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Pharmacological profile of JNJ-27141491, a non-competitive and orally active antagonist of the human chemokine receptor CCR2

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a) Running title

Identification of JNJ-27141491, a novel CCR2 antagonist

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d) Abbreviations

hCCR2: human CC chemokine receptor 2; MCP-1: monocyte chemoattractant protein-1, BAL: bronchoalveolar lavage; PBMC: peripheral blood mononuclear cells; *i.t.*: intratracheal; *b.i.d.*: bis in die*; q.d.*: quaque die.

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ABSTRACT

The interaction between CC chemokine receptor 2 (CCR2) with monocyte chemoattractant proteins, such as MCP-1, regulates the activation and recruitment of inflammatory leukocytes. In this study, we characterized JNJ-27141491, a thioimidazole derivative, as a non-competitive and orally active functional antagonist of human (h)CCR2. JNJ-27141491 strongly suppressed hCCR2-mediated *in vitro* functions, such as MCP-1-induced [^{35}S]GTPγS binding, MCP-1, -3 and -4-induced Ca^{2+} mobilization and leukocyte chemotaxis towards MCP-1 (IC_{50} = 7-97 nM), while it had little or no effect on the function of other chemokine receptors tested. The inhibition of CCR2 function was both insurmountable and reversible, consistent with a non-competitive mode of action. JNJ-27141491 blocked the binding of [^{125}I]MCP-1 to human monocytes (IC_{50} = 0.4 μM), but failed to affect MCP-1 binding to mouse, rat and dog cells (IC_{50} > 10 μM). Therefore, transgenic mice, in which the mouse (m)CCR2 gene was replaced by the human counterpart, were generated for *in vivo* testing. In these mice, oral administration of JNJ-27141491 dose-dependently (5-40 mg/kg, *q.d.* or *b.i.d.*) inhibited monocyte and neutrophil recruitment to the alveolar space 48h after intratracheal mMCP-1/LPS instillation. Furthermore, treatment with JNJ-27141491 (20 mg/kg, *q.d.*) significantly delayed the onset and temporarily reduced neurological signs in an experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis. Taken together, these results identify JNJ-27141491 as a non-competitive, functional antagonist of hCCR2, capable of exerting oral anti-inflammatory activity in transgenic hCCR2-expressing mice.

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INTRODUCTION

CCR2 is a G-protein coupled chemokine receptor, expressed on monocytes, macrophages, basophils, dendritic cells, natural killer cells and activated T lymphocytes, that mediates the activation and movement of responsive leukocytes along a chemotactic gradient. CCR2 has two isoforms (CCR2A and CCR2B) that are generated by alternative splicing and differ only in their carboxy-terminal tail. Leukocytes predominantly express the longer CCR2B variant (Tanaka et al., 2002). CCR2B (henceforth CCR2) is activated by several members of the CC-chemokine subfamily, comprising monocyte chemoattractant protein-1 (MCP-1/CCL2), MCP-2/CCL8, MCP-3/CCL7, MCP-4/CCL13 and MCP-5/CCL16. MCP-1 exclusively interacts with CCR2 and is therefore recognized as the prime CCR2 agonist. Engagement of CCR2 inhibits adenylyl cyclase, promotes intracellular calcium mobilization, stimulates mitogen-activated protein kinase and phosphatidylinositol 3-kinase pathways, and promotes chemotaxis (Jimenez-Sainz et al., 2003). Recent evidence suggests that CCR2 is critical for monocyte egress from the bone marrow (Tsou et al., 2007), though CCR2 has mostly been implicated in the pathophysiology of a wide range of both acute and chronic inflammatory conditions characterized by mononuclear cell infiltration, such as rheumatoid arthritis (RA), multiple sclerosis (MS), atherosclerosis, uveitis, asthma, chronic obstructive pulmonary disease, psoriasis, diabetes, inflammatory bowel disease and transplant rejection (Dawson et al., 2003, Belvisi et al., 2004, Feria and Diaz-Gonzalez, 2006). Evidence for CCR2 involvement in these diseases comes from clinical studies showing elevated expression of MCP-1 correlating with leukocyte infiltration (Ellingsen et al., 2001, Mahad and Ransohoff, 2003, Schober and Zernecke, 2007, Rose et al., 2003). Moreover, genetic deletion of either CCR2 or MCP-1, showed a selective defect in migration of macrophages to sites of inflammation (Kurihara et al., 1997, Lu et al., 1998) and protection in inflammatory disease models such as experimental autoimmune encephalomyelitis (EAE), (Fife et al., 2000, Izikson et al., 2000, Huang et al., 2001) and atherosclerosis (Boring et al., 1998, Gosling et

al., 1999). Furthermore, a reduction of disease in models of arthritis, MS and asthma was demonstrated after treatment with anti-MCP-1 antibodies (Ogata et al., 1997, Kennedy et al., 1998), an anti-CCR2 antibody (Mellado et al., 2007) or low molecular weight CCR2 antagonists (Brodmerkel et al., 2005, Higgins et al., 2007, Xia et al., 2007). These observations led to the hypothesis that CCR2 could represent a potentially attractive disease target.

By functional screening in a guanosine 5'-O-(γ-thio)triphosphate ([^{35}S]GTPγS) binding assay using hCCR2-expressing CHO cells, we hit upon a series of thioimidazole derivatives with micromolar CCR2 antagonistic potency. Subsequent targeted chemical synthesis led to compound JNJ-27141491 or (S)-3-[3,4-difluoro-phenyl)-propyl]-5-isoxazol-5-yl-2-thioxo-2,3-dihydro-1H-imidazole-4-carboxyl acid methyl ester (Fig. 1) (Van Lommen et al., 2005, Doyon et al., 2008). The present paper characterizes this compound as a potent, selective and non-competitive antagonist of the hCCR2. Oral activity is demonstrated in transgenic mCCR2 knock-out/hCCR2 knock-in mice, using a model measuring monocyte and neutrophil recruitment to the alveolar space after intratracheal (*i.t.*) MCP-1/LPS instillation and in the mouse EAE model of multiple sclerosis.

METHODS

Animals

C57Bl/6 mice and Lewis rats were obtained from Charles River (Sulzfeld, Germany). Beagle dogs were obtained from CEDS (Mézilles, France). mCCR2 knock-out/hCCR2 knock-in mice were generated as described (Supplemental Data) in collaboration with Lexicon Pharmaceuticals Incorporated (USA) using targeted 129Sv/Evbrd (LEX1) embryonic stem cell clones injected into C57Bl/6 mice (Supplemental Figure A). Expression of the hCCR2B transcript was confirmed by quantitative RT-PCR performed on spleen, kidney, thymus and lung total RNA from heterozygous and homozygous hCCR2B knock-in mice (Supplemental Figure B). Backcrossing into C57Bl/6 genetic background continued to the 8th generation. Transgenic mice were maintained in an SPF facility. Other mice strains, rats and dogs were housed in facilities that meet all Belgian and European requirements for animal care. Experimental procedures were carried out in accordance with the European Communities Council Directives (86/609/EEC) and were approved by the institute's animal care and use committee.

Cells

Human CCR2B-transfected CHO (hCCR2-CHO) cells (purchased from Euroscreen) were cultured in DMEM-NutF12 medium; human monocytic THP-1 cells (ATCC TIB-202) in RPMI-1640 medium and murine monocytic WEHI-274 cells (ATCC CRL-1679) in DMEM medium supplemented with 4.5 g/l glucose, 50 µM 2-mercaptoethanol, 1% Na-pyruvate. To all media, 10% fetal bovine serum (FBS), 1% *L*-glutamine and 1% penicillin/streptomycin was added (all from Invitrogen). Peripheral blood mononuclear cells (PBMC) from heparinized blood were isolated using Ficoll-Paque gradient centrifugation (GE Healthcare Bio-Sciences). Human monocytes were further separated using the Dynal® monocyte negative isolation kit (Invitrogen).

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Chemicals

JNJ-27141491 was synthesized at the medicinal chemistry department of J&J PRD (Beerse, Belgium) according to the methods described by Doyon et al. (2008). Reference compounds were synthesized as described; INCB3344 (WO2004050024, Brodmerkel et al, 2005), compound 7a (Xia et al., 2007) and UCB-102405 (Dasse et al., 2007). For *in vitro* experiments, compounds were dissolved at 5 mM in dimethyl sulfoxide (DMSO) and appropriately diluted in the indicated buffers with a final DMSO concentration of 1% in [^{35}S]GTPγS binding, calcium and binding assays and 0.2% in chemotaxis assays. For *in vivo* experiments, JNJ-27141491 was suspended in a vehicle solution consisting of DMSO:cremophor:0.9% NaCl saline (1:1:8) (Sigma) and orally administered to mice once or twice daily in a volume of 100 µl/10 g of body weight.

[^{35}S]GTPγS binding assay

To prepare a stock of membranes, hCCR2-CHO cells were centrifuged (10 min, 16.000 rpm) and pellets were resuspended in cell homogenization buffer A (15 mM Tris-HCl - pH 7.5, 2 mM $MgCl_2$, 0.3 mM EDTA, 1 mM EGTA). After homogenization, membranes were pelleted (25 min, 22.000 rpm, 4°C) and again resuspended in buffer A, homogenized and centrifuged (25 min, 22.000 rpm). Finally, the membrane pellet was resuspended in buffer B (7.5 mM Tris-HCl - pH 7.5, 12.5 mM $MgCl_2$, 0.3 mM EDTA, 1 mM EGTA, 250 mM sucrose), homogenized, aliquoted at 1700 µg/ml and stored at –70ºC until analysis. Membranes were diluted in incubation buffer (20 mM HEPES, 100 mM NaCl, 10 µM GDP, 1 mM $MgCl_2$, pH 7.4) supplemented with 14.3 µg/ml saponin, and pre-incubated with compound for 30 min at 30ºC in 96-well plates (10 µg protein/well). Then, MCP-1 (100 nM) was added for 30 min. Finally, the mixture was incubated for another 30 min with [^{35}S]GTPγS (~1100 Ci/mmol, 2.5 nM, Amersham), before membranes were harvested on GF/B filter plates (Perkin Elmer) using a Packard Filtermate harvester. The plates were counted in a Topcount NXT (Packard) after loading with Microscint 0 (Packard).

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Calcium mobilization assays

Cells were fluorescently labelled with Fluo-3AM (Invitrogen) as described in Van Lommen et al. (2005), and pre-incubated with compound for 20 min before adding recombinant human MCP-1, MCP-2, MCP-3, MCP-4 or murine (m)MCP-1/JE (R&D Systems). Cells were pre-incubated for 20 min in 1% DMSO when compound and agonist were added simultaneously and when compound was added alone. Changes in intracellular free [Ca^{2+}] were measured using the Fluorescent Imaging Plate Reader (Molecular Devices) or FDSS6000 (Hamamatsu).

Functional testing of chemokine receptor selectivity was performed at Euroscreen using their aequorin-based calcium mobilization method (AequoScreen™, www.euroscreen.com)

Chemotaxis

Chemotaxis assays were performed using Calcein-labeled human PBMC in disposable 96-well chemotaxis chambers (ChemoTx, NeuroProbe), as described in Van Lommen et al. (2005). PBMC were pre-incubated for 10 min at room temperature with or without compound, before adding 20 µl cell suspension (10.000 cells) in triplicate to the topside of the filter, of which the bottom wells were filled with buffer or 30 ng/ml MCP-1. After 2h at 37°C, non-migrated cells were removed from the top of the filter by gently wiping and migrated cells were measured using a fluorescent plate reader (λ_{ex}=485 nm; λ_{em}=538 nm).

Binding assays

In competition binding assays, cells were dissolved in binding buffer consisting of 25 mM HEPES, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1% NaN_3 and 0.5% protease-free bovine serum albumin (BSA) (pH 7.4). After pre-incubating the cells ($3.5x10^5$ cells/well) with compound for 30 min at 25°C, [^{125}I]hMCP-1 (0.08-0.21 nM) or [^{125}I]mMCP-1/JE (0.6-0.7 nM) (Perkin Elmer) was added for 60 min. The assay was terminated by filtration of the reaction mixture through GF/B filter plates (presoaked in 0.5% polyethyleneimine) using a Packard cell

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harvester. The filter plates were washed with binding buffer supplemented with 500 mM NaCl. Filter bound radioactivity was determined by liquid scintillation counting on a TopCount NXT™ (Packard).

Plasma and tissue kinetics in C57Bl/6 and transgenic CCR2 mice

JNJ-27141491 was orally dosed by gavage at a final dose of 20 mg/kg. At 0.5, 1, 2, 4, 8 and 24h after administration, animals (n=3 per time point) were sacrificed by decapitation and blood was collected by exsanguination into K_3EDTA Vacutainer tubes (BD Biosciences). Plasma was obtained following centrifugation at 4°C for 10 min at 1900xg. Plasma samples were analyzed using a LC-MS/MS method. Chromatography was performed using a Hypersyl Keystone C18 column (50 x 4.6 mm, 3 μm) while the mass detector was an API4000 (Applied Biosystems). The MRM transition 378→223 Da in negative mode was used. The lower limit of quantification was 1-10 ng/ml for plasma. A limited non-compartmental pharmacokinetic analysis was used for all oral data using WinNonLin™ (version 3.3, Pharsight Ltd).

MCP-1-induced monocyte recruitment to the alveolar space of mice

Resident alveolar macrophages (rAM) were fluorescently labeled by injecting 200 μl PKH26-PCL dilution (50 μM, Sigma) in the tail vein. After 24h, mice were orally treated with vehicle or JNJ-27141491 (5-40 mg/kg). 1h later, mice were intraperitoneally anesthetized with 100 mg/kg ketamine hydrochloride and 5 mg/kg xylazine hydrochloride and placed on a holder with the upper incisors hooked onto an elastic band to facilitate cannulation of the trachea with a blunted, 20-gauge gavage needle. Then, a 50 μl aliquot containing PBS, 10 ng *E.coli* LPS, 20 μg recombinant mMCP-1/JE or 20 μg recombinant mMCP-1/JE + 10 ng *E.coli* LPS, all containing 0.2% BSA was instilled directly into the trachea via a catheter in the gavage needle. This method required 2-5 min to treat each mouse. Mice were allowed to recover from anesthesia and returned to their cages with free access to food and water.

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Mice were orally treated once or twice daily with vehicle or JNJ-27141491. After 48h, mice were euthanized by cervical dislocation under isoflurane anesthesia and the thoracic cavity was opened by midline incision. Whole bronchoalveolar lavage (BAL) was performed using 15 ml ice-cold PBS containing 3 mM EDTA. Total cell counts in the BAL fluid were performed using a cell counter (Cytoron, Orthodiagnostics) and cell differentiation was performed using flow cytometry and cytospin analysis after cell sorting and staining with DiffQuik.

Flow cytometry

After BAL, isolated cells were centrifuged for 5 min at 1.400 rpm at 4°C and resuspended in PBS containing 5% FBS and 0.02% NaN_3. Cells were blocked with anti-CD16/CD32 and stained with anti-CD14 or anti-Ly6G/6C FITC-conjugated antibodies (BD Biosciences) or left unstained before analysis on a FACS Calibur. rAM, monocytes and neutrophils were gated based on their forward scatter (FSC) or FL1 *versus* FL2 (PKH-26) characteristics.

EAE induction and clinical evaluation

Female hCCR2 knock-in mice (9–11 weeks old) were immunized with 100 µg myelin oligodendrocyte glycoprotein peptide, $MOG_{35–55}$ (MEVGWYRSPFSRVVHLYRNGK) (Eurogentec, Seraing, Belgium), in 100 µl saline emulsified in an equal volume of CFA that had been supplemented with 4 mg/ml *Mycobacterium tuberculosis* (strain H37 RA; DIFCO™) (2x50 µl distributed over two sites of the back). At the time of immunization and 48h later, the mice received an intraperitoneal injection of 100 µl saline containing 200 ng *Bordetella pertussis* toxin (Sigma). The animals were observed daily and disease severity was scored from 0 = no overt signs of disease, 1 = hind limp weakness, 2 = unilateral hind limb paralysis, 3 = bilateral complete hind limb paralysis, 4 = total paralysis to 5 = moribund state or death, using 0.5 point for intermediate clinical findings. The onset of EAE was defined as the day an animal showed a clinical score ≥ 0.5.

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Statistics

For [^{35}]GTPγS, calcium, chemotaxis and radioligand binding experiments, IC_{50} values were calculated using non-linear regression in GraphPad Prism. Results of cell recruitment after *i.t.* mMCP-1 installation and mean day of EAE onset were analyzed using Student t-test statistic. Clinical EAE disease scores were analyzed using Wilcoxon-Mann-Whitney test statistics.

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RESULTS

JNJ-27141491 inhibits hCCR2 activation *in vitro*

Nanomolar concentrations of JNJ-27141491 inhibited MCP-1-induced [^{35}S]GTPγS binding to hCCR2-CHO cell membranes (IC_{50} = 38 $\pm$ 9 nM, Fig. 2A) and reduced MCP-1-induced Ca^{2+} mobilization in hCCR2-CHO cells (IC_{50} = 13 $\pm$ 1 nM), in THP-1 cells (IC_{50} = 13 $\pm$ 2 nM) and in human blood monocytes (43 $\pm$ 4 nM) (Fig. 2B-D). For comparison, in the MCP-1-induced Ca^{2+} mobilization assay in THP-1 cells, the known experimental CCR2 antagonists INCB3344 (Brodmerkel et al, 2005), compound 7a (Xia et al., 2007) and UCB-102405 (Higgins et al., 2007) suppressed the reaction with IC_{50} values of 13, 13 and 0.8 nM, respectively. JNJ-27141491 also inhibited chemotaxis of human PBMC towards MCP-1 with an IC_{50} of 97 $\pm$ 16 nM (Fig. 2E). To assess the antagonistic effect of JNJ-27141491 on other CCR2 agonists, its activity was determined on Ca^{2+} mobilization in hCCR2-CHO cells stimulated by MCP-3 and MCP-4, and in THP-1 cells stimulated with murine MCP-1. As shown in Fig. 2B and C, JNJ-27141491 reduced Ca^{2+} influx with comparable potency against all agonists (IC_{50} = 7 $\pm$ 1, 7 $\pm$ 2 and 20 $\pm$ 4 nM respectively). Incubation of JNJ-27141491 alone (1 μM) with THP-1 cells or human monocytes did not induce Ca^{2+} mobilization, indicating that the compound does not exert intrinsic agonist activity.

JNJ-27141491 competes with the binding of MCP-1 to hCCR2

In competition binding experiments, JNJ-27141491 blocked the binding of [^{125}I]MCP-1 to hCCR2-CHO cells, THP-1 cells and human PBMC with IC_{50} values of 95 $\pm$ 9 nM, 390 $\pm$ 110 μM and 380 $\pm$ 80 μM respectively (Fig. 2F).

JNJ-27141491 acts as a non-competitive antagonist of the CCR2 receptor

To examine the marked potency difference between the compound's functional and binding antagonism of CCR2, we performed Schild analysis in MCP-1-induced Ca^{2+} mobilization

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experiments in THP-1 cells. The MCP-1 dose response curves shown in Fig. 3, measured after 20 min pre-incubation with 0, 3, 9 or 15 nM JNJ-27141491 indicate that JNJ-27141491 acts as an insurmountable antagonist of CCR2. Indeed, the maximal response (E_{max}) to MCP-1 in the presence of 3, 9 or 15 nM JNJ-27141491 was reduced to 98, 81 and 57% respectively of the E_{max} measured in the absence of compound, whereas MCP-1 potency was only slightly affected (EC_{50} values of 4, 8, 14 and 14 nM respectively). The decline was near to complete after pre-incubation with 50 nM JNJ-27141491 (data not shown). To find out whether an insurmountable antagonist is competitive or not, it is essential for the receptors to be exposed to the antagonist and agonist simultaneously. A decrease of the maximal agonist-evoked response is only to be expected in the case of non-competitive antagonism (Vauquelin et al., 2002). As shown in Fig. 4, the E_{max} values of the MCP-1 dose response curves were reduced to 99, 92, 73 and 44% after co-incubation with 0.5, 1, 3 or 10 μM JNJ-27141491 (albeit >100-fold higher than in the pre-incubation experiments), whereas MCP-1 potency was only slightly affected; EC_{50} values were 6, 8, 12 and 23 nM respectively (4 nM without compound). Furthermore, the observation that the effect of JNJ-27141491 was readily lost after cell washings, supported the reversible character of CCR2 antagonism (data not shown). In addition, IC_{50} values determined in pre-incubation experiments in the presence of different stimulatory quantities of MCP-1 (EC_{70}, EC_{80}, EC_{90} and EC_{95}) showed similar inhibitory potency of JNJ-27141491 (IC_{50} = 8-28 nM). Therefore, these data indicate that compound JNJ-27141491 is a reversible, non-competitive antagonist of hCCR2.

JNJ-27141491 shows high receptor selectivity

Functional selectivity of JNJ-27141491 was evaluated by measuring its effect on intracellular Ca^{2+} mobilization in chemokine-activated human CCR1-8 and CXCR1-3 expressing-CHO cells. Besides strong inhibition of MCP-1 and MCP-3-induced hCCR2 activation (IC_{50} = 40 and 50 nM, respectively), JNJ-27141491 produced a moderate activity

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on MIP-1α-activated CCR1 (IC_{50} = 0.6 μM). There was no effect ($IC_{50} \geq 5$ μM) on Ca^{2+} mobilization induced via CCR3, CCR4, CCR5, CCR6, CCR7, CCR8, CXCR1/2 and CXCR3 by eotaxin, MDC, RANTES, MIP-3α, MIP-1β, I-309, IL-8 and I-TAC respectively (Table 1).

CCR2 antagonism is restricted to the human CCR2

To determine the species selectivity of JNJ-27141491, [^{125}I]MCP-1 competition binding assays were performed using human, dog and rat PBMC and murine WEHI cells. As shown in Fig. 5, JNJ-27141491 inhibited the binding of [^{125}I]MCP-1 to human PBMC (IC_{50} = 0.38 μM), but it produced no blocking effect on binding of MCP-1 to murine WEHI cells, rat or dog PBMC when tested up to 10 μM. These data indicate that the antagonism of JNJ-27141491 is restricted to hCCR2. Therefore, *in vivo* studies were done in transgenic mice in which the mCCR2 gene was knocked out and replaced by the human orthologous gene (Supplemental Data).

Pharmacokinetics in transgenic hCCR2 knock-in mice

Oral exposure of JNJ-27141491 in the hCCR2 knock-in mice was determined at different time points after the mice were orally administered with 20 mg/kg compound. The maximum plasma concentration (C_{max} = 2.5 μM, n=3) was already observed at 30 min post dose (T_{max}), indicating a fast absorption of the compound from the intestinal track. $T_{1/2}$ and $AUC_{0\text{-}inf}$ values were respectively 2h and 1240 ng·h/ml. At 8h post dose, the plasma concentration was 0.04 μM (n=3), whereas after 24h all individual levels were below the limit of quantification. Based on these pharmacokinetic data, mice were treated *q.d.* and/or *b.i.d.* in further *in vivo* experiments. In addition, oral exposure in transgenic mice was comparable with data obtained in wild type C57Bl/6 mice (data not shown).

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JNJ-27141491 inhibits recruitment of monocytes and neutrophils into the alveolar air space of transgenic hCCR2 knock-in mice.

The *in vivo* activity of JNJ-27141491 was tested in a mouse model that permits the identification and quantification of resident alveolar macrophages (rAM) and recruited monocytes and neutrophils in the lungs after *i.t.* instillation of PBS; 10 ng LPS or 20 µg mMCP-1 in either absence or presence of LPS (Maus et al., 2002a). No cell influx was observed after *i.t.* instillation of PBS; only resident rAM were detected in the BAL fluid. Bronchoalveolar deposition of mMCP-1 alone provoked a monocyte influx, which peaked at 48h and LPS alone provoked sole neutrophil influx that peaked at 12h (data not shown). Upon alveolar co-challenge with mMCP-1 and a low dose of LPS, the magnitude of the neutrophil influx (early phase) and monocyte influx (later phase) into the alveolar compartment was strongly increased as compared to the respective mono-stimuli. Using flow cytometry, recruited monocytes and neutrophils could easily be discriminated from rAM in the BAL fluid, as the rAM strongly accumulated the red fluorescent dye PKH26. The gating of cell populations based on FL1 or FSC *versus* FL2 (PKH26) properties as described by Maus et al. (2001), was confirmed by positive staining of monocytes for CD14 and neutrophils for Ly6G/6C and cytospin analysis (data not shown). Fig. 6 shows representative dot plots of BAL cells from a vehicle-treated (A) and a JNJ-27141491-treated mouse (20 mg/kg *b.i.d.*) (B), 48h after mMCP-1/LPS instillation. The BAL fluid of vehicle-treated mice contained 792 $\pm$ 47 x10^3 cells (mean $\pm$ S.E.M., n=16); consisting of 23 $\pm$ 5% monocytes, 21 $\pm$ 7% neutrophils and 56 $\pm$ 11% rAM (mean $\pm$ stdev), whereas the total number was significantly reduced to 636 $\pm$ 34 x10^3 (n=5) after *b.i.d.* JNJ-27141491 treatment (20 mg/kg) ($p<0.05$). This reduction was solely due to reduced numbers of recruited monocytes and neutrophils, comprising respectively 7 $\pm$ 1% and 14 $\pm$ 4% of the BAL cells (both $p<0.05$), with no significant effects on the total number of rAM. Fig. 7 demonstrates that the effect of JNJ-2714149 treatment on the number of infiltrated monocytes and neutrophils was dose-dependent. Once daily oral treatment with 40, 20, 10

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or 5 mg/kg inhibited the monocyte influx with 77, 57, 49 and 27% respectively as compared to vehicle-treatment, while this was 74 and 22% after twice daily oral treatment with 20 or 5 mg/kg (n=5-16 mice per group). The neutrophil influx was also reduced; neutrophil numbers were decreased with 56, 45, 20 and 8% after 40, 20, 10 or 5 mg/kg *q.d.* treatments and with 45 and 20% after 20 and 5 mg/kg *b.i.d.* treatments (Fig. 7). The amount of rAM did not significantly differ in any of the tested groups (data not shown). As expected, treatment of wild type C57Bl/6 mice with 20 mg/kg JNJ-27141491 (*b.i.d.*) had no inhibitory effect on mMCP-1/LPS-induced monocyte nor neutrophil pulmonary influx (data not shown).

JNJ-27141491 delays EAE in transgenic CCR2 mice

Fig. 8 shows a representative MOG_{35-55}-induced EAE experiment in hCCR2 knock-in mice. One group of mice (n=8) were orally treated with JNJ-27141491 (20 mg/kg) once daily from day 0 till day 16, while the control animals (n=8) received vehicle at the same time points. Fig. 8A shows that oral treatment with JNJ-27141491 significantly ($p<0.05$) reduced the clinical disease scores on day 7 (from 0.6 ± 0.2 to 0.1 ± 0.1), day 8 (from 0.8 ± 0.2 to 0.3 ± 0.2), day 9 (from 1.3 ± 0.3 to 0.4 ± 0.2) and on day 10 (from 1.3 ± 0.3 to 0.6 ± 0.2). Furthermore, JNJ-27141491 treatment significantly delayed the start of clinical signs of disease (mean day of onset 9.6 ± 0.7 in the compound-treated group *versus* 7.5 ± 0.7 in the vehicle-treated group, $p<0.05$) with lower disease grades (Fig. 8B). However, compound treatment failed to significantly attenuate clinical disease day 11 onward, resulting in a mean clinical score of 1.9 ± 0.3 in the treated group *versus* 2.3 ± 0.3 in the control group on day 16.

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DISCUSSION

Numerous studies support the important role of the interaction between MCP-1 and CCR2 in the pathophysiology of a wide range of inflammatory conditions, which has put forward CCR2 as a potential therapeutic target. In this study, we characterized JNJ-27141491 as a potent, selective and non-competitive antagonist of hCCR2 without intrinsic agonist activity, as determined by its inability to induce Ca^{2+} mobilization. JNJ-27141491 inhibited hCCR2 function *in vitro* with IC_{50} values of 7-97 nM as measured in [^{35}S]GTPγS binding assays, Ca^{2+} mobilization assays and chemotaxis assays using either hCCR2-CHO cells, THP-1 cells or human PBMC. The compound showed high selectivity against 9 other chemokine receptors tested (5 μM), with minimal functional activity on the MIP1-α/CCR1 interaction (IC_{50}=0.6 μM). Markedly higher compound concentrations were required for inhibiting [^{125}I]MCP-1 binding to hCCR2 (IC_{50}=0.38-0.40 μM). These findings are reminiscent of the non-competitive CCR1/CCR3 antagonist UCB-35625 and CXCR1 antagonist Repertaxin. Both compounds inhibit chemokine receptor function with low effect on binding (Sabroe et al., 2000, Bertini et al., 2004). Our results, showing that JNJ-27141491 behaves as a reversible inhibitor of hCCR2 and as an insurmountable antagonist of hCCR2 in Schild analyses in both pre- and co-incubation settings (Vauquelin et al., 2002), suggest that JNJ-27141491 is a non-competitive CCR2 antagonist. Insurmountable antagonists exhibiting adequate receptor selectivity, may have great potential as therapeutic drugs, since they exert their blocking action irrespective of the concentration of the natural agonist. In addition, in terms of potency, our compound proved to be comparable to that of other reported CCR2 antagonists such as INCB3344 (Brodmerkel et al, 2005), compound 7a (Xia et al., 2007) and UCB-102405 (Higgins et al., 2007), which suppressed MCP-1 induced Ca^{2+} mobilization in THP-1 cells with IC_{50} values of 13, 13 and 0.8 nM, respectively.

Despite good activity towards hCCR2, the compound had no effect on the binding of [^{125}I]MCP-1 to murine, rat or dog leukocytes (IC_{50}>10 μM). *In vivo* experiments were

therefore carried out using transgenic mice in which the mCCR2 gene was replaced by the human counterpart. PK analysis after oral dosing with 20 mg/kg showed good absorption and bioavailability, with no differences as compared to wild type C57Bl/6 mice.

In these transgenic animals, once or twice daily oral treatment with JNJ-27141491 dose-dependently (5-40 mg/kg) inhibited the recruitment of monocytes and neutrophils into the alveolar space, 48h after *i.t.* mMCP-1/LPS instillation. In this pharmacodynamic model, mice were co-challenged with mMCP-1 and a low dose of LPS, as this increased the magnitude of the neutrophil influx (early phase) and the monocyte influx (later phase) largely as compared to the respective mono-stimuli. Although both mMCP-1 and hMCP-1 induced neutrophil and monocyte recruitment in the presence of LPS in the transgenic CCR2 mice, we choose mMCP-1 for testing JNJ-27141491 at different doses, because it was considered more compatible with the murine system. The mechanism underlying the synergism of MCP-1 and LPS in eliciting inflammatory lung events could be explained in different ways. Alveolar deposited or locally liberated MCP-1 might directly pass the epi-/endothelial barrier to bind to and chemoattract circulating CCR2-expressing monocytes from the vascular to the alveolar compartment. Alternatively, rAM located at the air-tissue interface of the lung are strategically positioned to respond to the local appearance of microbial or inflammatory agents. As rAM constitutively express CCR2, they might be directly involved in the MCP-1-driven monocyte recruitment process. *In vitro* studies have shown that alveolar macrophage-derived products like TNF-α can activate alveolar epithelial cells to release chemokines such as IL-8 and MCP-1, which may then promote both alveolar neutrophil and monocyte recruitment. Furthermore, alveolar-recruited and activated neutrophils are well known to be capable of releasing MCP-1 in the alveolar air space, implying a potential role of co-recruited neutrophils in the alveolar monocyte traffic (Maus et al., 2002b). Using anti-CCR2 antibodies and $CCR2^{-/-}$ mice, Maus et al. (2002a) have demonstrated that CCR2 is necessary not only for the monocyte influx after *i.t.* mMCP-1/LPS instillation, but also for the earlier accumulation of neutrophils. Blocking

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CCR2 with a small molecule CCR2 antagonist confirmed these findings, as JNJ-27141491 also inhibited both the influx of monocytes and neutrophils in this model.

The efficacy of JNJ-27141491 was also tested in an animal model of chronic inflammation. EAE, the animal model that closely resembles MS (Owens and Sriram, 1995), can be induced by immunization with central nervous system (CNS)-specific antigens, such as MOG_{35-55} peptide, and is characterized by leukocyte infiltration, demyelination and paralysis. A clear role for MCP-1 and CCR2 in monocyte trafficking to the CNS and in the pathogenesis of EAE was established by studies of Fife et al. (2000), Izikson et al. (2000) and Huang et al. (2001). When EAE was induced in the hCCR2 knock-in mice, daily oral treatment with 20 mg/kg JNJ-27141491 significantly delayed the day of onset of neurological signs and temporarily reduced the clinical scores. However, from day 11 post immunization, all mice showed signs of EAE, with no significant difference in clinical grade in control and compound-treated groups. These data might be in agreement with a study of Gaupp et al. (2003), demonstrating that mice with CCR2 deletions remain susceptible to EAE. In contrast to the studies of Fife et al. (2000) and Izikson et al. (2000), these $CCR2^{-/-}$ mice showed a modified EAE disease course, but CNS lesions in which the process of demyelination was not affected. The authors suggested that the lack of CCR2 was compensated by an altered chemokine/chemokine receptor environment and CNS infiltrate (more neutrophils).

Because of the redundancy of the chemokine system, it might be naive to think that the blockade of a single chemokine can abrogate the recruitment of leukocytes to inflammatory foci in human pathology (Sorensen et al., 2004). In accordance with this, phase II clinical trials in RA patients, testing systemic inhibition of CCR2 using MLN1202, a humanized anti-CCR2 monoclonal antibody (Vergunst et al., 2006) and MK-0812, a small molecule CCR2 antagonist (Braddock et al., 2007) showed - despite good safety - disappointing efficacy.

In conclusion, we have characterized JNJ-27141491 as a potent, non-competitive antagonist of hCCR2 with high selectivity among other chemokine receptors. This

compound showed good oral bioavailability and dose-dependent efficacy on inhibition of monocyte and neutrophil recruitment to the lungs in a pharmacodynamic model using hCCR2 expressing mice. Furthermore, oral administration of JNJ-27141491 (20 mg/kg) could significantly delay and temporarily reduce neurological signs in EAE. However, no disease improving effects on the longer term could be demonstrated. Accepting the predictability of the EAE model, the use of JNJ-27141491 in MS patients is likely going to be unsuccessful.

ACKNOWLEDGEMENTS

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LEGENDS FOR FIGURES

Fig. 1 Structure of compound JNJ-27141491

Fig. 2 *In vitro* inhibitory activity of JNJ-27141491 on MCP-1-induced [^{35}S]GTPγS binding using hCCR2-CHO cell membranes (A); MCP-1, MCP-3, MCP-4 or mMCP-1-induced Ca^{2+} mobilization in hCCR2-CHO cells (B), THP-1 (C) cells and human monocytes (D) and chemotaxis of human PBMC towards MCP-1 (E). Data shown are mean ± S.E.M. inhibition of 2-6 independent experiments carried out in duplo or triplicate. Cells were stimulated with a MCP-1, -3, -4 or mMCP-1 concentration that caused 80% of the maximal agonist-induced response (EC_{80}). MCP-2 induced only weak $[Ca^{2+}]_i$ increases in hCCR2-CHO cells, precluding inhibition testing with compound. Effect of JNJ-27141491 on [^{125}I]MCP-1 receptor binding to hCCR2-CHO cells, THP-1 cells or human PBMC is shown as mean ± S.E.M. % [^{125}I]MCP-1 bound of 2-3 independent experiments carried out in duplo (F).

Fig. 3 Concentration-response curves of MCP-1-induced Ca^{2+} mobilization in THP-1 cells after 20 min pre-incubation with 0, 3, 9 or 15 nM JNJ-27141491. Results are expressed as mean ± S.E.M. relative fluorescent units (RFU) from triplicate determinations using FLIPR technology. This experiment is representative for at least 3 independent ones.

Fig. 4 Concentration-response curves of MCP-1-induced Ca^{2+} mobilization in THP-1 cells where JNJ-27141491 (0, 0.5, 1, 3 or 10 μM) and MCP-1 were added simultaneously to the cells (pre-incubated for 20 min with 1% DMSO). Results are expressed as mean ratio ± S.E.M from triplicate determinations measured using FDSS6000 technology.

Fig 5 Effect of JNJ-27141491 on binding of [^{125}I]hMCP-1 to human and dog PBMC and [^{125}I]mMCP-1 to WEHI cells and rat PBMC. Results are expressed as mean ± S.E.M % bound [^{125}I]MCP-1 of duplicate determinations from 2-3 independent experiments.

Fig. 6 Representative flowcytometric analysis of 10.000 cells in the BAL fluid of hCCR2 knock-in mice, 48h after *i.t.* instillation of 20 µg mMCP-1 and 10 ng LPS. Resident alveolar macrophages, recruited monocytes and neutrophils were discriminated based on FSC, FL1 (CD14 or Ly6G/6C) and FL2 (PKH26) properties. Twice daily oral treatment with 20 mg/kg JNJ-27141491 inhibited monocyte and neutrophil recruitment in mice bearing a humanized CCR2 (B) as compared to treatment with vehicle (A).

Fig. 7 Amount of monocytes and neutrophils in the BAL fluid of hCCR2 knock-in mice, 48h after *i.t.* instillation of 20 µg mMCP-1 and 10 ng LPS and treated once or twice daily with 5, 10, 20 or 40 mg/kg JNJ-27141491. Significant differences as compared to vehicle-treated animals are indicated with an asterisk (*$p<0.05$, **$p<0.01$, ***$p<0.001$, n=5-16 mice/group). Total amounts of rAM did not differ in any of the tested groups (data not shown).

Fig. 8 MOG_{35-55}-induced EAE in hCCR2 knock-in mice. Animals were once daily orally treated with vehicle or 20 mg/kg JNJ-27141491 from day 0 till 16. Results are represented as: A) mean clinical disease score ± S.E.M. (n=8 animals/treatment group; *$p < 0.05$) and B) disease incidence, calculated as percentage of mice with disease score > 0.5.

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Table 1: Effect of JNJ-27141491 on other chemokine-chemokine receptor functional interactions*.

Receptor	Chemokine	IC_{50} (μM)
hCCR1	MIP-1α	**0.6**
hCCR2	MCP-1	**0.04**
hCCR2	MCP-3	**0.05**
hCCR3	eotaxin	>5
hCCR4	MDC	>5
hCCR5	RANTES	~5
hCCR6	MIP-3α	>5
hCCR7	MIP-1β	>5
hCCR8	I-309	~5
hCXCR1/2	IL-8	>5
hCXCR3	I-TAC	>5

**Results from experiments carried out by Euroscreen using their aequorin-based calcium mobilization method for testing functional GPCR activity (AequoScreen™, www.euroscreen.com). Results are represented as IC_{50} values of duplicate determinations.*

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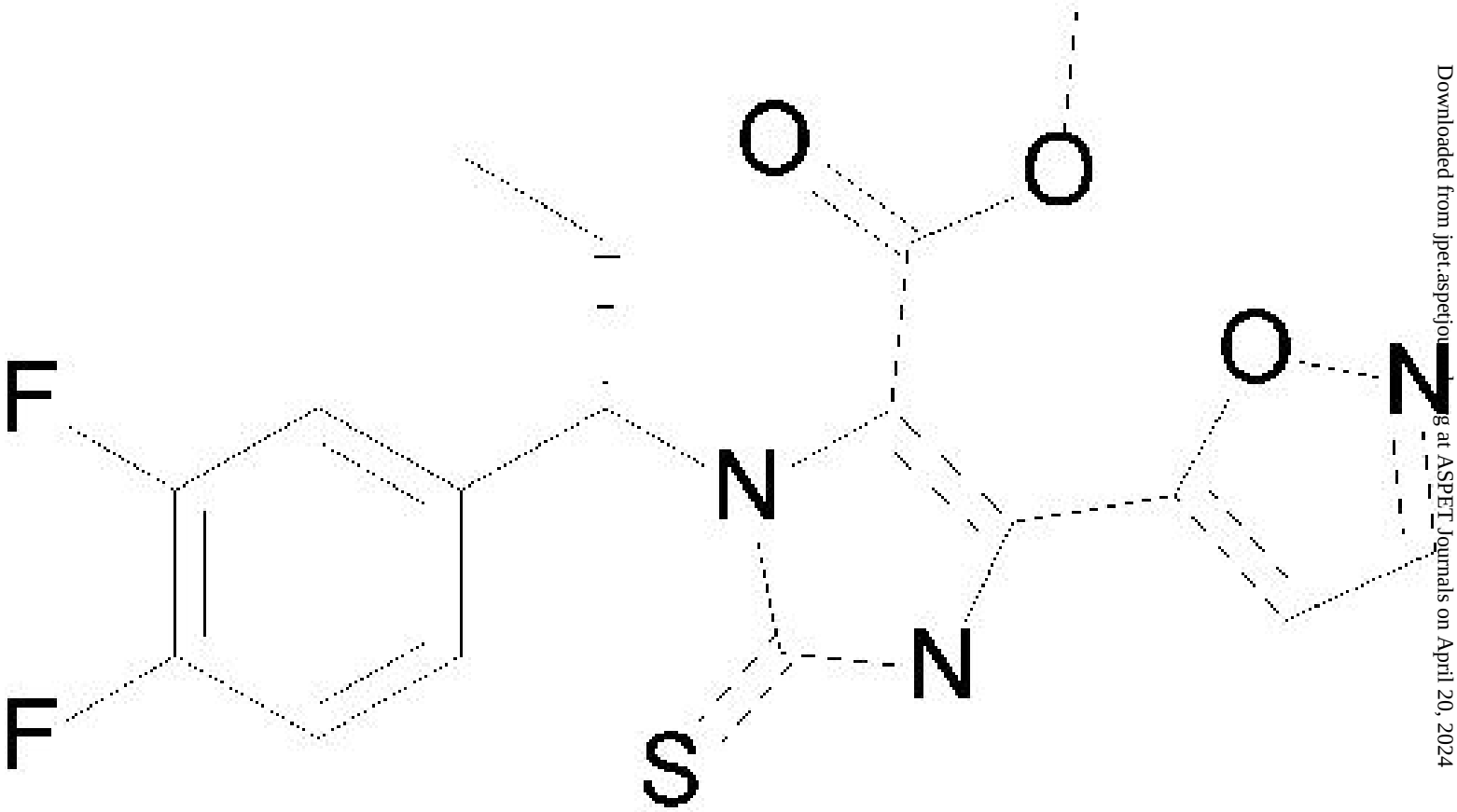

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Fig. 2A

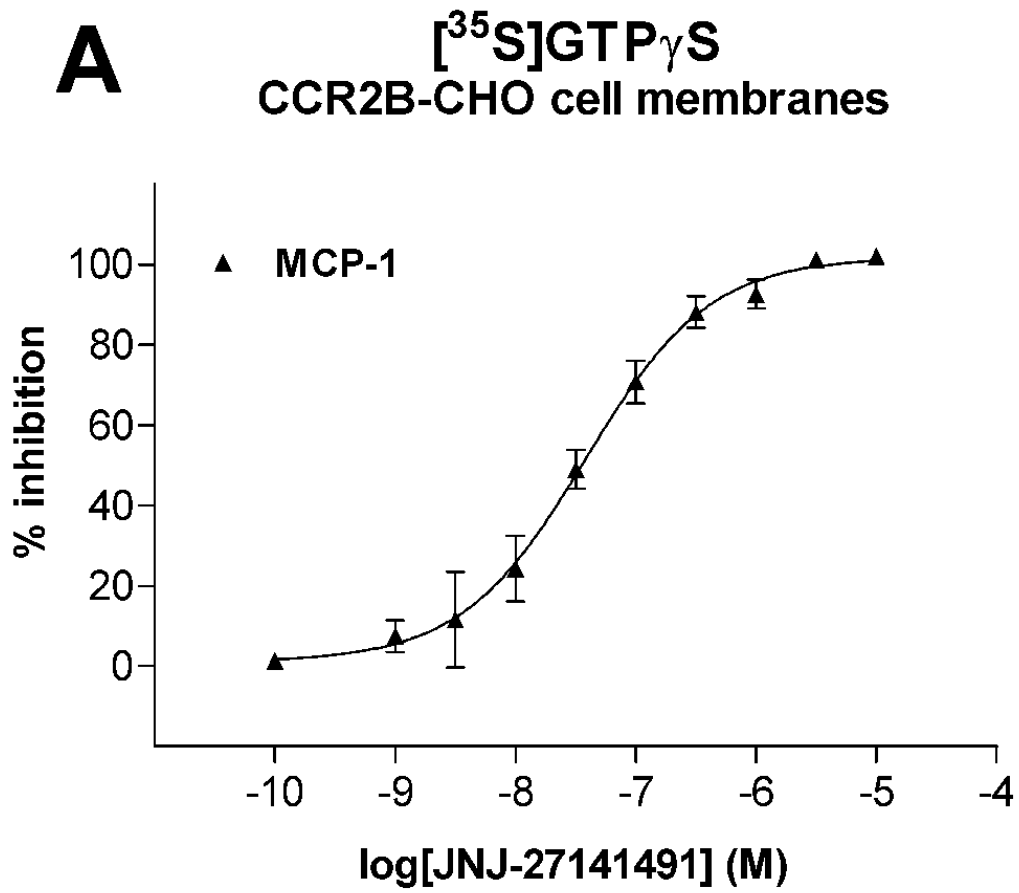

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Fig. 2B

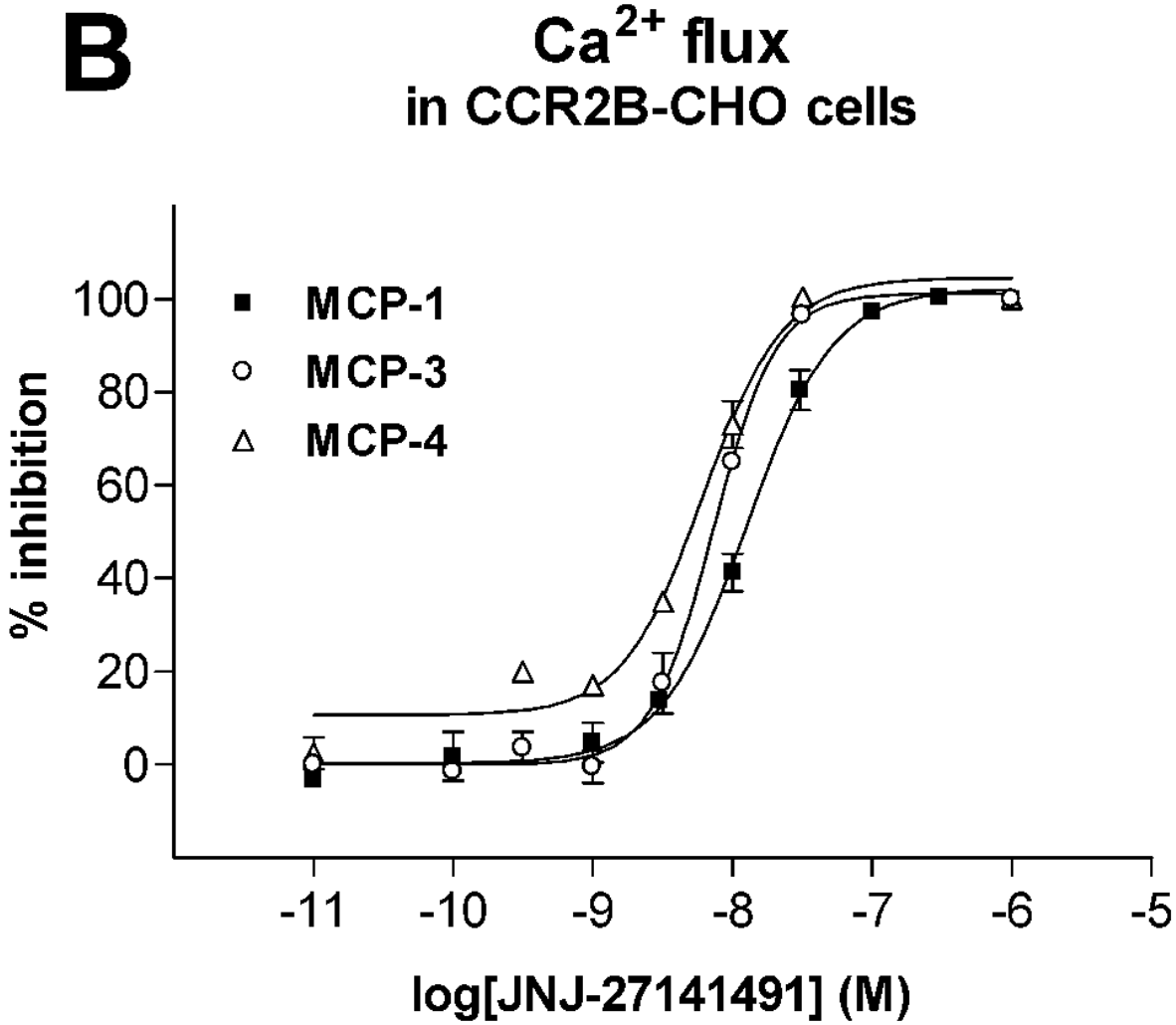

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Fig. 2C

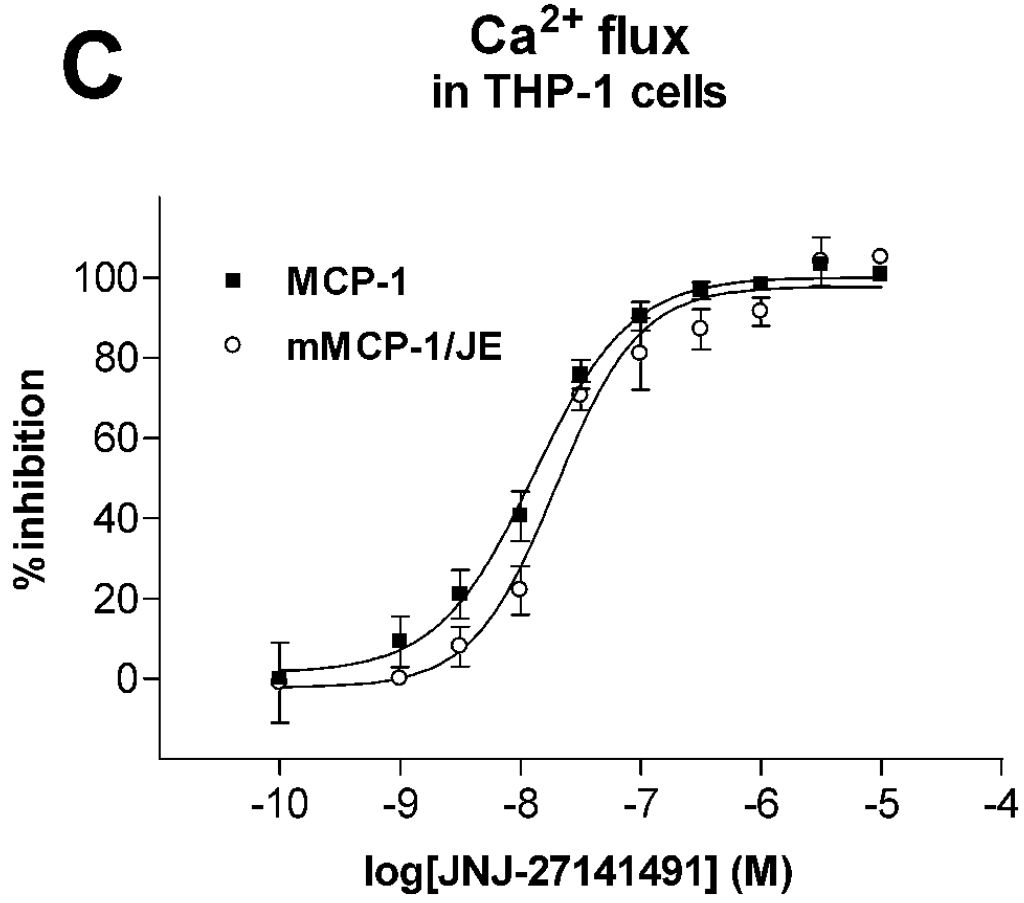

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Fig. 2D

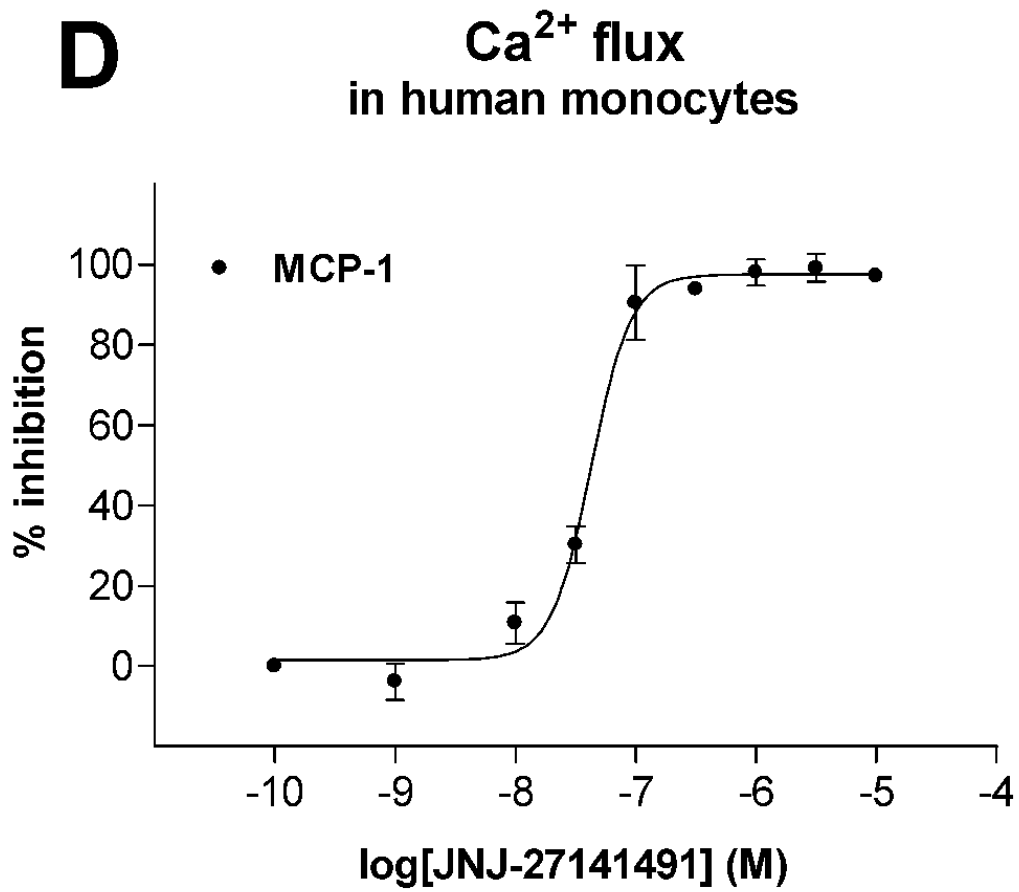

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Fig. 2E

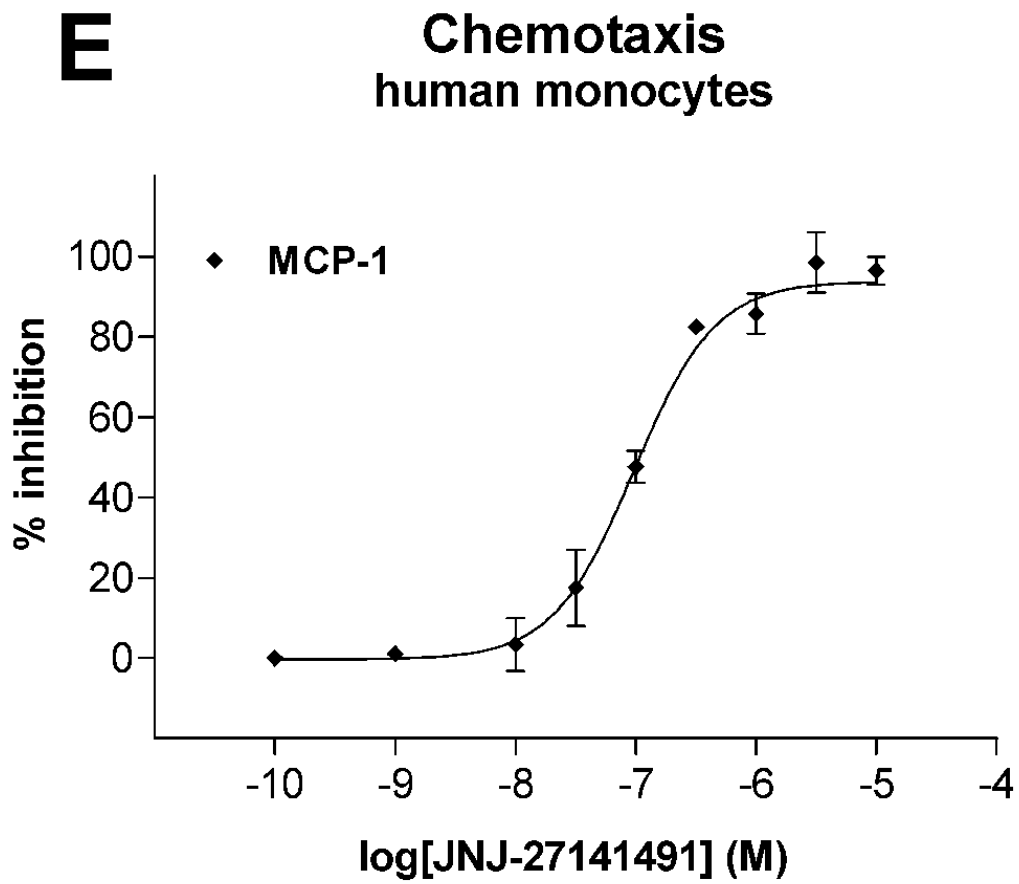

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Fig. 2F

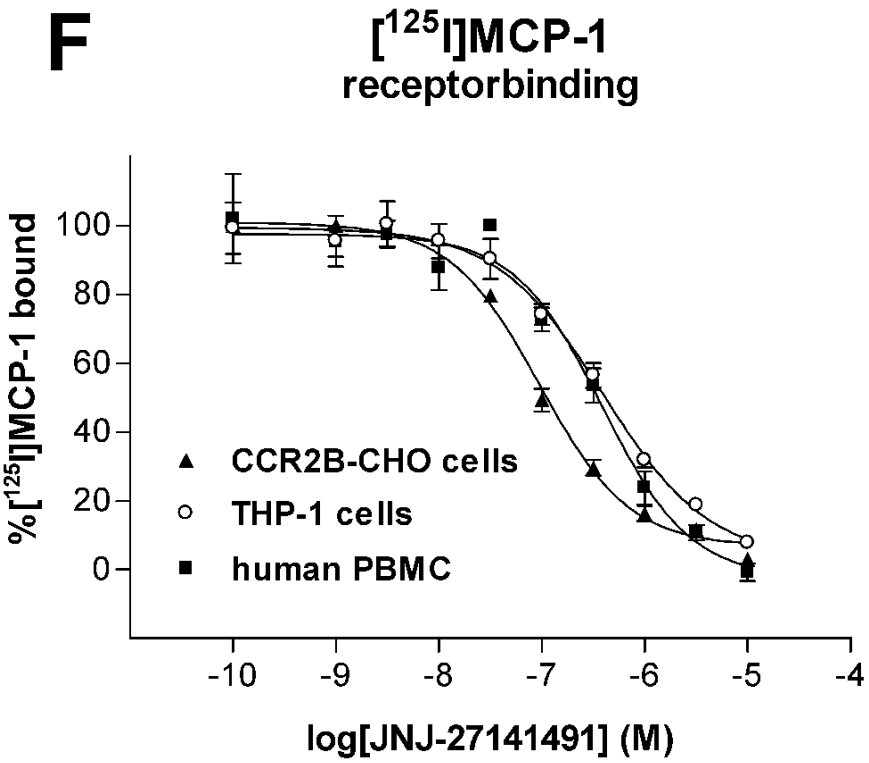

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Fig. 3

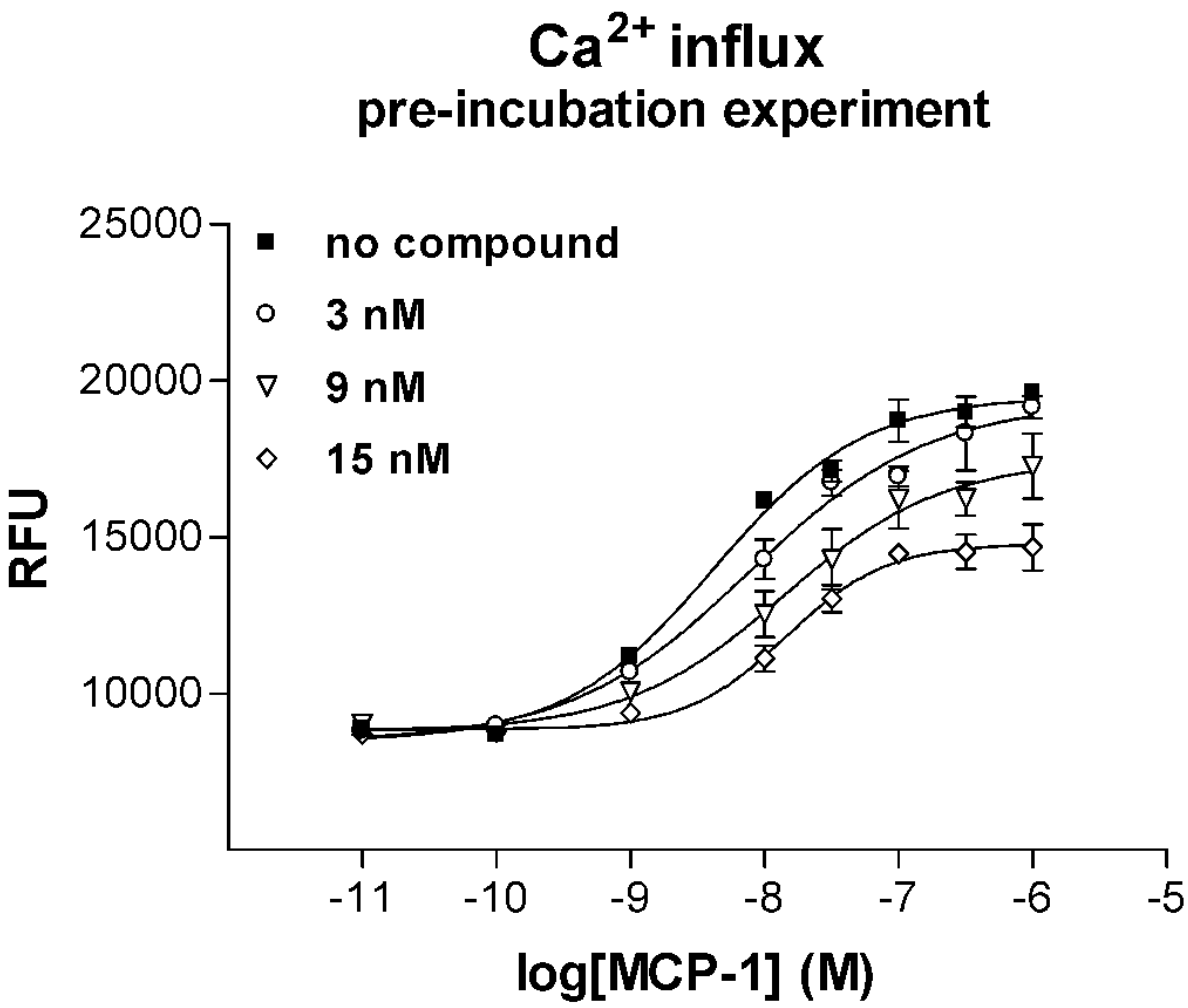

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Fig. 4

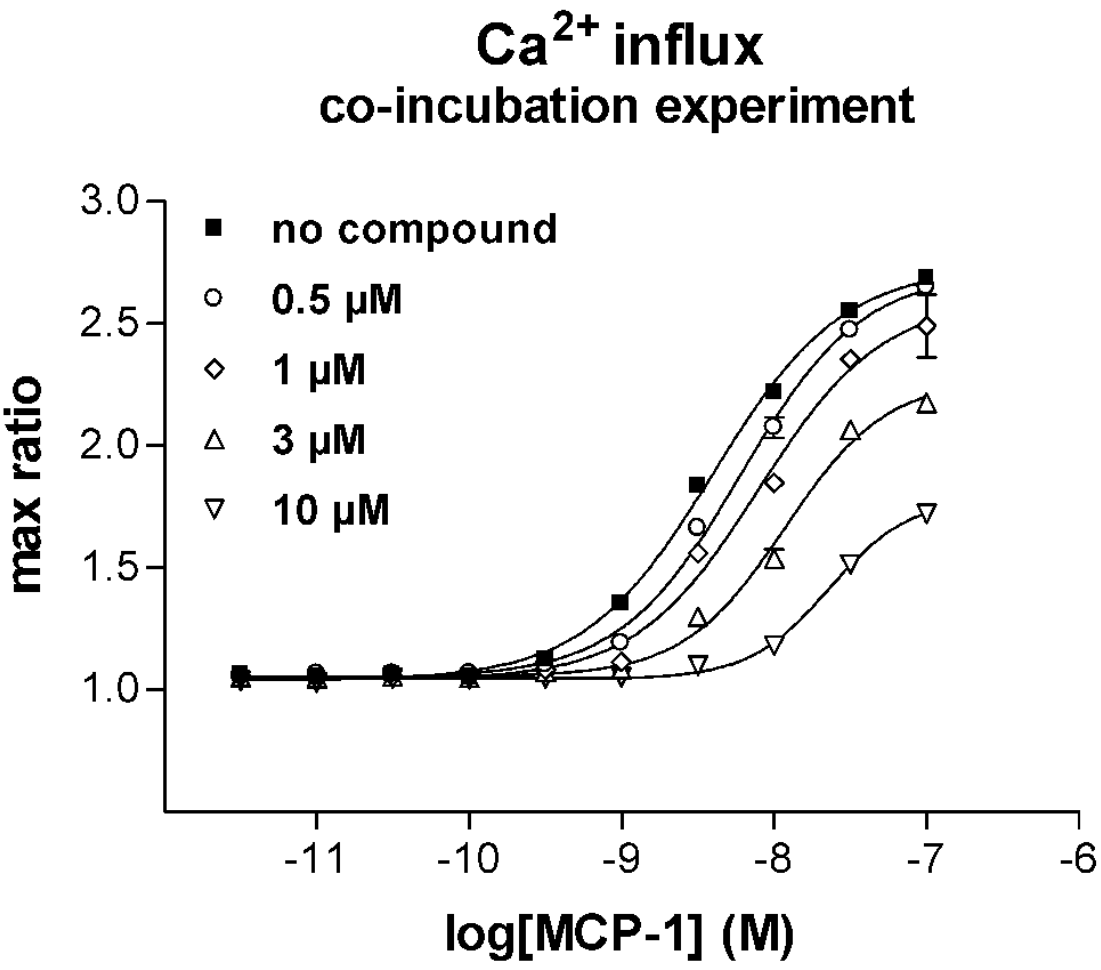

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Fig. 5

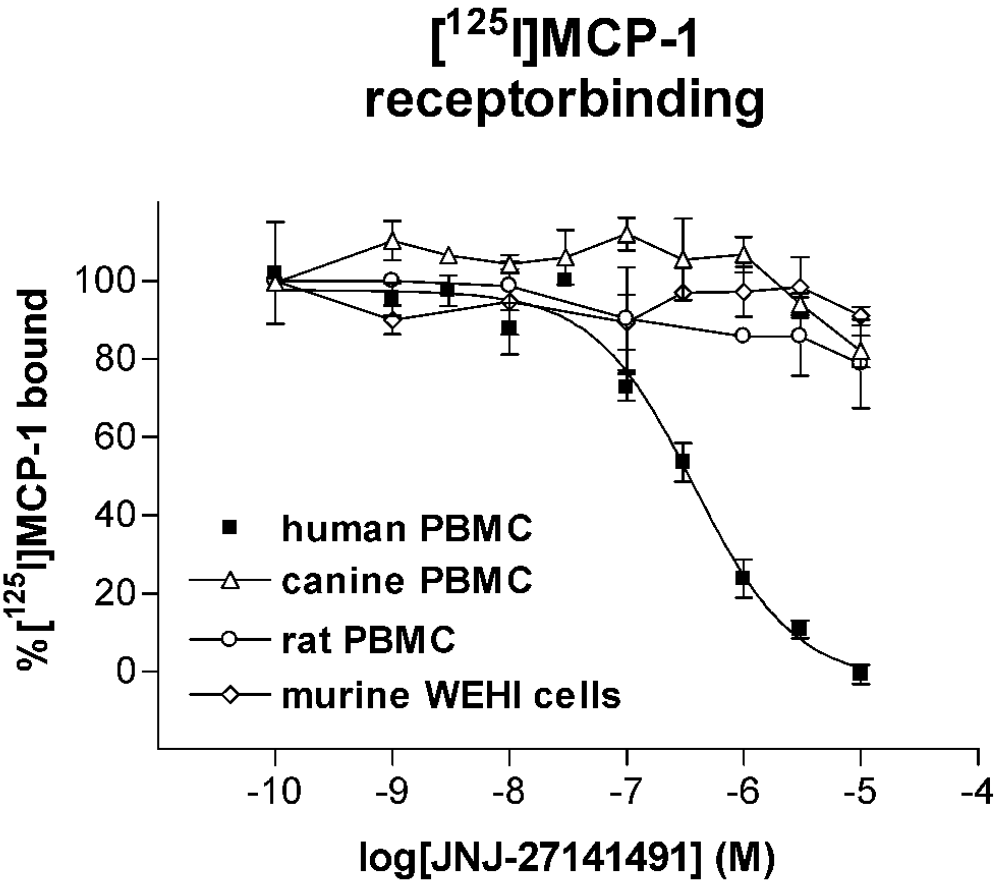

Fig. 6

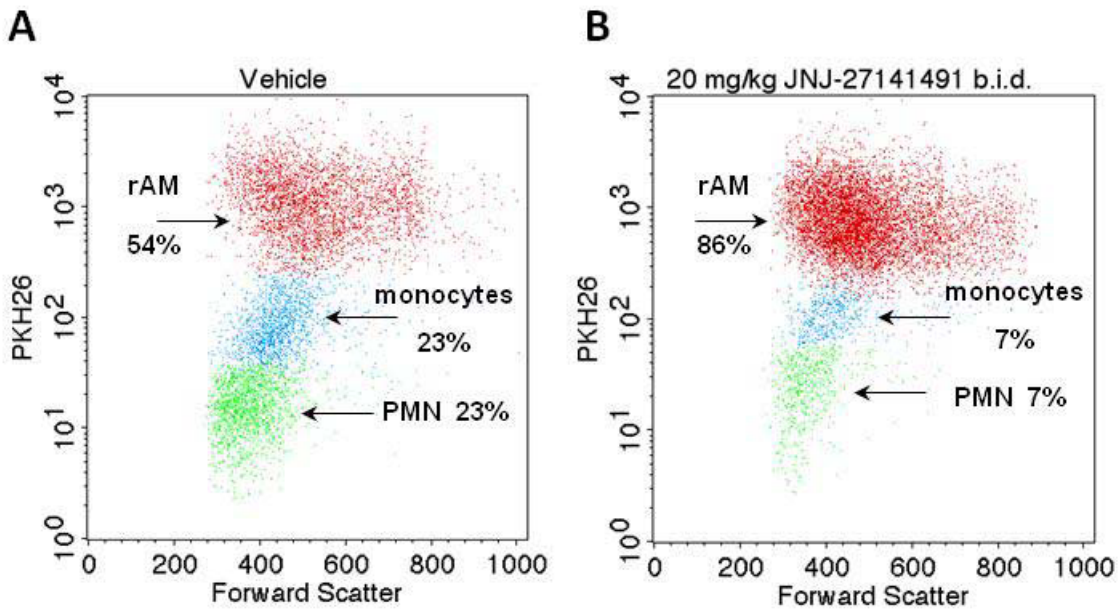

Fig. 7

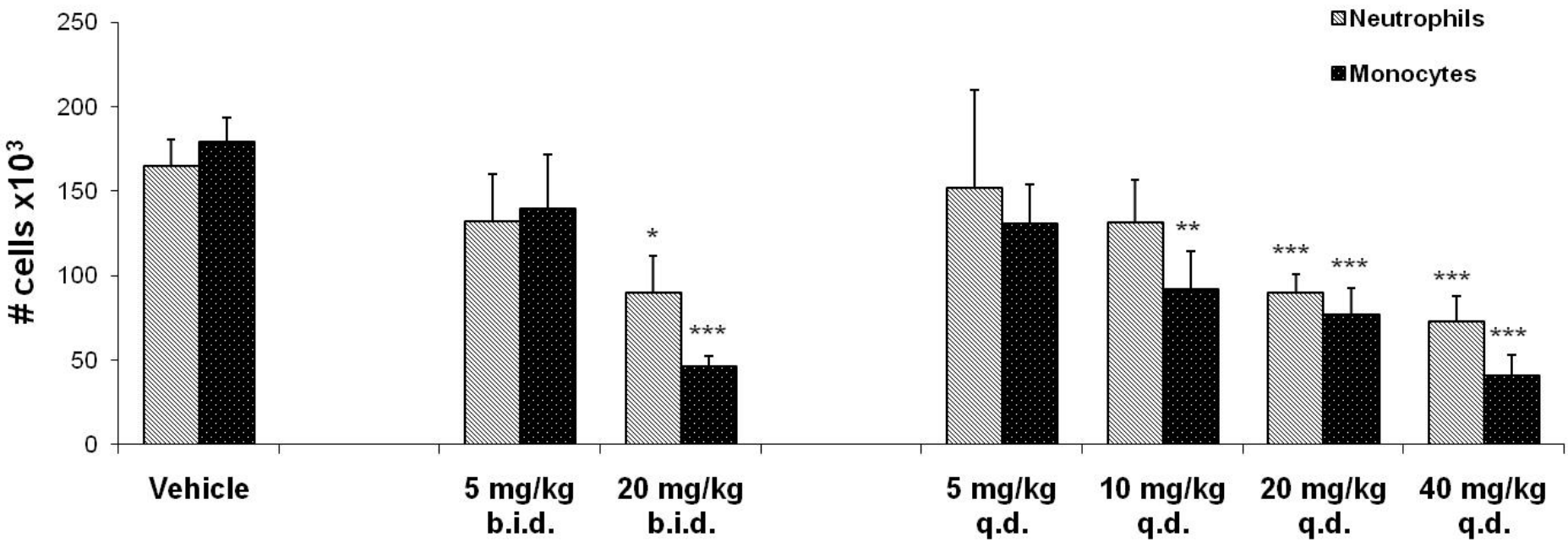

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Fig. 8A

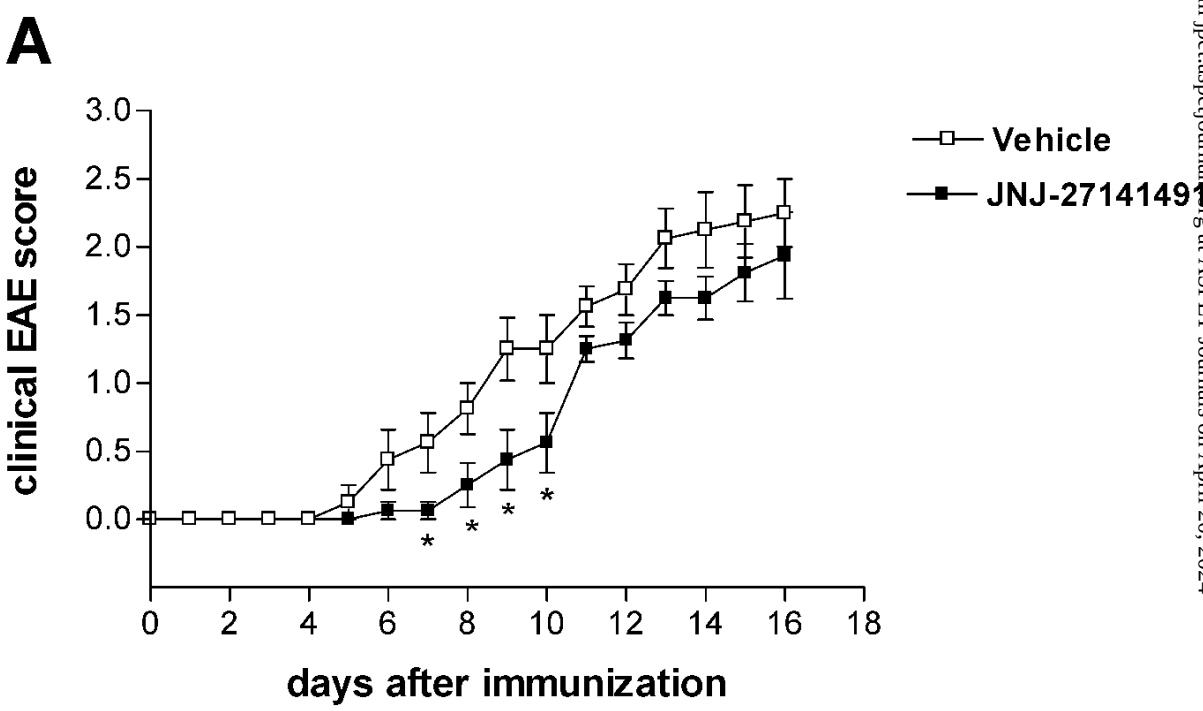

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Fig. 8B