV promoter for transient and stable mammalian cell expression studies. The authenticity of the CCR5 sequence was confirmed by nucleotide sequencing (Lark Technologies, Takely, UK).

2.4. Generation of mCCR5 cell line

The pcDNA3.1(−) plasmid containing macaque CCR5 cDNA was transfected into HEK-293 cells, using LipofactAMINE reagent and protocol (Invitrogen). The cells were grown in Dulbecco's modified Eagle medium (DMEM, Invitrogen) supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine, 1 × non-essential amino acids (Invitrogen) and geneticin (0.8 mg/ml) for approximately 2 weeks. Cells expressing the macaque CCR5 gene survived as individual cell foci and were subject to selection using a Fluorescence Activated Cell Sorter (FACS). Cells were isolated by their ability to bind biotinylated human recombinant MIP-1β, thereby gating via SPA-fluorescence. Individual cell clone populations were then expanded and screened for responsiveness to human recombinant MIP-1β in a calcium flux assay using a Fluorometric Imaging Plate Reader (FLIPR, Molecular Devices, Wokingham), as previously described by Combadiere et al., 1996 [7].

2.5. Functional assays

HEK-293 cells expressing macaque CCR5 were seeded into 96-well black-wall clear bottomed poly-D-lysine coated plates (Fisher Scientific) at a density of 7.5×10^4 cells/well and cultured overnight in a 5% CO_2 humidified incubator at 37 °C. After this time the cells were washed in HANKS buffer containing 1.6 mM $CaCl_2$ and 10 mM HEPES, pH 7.2 (calcium flux buffer) and then incubated for 2 h in Calcium Plus Assay dye (Molecular Devices, UK). Cells were then washed, as before, and 160 μl of calcium flux buffer added to each well. Plates were loaded into a FLIPR and calcium release was monitored before, during and after the addition of MIP-1β (20 nM) at an excitation wavelength of 488 nm and an emission wavelength of 530 nm. Inhibition of MIP-1β mediated elevation of intracellular calcium by maraviroc (0.51–1 μM) was assessed in quadruplicate. MIP-1β (20 nM in well) was added following a 4 min incubation period with either antagonist or vehicle. Fluorescence was measured over a total of 8 min to investigate any direct effects of maraviroc on cell signalling, and inhibitory effects on MIP-1β-mediated signalling.

2.6. Membrane preparation for binding experiments

Cells were grown to until they were 80% confluent before being harvested into ice-cold Dulbecco's phosphate buffered saline (Invitrogen) and centrifuged at 350 × *g* for 10 min at 4 °C using a bench top centrifuge. The supernatant was discarded and the pellet resuspended in lysis buffer (20 mM HEPES containing 1 mM $CaCl_2$ and protease inhibitor cocktail (Boehringer Mannheim COMPLETE protease inhibitor tablet)). The cells were then homogenised with an Ultra-Turrax T25 (3 × 10 s bursts) and centrifuged for 20 min at 48,000 × *g* at 4 °C. The supernatant was discarded and the pellet resuspended in lysis buffer and centrifuged as before. The final pellet was resuspended in lysis buffer, aliquoted and stored at −80 °C. The protein concentration was determined using a Coomassie Blue-based assay kit (Sigma). Membranes were also prepared from HEK-293 cells stably expressing the human recombinant CCR5 (previously constructed at Pfizer [24]) cultured and harvested as described for HEK-293 cell expressing macaque CCR5.

2.7. Radioligand binding studies

[^{125}I]-MIP-1β binding was performed as described by Combadiere et al. [7] in 50 mM HEPES buffer containing 1 mM $CaCl_2$, 5 mM $MgCl_2$ and 0.5% bovine serum albumin (pH 7.4) at room temperature for 2 h in a total volume of 100 μl using 7.5–10 μg of membrane protein per well. Non-specific binding (NSB) was defined using 100 nM MIP-1β. For [^{125}I]-MIP-1β competition binding studies with maraviroc, non-specific binding was defined using 10 μM UK-396,794, a similar small molecule CCR5 antagonist synthesised at Pfizer laboratories. [^{3}H]-maraviroc binding was performed as described for [^{125}I]-MIP-1β binding using 2.5–5 μg of membrane protein per well. Saturation binding was performed in duplicate wells using 12 concentrations of [^{125}I]-MIP-1β (0.01–1 nM) or [^{3}H]-maraviroc (0.25–35 nM). Assays were terminated by separation of bound and free radioligand by filtration through GF/B filters presoaked in 0.3% polyethylenimine (PEI). Filters were counted on a Cobra II gamma counter. Competition binding experiments were performed using a sub-K_d concentration of [^{125}I]-MIP-1β (0.1 nM), in the presence and absence of different chemokines or maraviroc. IC_{50} values were determined using 10 half-log serial dilutions in duplicate. Assays were terminated by separation of bound and free radioligand by filtration through GF/B Packard Unifilter plates presoaked in 0.3% PEI. Unifilters were counted on a Topcount Liquid scintillation counter (Packard). For association experiments membrane homogenates were incubated with 0.1 nM [^{125}I]-MIP-1β or 5 nM [^{3}H]-maraviroc for various times from 1 min to 3 h in the presence and absence of 100 nM unlabelled MIP-1β or 10 μM maraviroc. For [^{125}I]-MIP-1β dissociation experiments, membrane homogenates were incubated with 0.1 nM [^{125}I]-MIP-1β for 2 h to allow association of [^{125}I]-MIP-1β. After this time an excess (100 nM) of unlabelled MIP-1β was added to each well at various times, and dissociation followed from 1 min to 4 h. Plates for kinetic experiments were processed as described for competition assays. For [^{3}H]-maraviroc dissociation experiments, membrane homogenates were incubated with 5 nM [^{3}H]-maraviroc for 2 h and dissociation was initiated by the addition of 50 μM maraviroc. Initially, dissociation assays with [^{3}H]-maraviroc were performed over 8 h at room tempertaure, but were extended to 51 h for the human CCR5 due to incomplete dissociation after 8 h. In these experiments, the assay buffer included antibiotics (penicillin (5 U/mL) and streptomycin (0.5 mg/mL) and protease inhibitors (Boehringer

Mannheim COMPLETE protese inhibitor cocktail) to prevent degradation of the membrane homogenates.

2.8. Data analysis

Data are reported as mean ± S.E. mean of n experiments. Functional responses were measured as peak fluorescence intensity minus basal. Data were analysed using the non-linear regression functions within PRISM (GraphPad Software, San Diego, CA, USA). The equilibrium dissociation constant (K_d) and the binding site density (Bmax) were derived from radioligand saturation curves using the Langmuir equation RL = RtL/(K_d + L) where L is the free ligand concentration, RL is the concentration of receptor-bound ligand at equilibrium, and Rt is the total receptor concentration. IC_{50} (the concentration of compound producing 50% inhibition of specific binding) and Hill Coefficients were derived from fitting to a four-parameter logistic equation. Curve fitting was not constrained when data fully reached maximum and minimum plateaus, but was constrained where compounds had low affinity and did not fully inhibit binding. Apparent K_i values were derived when Hill slopes were not different from unity using the Cheng and Prusoff equation; $K_i = IC_{50}/(1+[L]/K_d)$ where [L] is the free concentration of radioligand and K_d is the appropriate equilibrium dissociation constant derived from the saturation analysis [31]. In kinetic experiments, the observed association rate constant (k_{obs}) and the association and dissociation half-lives were derived directly from the one-phase non-linear exponential models within PRISM. The dissociation rate constant (k_{off} in units of time^{-1}) was calculated as $0.693/t_{1/2\ off}$, The association rate constant (k_{on} in units of time^{-1} nM^{-1}) was derived by the pseudo first-order method [32] which takes into account the concentration of radioligand used, the observed association rate constant (k_{obs} in units of time^{-1}) and the dissociation rate constant (k_{off}) such that ($k_{on} = k_{obs} - k_{off}$)/[L]. The ratio of k_{off}/k_{on} was also used to estimate K_d. Binding data from saturation and kinetic experiments were compared using two-sample, unpaired, equal variance Student's t-tests. Differences were considered to be statistically significant when P-values <0.05.

3. Results

3.1. Macaque CCR5 sequence and creation of stable cell lines

Analysis of the macaque CCR5 gene sequence showed that it encodes 352 amino acids. As previously reported [26–29] comparison with the human CCR5 gene sequence reveals close homology and a difference of only eight amino acids; the majority of these substitutions being of similar amino acids. The locations of the amino acid differences are shown in Fig. 1. To characterise the pharmacological properties of the macaque CCR5, cDNA encoding the receptor sequence was stably expressed in HEK-293 cells. As human MIP-1β is known to bind to macaque CCR5 [28,33] cells transfected with pcDNA3.1(−) plasmid containing the macaque CCR5 cDNA were sorted on their ability to bind biotinylated human recombinant MIP-1β using FACS analysis. This process led to the selection of 18 clones that were subsequently used for functional assessment in an intracellular calcium release assay. Of these, cells from clone 7, demonstrated the greatest response to MIP-1β and were expanded for further characterization (Fig. 2). Untransfected parental HEK-293 cells failed to respond to MIP-1β confirming that the responses in transfected cells was mediated through the macaque CCR5.

3.2. [^{125}I]-MIP-1β binding studies

Using membranes prepared from homogenates of HEK-293 cells, the kinetic and equilibrium binding properties of [^{125}I]-MIP-1β at macaque and human CCR5 were compared. Fig. 3 shows that the time-course of association and dissociation of [^{125}I]-MIP-1β at human and macaque CCR5 were similar. Specific binding of [^{125}I]-MIP-1β was time-dependent and fully reversible and was achieved after the addition of an excess of unlabelled MIP-1β (100 nM) once equilibrium had been reached. Saturation binding analysis confirmed that [^{125}I]-MIP-1β bound to CCR5 from macaque and human with the same high affinity. Table 1 shows that K_d values estimated from saturation analysis were also in good agreement with the K_d obtained from the ratios of k_{off}/k_{on} for both human and

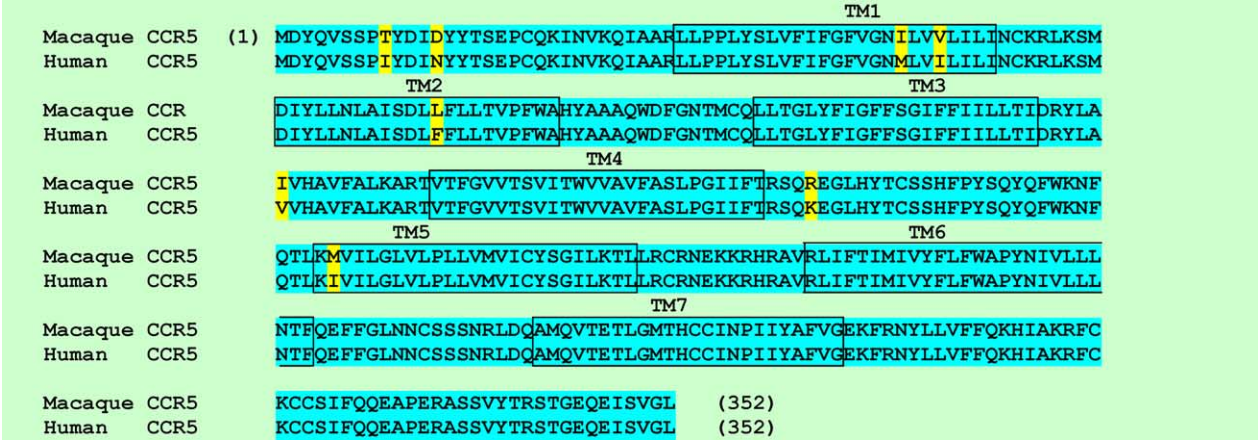

Fig. 1 – Comparison of peptide sequences from macaque CCR5 sequence obtained in the present study with the human CCR5 from Gen Bank (AF005660). TM, putative transmembrane domains.

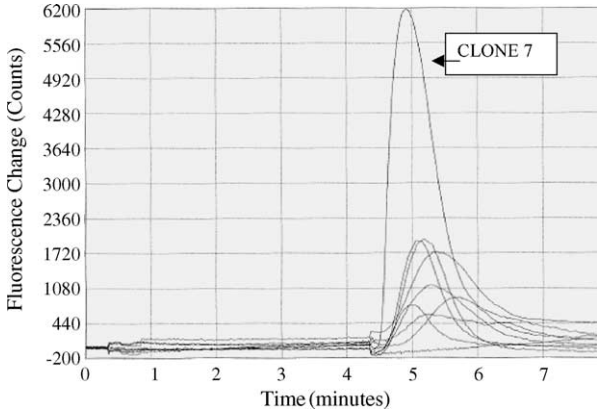

Fig. 2 – MIP-1β (20 nM) induced increases in intracellular calcium concentration in various clonal cells expressing macaque CCR5, as shown by FLIPR. Clone 7 showed the largest response. The data shown are from a single experiment.

Table 1 – Binding properties of [^{125}I]-MIP-β at macaque and human recombinant CCR5

	Macaque CCR5	Human CCR5
$t_{1/2}$ (on) (min)	12.77 ± 0.94*	8.54 ± 0.78
$t_{1/2}$ (off) (min)	17.86 ± 1.84	18.50 ± 2.46
k_{off} (min^{-1})	0.04 ± 0.01	0.04 ± 0.01
k_{on} (min^{-1} nM^{-1})	0.14 ± 0.03*	0.39 ± 0.07
k_{off}/k_{on} (nM)	0.35 ± 0.08	0.12 ± 0.03
K_d (nM)[a]	0.24 ± 0.05	0.23 ± 0.05

Data expressed as mean ± S.E. mean of at least three separate experiments.
[a] From saturation experiments.
* $P < 0.05$ compared to human.

macaque CCR5. The kinetic binding properties of [^{125}I]-MIP-1β binding to human and macaque CCR5 were also comparable; the off-rate and dissociation half-life values were very similar, while on-rate and association half-life values were less than 3-fold different. The apparent faster association rate may be related to the higher level of receptor expression in cells expressing the human CCR5, as indicated by a higher Bmax value (1979 ± 230 fmol/mg protein) compared with cells expressing the macaque CCR5 (452 ± 100 fmol/mg protein).

Competition experiments were performed to compare the ability of a panel of chemokines to inhibit the binding of [^{125}I]-MIP-1β to homogenates of HEK-293 cells expressing either the macaque or human CCR5. Chemokines were first tested for their ability to inhibit [^{125}I]-MIP-1β binding at 100 nM. Those inhibiting binding by more than 50% (MIP-1α, MIP-1β, RANTES, MCP-1, MCP-2, MCP-3, HCC-1) were analysed over a wider concentration range to determine pK_i values. As Fig. 4 shows the classical CCR5 chemokines, MIP-1α, MIP-1β, and RANTES as well as HCC-1, MCP-1, MCP-2, MCP-3, significantly inhibited binding (>50%) of [^{125}I]-MIP-1β to macaque and human CCR5 at 100 nM. The rank order of affinity of the chemokines for

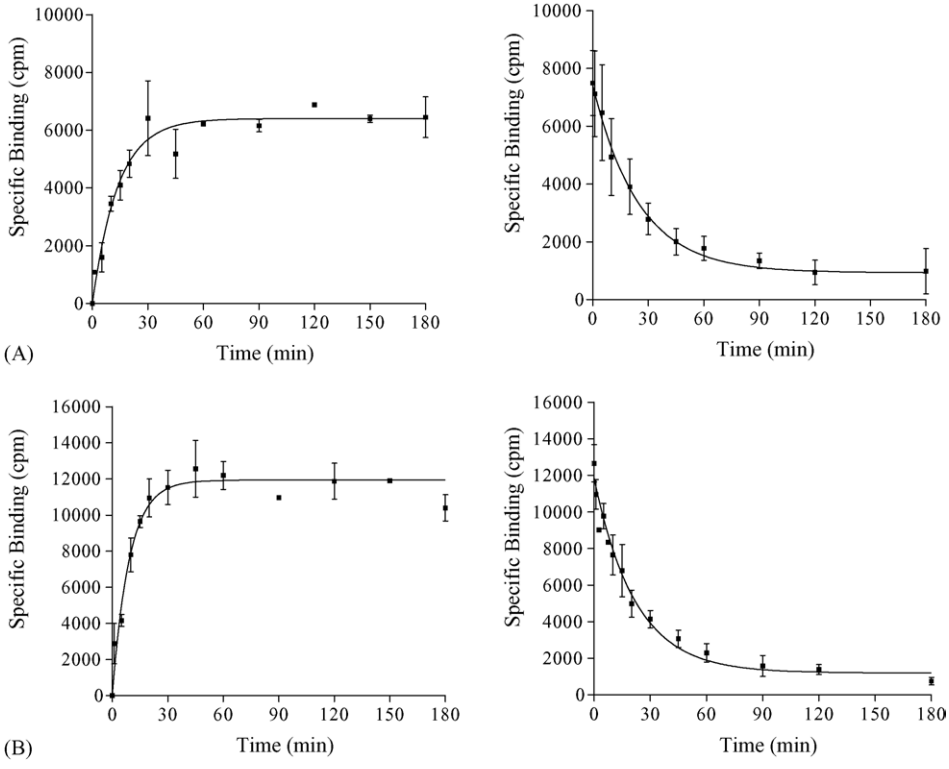

Fig. 3 – Binding kinetics of [^{125}I]-MIP-1β to HEK-293 cell membranes expressing the macaque (A) and human (B) recombinant CCR5. Association time-course of 0.1 nM [^{125}I]-MIP-1β binding to 10 μg membrane protein per well (*left*). Dissociation time-course of [^{125}I]-MIP-1β (0.1 nM) initiated by 100 nM MIP-1β (*right*). The data are expressed as mean ± S.E.M of 4–5 individual experiments.

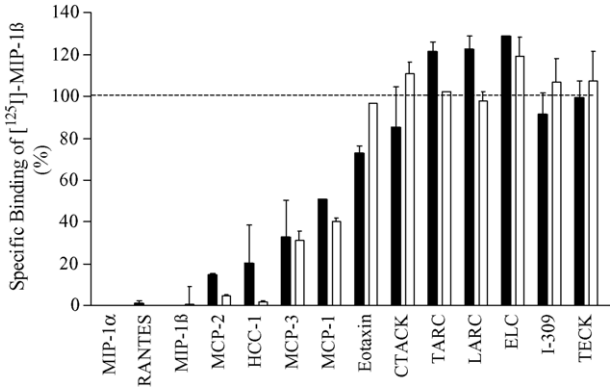

Fig. 4 – Inhibition of [^{125}I]-MIP-1β binding to HEK-293 cell membranes expressing human (black bars) and macaque (white bars) CCR5 by various chemokines (100 nM). The data are expressed as the mean of duplicate wells from a single experiment.

macaque CCR5 was similar to human CCR5; MIP-1α ≥ MIP-1β > RANTES > MCP-2, ≫MCP-1, MCP-3, HCC-1 > eotaxin, CTACK, TARC, LARC, ELC, I-309, TECK (Table 2, Fig. 5). Although MCP-2 had high affinity (IC_{50} values of 1.62 and 0.67 nM for human and macaque, respectively) the slopes of the competition curves were shallow (−0.46 and −0.72) and for this reason K_i values were not calculated.

3.3. *Binding and functional antagonism of maraviroc, a small molecule CCR5 antagonist*

The binding of maraviroc to human and macaque CCR5 was compared in a standard [^{125}I]-MIP-1β competition binding assay. As Fig. 6 shows, maraviroc was a potent inhibitor of [^{125}I]-MIP-1β binding to the macaque CCR5 with an IC_{50} of 17.50 ± 1.24 nM (n = 5), a value that was within ~2-fold of that obtained at the human CCR5 (7.18 ± 0.93 nM, n = 17). As slope factors were consistently steep (i.e. >1.2) in all competition

Table 2 – Apparent equilibrium inhibition constants (pK_i) for chemokines at the macaque and human recombinant CCR5

Chemokine	Macaque CCR5 (pK_i)	Human CCR5 (pK_i)
MIP-1α[a]	10.05 ± 0.06	9.56 ± 0.09
MIP-1β[a]	9.76 ± 0.06	9.55 ± 0.12
RANTES[a]	9.04 ± 0.10	9.15 ± 0.11
MCP-1[a]	7.43 ± 0.07	7.51 ± 0.17
MCP-3[a]	7.90 ± 0.06	7.48 ± 0.14
HCC-1[a]	7.57 ± 0.09	7.24 ± 0.23
Eotaxin[b]	<7	<7
CTACK[b]	<7	<7
LARC[b]	<7	<7
I-309[b]	<7	<7
TECK[b]	<7	<7

[a] Data expressed as mean ± S.E. mean of at least three separate experiments.
[b] Estimated from a single experiment performed in duplicate where chemokines were tested at 100 nM.

Table 3 – Binding properties of [^{3}H]-maraviroc at macaque and human recombinant CCR5

	Macaque CCR5	Human CCR5
$t_{1/2}$ (on) (h)	0.14 ± 0.008	0.17 ± 0.03
$t_{1/2}$ (off) (h)	1.54 ± 0.12*	15.95 ± 1.97
k_{off} (h^{-1})	0.45 ± 0.036*	0.043 ± 0.005
k_{on} (h^{-1} nM^{-1})	0.80 ± 0.08	0.61 ± 0.06
k_{off}/k_{on} (nM)	0.51 ± 0.016*	0.071 ± 0.0042
K_d (nM)[a]	1.36 ± 0.07*	0.86 ± 0.08

Data expressed as geometric mean ± S.E. geometric meanof at least three separate experiments.
[a] From saturation experiments.
* $P < 0.05$ compared to human.

binding experiments with maraviroc, pK_i values were not calculated. In FLIPR experiments, maraviroc was shown to be a potent functional antagonist at macaque CCR5 with an IC_{50} value for inhibition of MIP-1β induced intracellular calcium release of 17.50 ± 3.30 nM (n = 4). This value was similar to the IC_{50} of 12.07 ± 1.89 nM (n = 8) obtained for the human CCR5.

3.4. *[^{3}H]-Maraviroc binding studies*

The direct binding properties of [^{3}H]-maraviroc at CCR5 from macaque and human were compared using similar methods as described for [^{125}I]-MIP-1β binding. As Table 3 shows, saturation binding analysis confirmed that [^{3}H]-maraviroc bound to CCR5 from macaque and human with similar high affinity. In addition, the time course of association of [^{3}H]-maraviroc to macaque and human CCR5 was also similar with

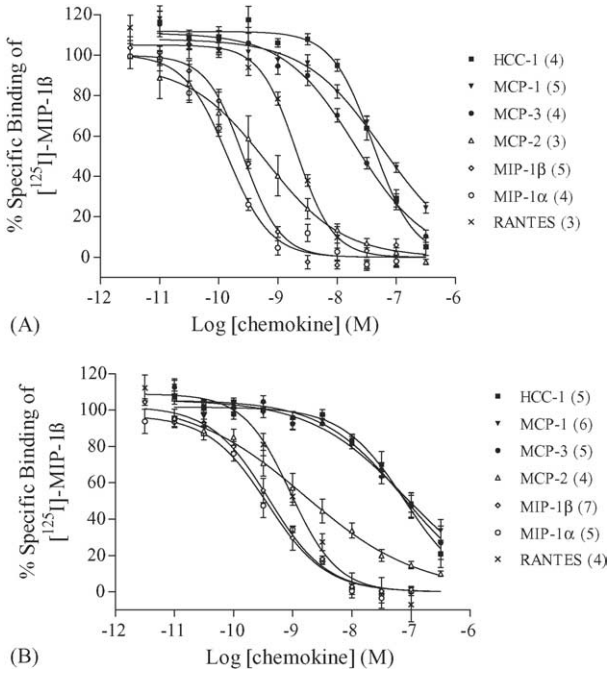

Fig. 5 – Competition binding of various chemokines against [^{125}I]-MIP-1β binding to HEK-293 cell membranes expressing macaque (A) and human (B) CCR5. The data are expressed as the mean ± S.E.M. of at least three experiments performed in triplicate.

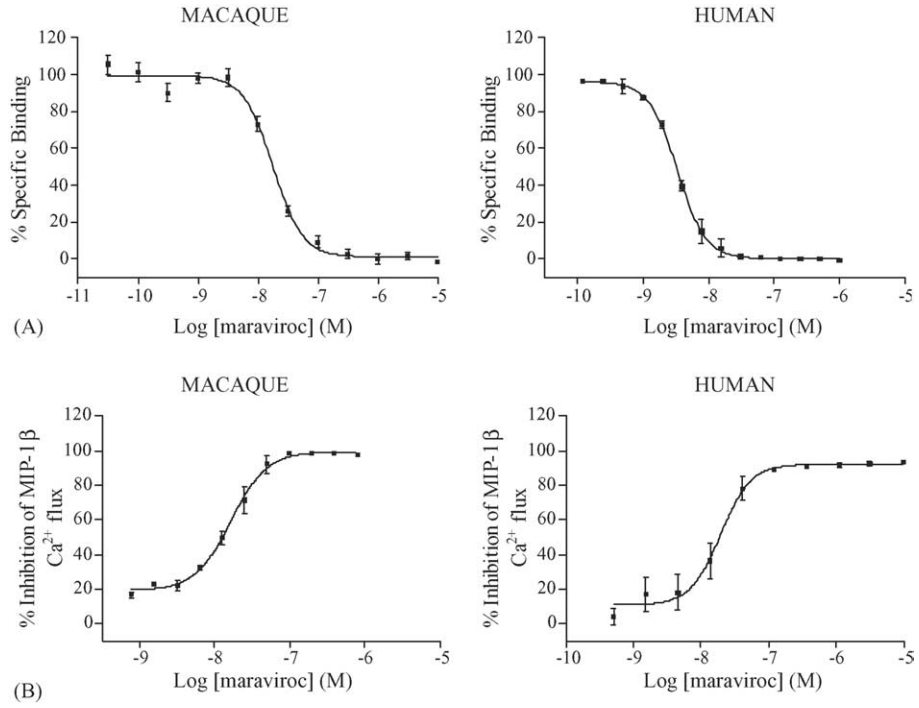

Fig. 6 – (A) Inhibition of [^{125}I]-MIP-1β binding to HEK-293 cell membranes expressing macaque and human CCR5 by maraviroc. (B) Inhibition of MIP-1β (20 nM) induced increases in intracellular calcium concentration in HEK-293 cells expressing macaque and human CCR5 by maraviroc. The data are expressed as the mean ± S.E.M. of *n* = 3–8 individual experiments.

binding reaching a plateau after 60 min (Fig. 7). However, although the association of [^{3}H]-maraviroc to macaque and human CCR5 appeared similar, dissociation of [^{3}H]-maraviroc from human CCR5 was significantly slower than from macaque CCR5. Dissociation of [^{3}H]-maraviroc from macaque CCR5 was complete after 8 h following addition of an excess of unlabelled maraviroc, while in contrast there was still more than 50% of added [^{3}H]-maraviroc bound to human CCR5. Even

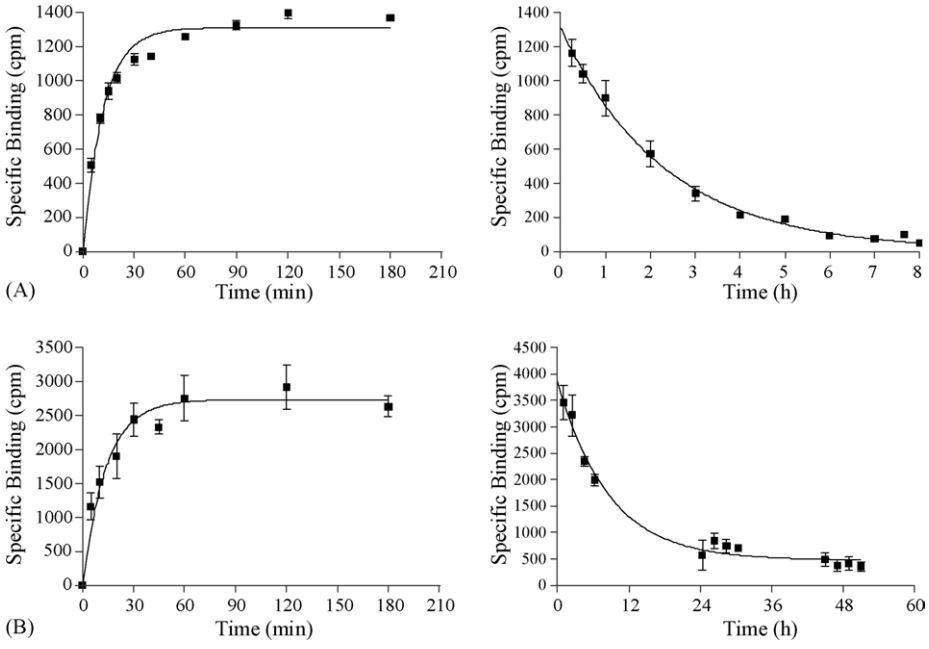

Fig. 7 – Binding kinetics of [^{3}H]-maraviroc to HEK-293 cell membranes expressing the macaque (A) and human (B) recombinant CCR5. Association time-course of 5 nM [^{3}H]-maraviroc binding to 5 μg membrane protein per well (*left*). Dissociation time-course of [^{3}H]-maraviroc (5 nM) initiated by 50 μM maraviroc (*right*). The data are expressed as mean ± S.E.M. of 3–6 individual experiments.

after 51 h there was still approximately 15% remaining. The K_d value estimated from k_{off}/k_{on} for macaque CCR5 was in reasonable (within 3-fold) agreement of that estimated from saturation analysis, but for human CCR5 was approximately 10-fold lower than that estimated from saturation analysis. This difference is almost certainly related to equilibrium conditions not being reached in saturation experiments due to the slow-offset of [^{3}H]-maraviroc from human CCR5. The K_d value estimated from k_{off}/k_{on} may actually be a more reliable estimate of the true affinity of maraviroc for the human receptor. The slow dissociation rate of maraviroc from the human CCR5 would also mean that it is highly unlikely that the plateau in the association time course shown in Fig. 7 represents establishment of equilibrium. Indeed, the mathematical model described by Motulsky and Mahan [34] predicts that the time take to reach equilibrium using a K_d concentration of radioligand, is 5 times the dissociation half-life. At concentrations $>K_d$ this is halved. Thus, an incubation time in excess of 35 h would be needed for [^{3}H]-maraviroc to reach equilibrium at human CCR5. It is also likely that this accounts for the steep slope factors observed in [^{125}I]-MIP-1β competition binding experiments with maraviroc.

4. Discussion

In this study, cDNA encoding the rhesus macaque chemokine CCR5 was isolated from PMK cells and expressed in HEK-293 cells as a stable cell line. Our data confirms previous reports that the rhesus macaque CCR5 sequence encodes 352 amino acids and shows close homology (98%) to the human CCR5 sequence [26–29]. As most chemokine receptors, including CCR5, are coupled to pertussis-sensitive heterotrimeric G proteins that signal through intracellular calcium mobilization and inositol-1,4,5-triphosphate formation [35] we used MIP-1β evoked increases in intracellular calcium to confirm functional activity. In addition, we confirmed that these responses were mediated through the macaque CCR5 by demonstrating that non-transfected parental HEK-293 cells were unresponsive to MIP-1β.

Having confirmed that our cell line expressing recombinant CCR5 was functionally active we then investigated if the direct binding properties of [^{125}I]-MIP-1β at macaque CCR5 were similar to human CCR5. Therefore, the equilibrium and kinetic binding properties of [^{125}I]-MIP-1β at human and macaque CCR5 were determined. These experiments demonstrated that human [^{125}I]-MIP-1β binds to macaque CCR5 with the same high affinity as human CCR5 and with similar kinetic constants. Overall, these data suggest that the binding site for MIP-1β is similar across these species and that differences in the amino acid sequences in the receptors do not appear to influence the binding affinity of MIP-1β.

Competition experiments were performed to compare the specificity of the recombinant macaque CCR5 for chemokines. Our data confirm that MIP-1α, MIP-1β and RANTES, had the highest affinity for macaque CCR5, consistent with the known pharmacology of human CCR5 [6,36,37]. Of notable exception was MCP-3, which is reported to bind to human CCR5 with greater affinity than our data indicates [36]. Our data show that HCC-1, MCP-1, MCP-2 and MCP-3 bind to macaque and human CCR5 with relatively moderate affinity and confirms the known promiscuous nature of chemokine receptors [6,36,37]. All other chemokines tested bound with lower affinity (<50% inhibition at 100 nM). Overall, the relative affinities of the chemokines tested for the macaque CCR5 were similar to those obtained with human CCR5.

The finding that the direct binding properties of MIP-1β at human and macaque CCR5 are similar is consistent with the known close homology between the receptor gene sequences [26–29] and the conserved nature of the chemokines expressed in human and primates. Rhesus macaque simian immunodeficiency virus (SIV) is often used as a model system for HIV-1 infection because macaque CCR5 supports entry of HIV-1 [26,38,39]. Furthermore, recombinant human RANTES, MIP-1α and MIP-1β are known to bind to macaque CCR5 and suppress simian immunodeficiency virus (SIV) replication [28,33]. Previous studies have shown that the second extracellular loop of human CCR5 is critical for high affinity binding and for signalling of MIP-1α, MIP-1β and RANTES [40]. Our data suggest that the single amino acid difference at position 171 (arginine in macaque, lysine in human) does not affect the affinity, specificity or kinetics of chemokine binding. Although amino acid differences between macaque and human CCR5 do not appear to drastically affect the affinity of chemokines for these receptors, the same may not hold true for small molecule CCR5 antagonists. Indeed, a single amino acid difference (isoleucine at position 198 in human, methionine at the same position in macaque) has been shown to determine the sensitivity of macaque CCR5 to the antiviral activity of the small molecule inhibitor SCH-C [29]. Given that sequence differences between macaque and humans may be important for small molecule inhibitors we wanted to verify if the similar binding properties of human and macaque CCR5 observed with chemokines would also hold for maraviroc. As non-clinical testing of maraviroc revealed that it has low affinity for CCR5 expressed in the standard toxicological species; mouse, rat and dog, we set out to identify an alternative species that could be used to evaluate mechanism-related safety and toxicology of the compound. To validate macaque as a relevant species we needed to demonstrate that the pharmacological profile of macaque CCR5 is similar to that of human CCR5. In competition binding experiments at the macaque CCR5 our data confirmed that maraviroc has similar low nanomolar binding affinity to that observed with the human CCR5. Furthermore, as with the human receptor, the binding affinity at the macaque CCR5translates into potent functional antagonism as demonstrated by dose-dependent inhibition of MIP-1β-induced intracellular calcium release.

Direct binding studies using [^{3}H]-maraviroc confirmed that the compound has high affinity for macaque and human CCR5. However, when the binding kinetics of [^{3}H]-maraviroc at macaque and human CCR5 were compared, significant differences in the dissociation rate emerged. The dissociation half-life of maraviroc from human CCR5 was approximately 10-fold longer than from macaque. This slow rate of dissociation of maraviroc from human CCR5 confirms the findings of Watson et al. [41] who reported that the dissociation rate of maraviroc and other CCR5 antagonists, including TAK779, Sch-C, Sch-D and GW873140 (aplaviroc) from human CCR5 is significantly slower than MIP-1α. In the

Watson study, an indirect measure of maraviroc dissociation was made by following the association of [^{125}I]-MIP-1α at 4 °C to CCR5 after pre-treatment with maraviroc. These methodological differences, in particular, assay temperature, as kinetics are temperature dependent [32], may explain the longer dissociation half-life for maraviroc (>136 h) reported by Watson. Despite the differences in absolute values, these data indicate that a slow dissociation from human CCR5 is not a unique property of maraviroc, and is common to other CCR5 antagonists. With such a slow rate of dissociation from human CCR5, mass action principles do not apply, i.e. the rate of on-set does not ever equal the rate of off-set. Thus, with increasing time and as long as there are available receptors, maraviroc will continue to associate until either ligand is depleted or receptors are saturated. Such properties could beneficial for antiviral drugs. The finding that the dissociation of MIP-1β from macaque and human CCR5 were similar but that maraviroc dissociation was 10 times slower from human CCR5, suggests that while differences in amino acid sequence between macaque and human do not affect endogenous chemokine binding they may affect the binding of small molecule antagonists. A consequence of these data is that in vivo the duration of action of maraviroc at the macaque CCR5 receptor may not be as sustained as in human. Therefore, receptor occupancy would need to be sustained through adequate plasma concentrations of drug.

Overall, these data confirm that the direct binding properties of [^{125}I]-MIP-1β at macaque CCR5 are similar to the human receptor. Compared to CCR5 from human, maraviroc was shown to dissociate more quickly from macaque however, as with the human receptor, maraviroc was shown to be a high affinity, potent functional antagonist of macaque CCR5 thereby indicating that the macaque should be a suitable species in which to evaluate mechanism related toxicology of CCR5 antagonists like maraviroc.

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