



DF2755A, a novel non-competitive allosteric inhibitor of CXCR1/2, reduces inflammatory and post-operative pain



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ARTICLE INFO

Article history:

Received 27 August 2015

Received in revised form

11 November 2015

Accepted 13 November 2015

Available online 28 November 2015

Keywords:

Chemokines

CXCR1/2

Inflammatory pain

Neutrophil

DF2755A

Neutrophil chemotaxis

Allosteric

ABSTRACT

The activation of CXCR1/2 has been implicated in the genesis of inflammatory and postoperative pain. Here, we investigated a novel orally acting allosteric inhibitor of CXCR1/2 (DF2755A) and evaluated its antinociceptive effect in several models of inflammatory and post-operative pain. DF2755A was tested in vitro for efficacy in the chemotaxis assay, selectivity and toxicity. In vivo, C57Bl/6 mice were treated orally with DF2755A and the following experiments were performed: pharmacokinetic profile; inflammatory hyperalgesia models using electronic pressure meter test; neutrophil migration assay assessed by myeloperoxidase assay. DF2755A selectively inhibited neutrophil chemotaxis induced by CXCR1/2 ligands without effect on CXCL8 binding to neutrophils. A single mutation of the allosteric site at CXCR1 abrogated the inhibitory effect of DF2755A on CXCL8-induced chemotaxis. DF2755A given orally was well absorbed (88.2%), and it was able to reduce, in a dose (3–30 mg/kg)-dependent manner, inflammatory hyperalgesia induced by carrageenan, LPS and CXCL1/KC as well as neutrophil recruitment and IL-1 β production. In addition, DF2755A was able to reduce post-incisional nociception. Therapeutic treatment with DF2755A reduced CFA-induced inflammatory hyperalgesia even when injected intrathecally. The present results indicate that DF2755A is a novel selective allosteric inhibitor of CXCR1/2 with a favorable oral pharmacokinetic profile. Furthermore, the results might suggest that DF2755A might be a candidate of a novel therapeutic option to control inflammatory and post-operative pain.

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1. Introduction

Hyperalgesia is one of the most significant symptoms of inflammatory diseases. Although it is effectively treated with

non-steroidal anti-inflammatory drugs (NSAIDs), they may cause several undesired side effects, which limit their use. Thus, there is still a significant need for novel alternative therapeutic options to control inflammatory hyperalgesia.

The physiopathology of inflammatory hyperalgesia is mainly based on two important events: sensitization of primary sensory neuron (peripheral sensitization) and second order nociceptive neurons (central sensitization). Peripheral sensitization is triggered by the action of directly acting hyperalgesic substances such as prostaglandins and sympathetic amines [1–3]. Furthermore, prostaglandins production in the central nervous system has been also demonstrated to play a role in the maintenance of hyperalgesia [4,5]. In this perspective, inhibition of prostaglandins synthesis by NSAIDs represents an effective therapeutic option for the management of inflammatory pain [6,7]. However, the eluci-

Abbreviations: CXCR1/2, chemokine (C-X-C motif) receptor 1/2; LPS, lipopolysaccharide; CFA, complete Freund's adjuvant; CXCL8, chemokine (C-X-C motif) ligand 8; CNS, central nervous system; PMN, polymorphonuclear cell family; HBSS, Hanks' balanced salt solution; PGE2, prostaglandin E2; TRPV1, transient receptor potential cation channel subfamily V member 1; CGRP, calcitonin gene related peptide; ERK, extracellular-signal-regulated kinases; NMDA, N-methyl-D-aspartate.

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<http://dx.doi.org/10.1016/j.yphrs.2015.11.005>

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ation of the molecular mechanisms underlying the inflammatory pain is required to identify alternative therapeutic approaches with improved specificity, efficacy, and possibly with fewer toxic effects.

In fact, other inflammatory mediators released at the site of inflammation and in the spinal cord take part in the induction and maintenance of inflammatory hyperalgesia [5]. Among these mediators, chemokines represent attractive therapeutic targets [8] in consideration of their pivotal role in the onset of the inflammatory process. Chemokines are multipotent cytokines that promote and orchestrate inflammation by inducing chemotaxis and cell activation of different inflammatory cell subtypes [9,10]. In particular, CXC chemokines acting on their CXCR1 and CXCR2 (CXCR1/2) receptors play a critical role in neutrophil migration and activation at the inflammatory site [11–13]. Inhibition of the CXCLs/CXCR1/2 pathway resulted in a potent antinociceptive effect associated with decreased neutrophilic infiltrate in several animal models of inflammation [12,14–16]. In this sense, the chemokine CXCL1 (keratinocyte-derived chemokine, KC) is locally triggered in response to several inflammatory pain stimuli, including carrageenan and lipopolysaccharide (LPS), mediating hyperalgesia and neutrophil influx through CXCR1/2 [12,17]. CXCL1/KC-induced mechanical hyperalgesia, when injected into the mice paw, is dependent on the production of sympathetic amines and activation of the IL-1 β /prostaglandins pathway [11]. Peripheral CXCL1/KC via CXCR1/2 also account for the genesis of post-operative pain dependent on the recruitment of neutrophil to the site of surgery [18]. Interestingly, recent studies concur to establish the hypothesis that this pathway may also exert a neutrophil-independent action in the genesis of pathological pain including inflammatory and post-operative pain [19,20]. In fact, above and beyond the peripheral role in the genesis of pathological pain, in a model of complete Freund adjuvant (CFA)-induced inflammatory pain, CXCLs were found produced at the spinal cord level and responsible for dorsal horn neurons sensitization [21]. These data might suggest selective inhibition of CXCR1/2 activation as an alternative promising approach to controlling inflammatory and post-operative pain.

Along the last ten years, the authors have been characterizing a novel class of small molecular weight (SMW) noncompetitive allosteric inhibitors of CXCR1 and CXCR2.

In this class, reparixin (formerly repertaxin) was selected as the first clinical candidate and is currently in an advanced clinical development stage for the prevention of early graft loss in transplantation [22]. The broad characterization of the molecular mechanism and the thorough medicinal chemistry studies allowed the generation of several lead compounds with optimized pharmacokinetic and pharmacodynamic characteristics and their pharmacological potential was investigated in a large set of inflammatory conditions [23,24]. A general characteristic of the above candidates is the poor distribution into the cerebrospinal fluid (CSF) since a central action was not so far envisaged for this class of molecules.

With the aim to test the hypothesis that peripheral and central activation of CXCR1/2 may concur to the onset and maintenance of inflammatory pain, in the present study DF2755A (Fig. 1), a new lead candidate with tailored pharmacological property is described.

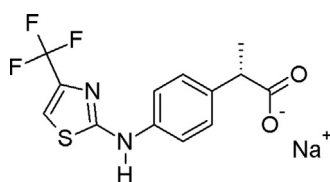


Fig. 1. Molecular structure of DF2755A (sodium (2S)-2-(4-((4-(trifluoromethyl)-1,3-thiazol-2-yl)amino)phenyl) propanoate).

The results of its characterization in *in vitro* cellular systems and *in vivo* pain models demonstrated that DF2755A provides an opportunity for the treatment of pain accompanying inflammation and surgery.

2. Materials and methods

2.1. Animals

All the animals used in these studies were caged under clean conditions at the animal facilities of the Dompé farmaceutici S.p.A (L'Aquila, Italy) or of Ribeirão Preto Medical School, University of São Paulo (Brazil). Animals had free access to pellets and water during a 12:12-h dark–light cycle (light from 6 a.m. to 6 p.m.). All the environmental conditions, as well as all the procedures adopted throughout the study for housing and handling the animals are in strict compliance with European Economic Community regulations and Italian Guidelines for Laboratory Animal Welfare.

For the mice used in pain modes, animal care and handling procedures were in accordance with the International Association for the Study of Pain guidelines for those animals used in pain research. Each animal was randomly distributed into experimental groups.

2.2. Cell isolation and culture

Human mononuclear cells and PMNs were obtained from buffy coats of heparinized peripheral blood from adult healthy volunteers through the courtesy of Centro Trasfusionale, Ospedale S. Salvatore, L'Aquila, Italy. Ethical clearance was obtained to perform these experiments. Mononuclear cells were obtained by centrifugation on Ficoll/Hipaque. Monocytes were separated by Percoll gradient centrifugation [25]. Human PMNs were prepared to 99% purity by dextran sedimentation followed by hypotonic lysis of contaminating red blood cells as previously described [26]. PMNs were washed once with saline and then resuspended at 1.5×10^6 /ml in HBSS for migration assay. Cell viability, as measured by trypan blue dye exclusion, was greater than 98%.

Murine, rat and guinea pig PMNs were isolated from peritoneal cavities injected with 1.5 mL (mouse) or 10 mL (rat and guinea pig) of 3% thioglycolate in saline. Four hours after injection, the animals were sacrificed by decapitation and peritoneal cavities were washed with saline. PMNs were recovered and centrifuged at $600 \times g$ for 10 min. The pellet was resuspended in HBSS; then the cells were counted using Türk's solution and diluted at 3×10^6 cell/ml. Rabbit PMNs were obtained from fresh heparinized rabbit blood as previously described [27] by dextran sedimentation followed by hypotonic lysis of contaminating red blood cells. PMNs were washed in PBS and finally resuspended at 1.5×10^6 /ml in PBS containing 1 mM Ca⁺⁺ and 1 mM Mg⁺⁺.

L1.2 cells were maintained as described previously in suspension at 37 °C with 5% CO₂ at a density of no more than 1×10^6 cells/ml [28]. Cells were transiently transfected by electroporation as previously described [29]. In brief, 1×10^6 cells per 3 microgram of DNA were electroporated and incubated overnight with medium supplemented with 5 mM sodium butyrate. Cells were harvested and assayed the following day. Cellular viability was >95% in all experiments, as measured by trypan blue dye exclusion. It is important to mention that these cells did not express constitutive CXCR2 (data not shown).

2.3. Generation of mutants

Human wtCXCR2 cDNA coding sequence (GenBank Acc. Num. M73969) was cloned using pcDNA3.1 expression vector (Invitrogen, USA) as Not I–Xho I fragment, sequence verified and used as templates to generate receptor mutants using the commercial

Site-Directed Mutagenesis kit (Clontech, Saint-Germain-en-Laye, France) following manufacturer's instructions. Each obtained mutant was fully sequenced to confirm the nucleotide substitution introduced [30].

2.4. Migration assay

Migration of human, rat, mouse, guinea pig and rabbit PMNs or human monocytes was evaluated by a microchamber technique in a 48 well microchemotaxis chamber, as previously described [23,31]. Briefly, twenty-five microliters of control medium (PBS for monocytes and HBSS for PMNs) or chemoattractant (CXCL1, CXCL8, rat CXCL2, mouse CXCL1, fMLP, C5a or CCL2) solutions were seeded in the lower compartment of the chemotaxis chamber. Fifty microliters of cell suspension (1.5×10^6 /ml for human and rabbit PMNs, 3.0×10^6 /ml for guinea pig, rat and mouse PMNs, 3×10^6 /ml for human monocytes) preincubated at 37 °C for 15 min in the presence or absence of different concentrations of DF2755A or vehicle were seeded in the upper compartment. The two compartments were separated by 5 μ m pre-size polycarbonate filter (polyvinylpyrrolidone-free for PMN chemotaxis). The chamber was incubated at 37 °C in air with 5% CO₂ for 45 min (PMNs) or 2 h (monocytes). At the end of incubation, filters containing migrated cells were removed, fixed, stained with Diff-Quik and five oil immersion fields at high magnification (100 \times ; Zeiss microscope) were counted after sample coding. Cell migration of L1.2 transfectants was evaluated in 5 μ m pore size Transwell filters [29]. Briefly, cells were pre-incubated at 37 °C for 15 min with vehicle or increasing concentration of DF2755A, seeded on the upper chamber and allowed to migrate in response to the appropriate chemokines seeded in the lower compartment at 10 nM. After 4 h at 37 °C, cells migrated in the lower compartment were recovered and counted in a Burkler chamber.

2.5. Radioligand binding assay

Isolated PMNs (10^7 ml⁻¹) were resuspended in RPMI 1640 and incubated at 37 °C for 15 min in the presence of DF2755A (1 μ M) or vehicle. After incubation, cell were resuspended (2×10^7 mL⁻¹) in binding medium (RPMI 1640 containing 10 mg mL⁻¹ BSA, 20 mM HEPES and 0.02% NaN₃) in the presence of DF2755A or vehicle. Aliquots of 0.2 nM of [¹²⁵I] CXCL8 and vehicle or serial dilution of unlabeled CXCL8 were added to 10^6 cells in 100 μ L of binding medium and incubated at room temperature for 1 h under gentle agitation. Unbound radioactivity was separated from cell-bound radioactivity by centrifugation through an oil gradient (80% silicon and 20% paraffin) on a microcentrifuge. Non-specific binding was determined by adding a 100-fold molar excess of unlabeled CXCL8. Scatchard analysis and all calculation were performed with the LIGAND program [32].

2.6. Peritoneal murine macrophage preparation and LPS-induced PGE₂ production

Peritoneal exudate cells were collected from peritoneal washings of male mice (Balb/c, C. River, Calco, Italy), 5 days after i.p. inoculum of 3% thioglycollate in saline (1.5 mL per mouse), as previously reported. Cells were placed at 1×10^6 mL⁻¹ in 96-well plates and non adherent cells removed by gentle washing 2 h later. DF2755A was then added to adherent macrophages 20 min before adding LPS (1 μ g mL⁻¹). Control cells received vehicle at the appropriate dilution. Total PGE₂ production was determined in the supernatant 24 h after LPS stimulation. PGE₂ levels were measured by EIA kit (sensitivity 2.5 pg per well).

2.7. Physicochemical Characterization

The pK_a, logP, logD_{7.4} and the solubility were determined by using the Sirius T3 instrument (Sirius-Analytical). The pK_a, logP and logD_{7.4} values of the sample were measured by Fast UV methods and pH-metrically. Solubility was measured pH-metrically. The samples were measured by titration in 1 mL of water-methanol using 50 ml aliquots of sample from 10 mM DMSO stock solution.

2.8. Protein Binding

Protein binding was measured at fixed concentration of 10 μ M in 100% of plasma from human and mouse by equilibrium dialysis method. Equilibrium dialysis was used to determine the extent of binding of the compound to plasma proteins. A semi-permeable membrane separates a protein-containing compartment from a protein-free compartment. The system was allowed to equilibrate at 37 °C for 16 h. The test compound present in each compartment was quantified by LC-MS/MS.

The molecular weight cut off (MWCO=5 KDa) of semi-permeable membrane was chosen to allow diffusion of a low molecular mass species (drug) but not of the high molecular mass molecules (plasma proteins and protein-drug complex).

2.9. Bidirectional permeation of DF2755A across Caco-2 cell line

Caco-2 cells (ECACC) were cultured in DMEM, 10% FCS, 1% NEAA, Hepes buffer 10 mM and a Pen/Strept mixture (100 U Penicillin and 100 μ g/ml Streptomycin), and split at confluence by trypsinization. For transport studies, 200,000 cells/well were seeded on Millicell 24-well cell culture plates. After 24 h of incubation at 37 °C and 5% CO₂, the medium was changed with Enterocyte Differentiation Medium with additives (Becton Dickinson), which allows Caco-2 cells to establish within three days a differentiated enterocyte monolayer. TEER, measured with a Millicell-ERS Millipore, was >1000 Ω .

The transport across the Caco-2 monolayer was determined with and without the P-gp inhibitor Verapamil 50 μ M in two ways: from apical to basolateral side (A-B) by adding a 10 μ M solution of compound in DMEM (0.2% final concentration of DMSO) to the apical side, and from basolateral to apical side (B-A) by adding a 10 μ M solution of test compound to the basolateral side; after 2 h incubation period at 37 °C and 5% CO₂, the basolateral side, the apical and the starting solutions were analyzed and quantified by LC-MS/MS. The experiments have been performed using buffers at different pH (6.5 apical vs. 7.4 basolateral) to better mimic the physiological conditions.

2.10. Pharmacokinetic studies

Pharmacokinetic experiments were performed in Balb/c mice (male, 17–23 g weight; Charles River, Calco, LC, Italy) in the animal facilities of Dompé farmaceutici S.p.A. (L'Aquila, Italy). The animals were food deprived 18 h before starting oral treatment. DF2755A was dissolved in saline and given in a single oral administration at the dose of 14.5 mg/kg by gavage. Blood sample from 3 to 4 mice/time were collected at 10 min, 1, 2, 4, 8, and 24 h after treatment. The acid derivative DF2755Y (the protonated acid form of DF2755A) was detected in plasma by HPLC.

2.11. Pain models

The experiments were performed in C57BL/6 male mice (Wild Type, WT, 20–25 g). All animals were housed in the animal care facility of the Ribeirao Preto Medical School, University of Sao Paulo.

Animals were taken to the testing room at least 1 h before experiments and were used only once. Behavioral tests were performed on animals in a randomized order in a double-blind fashion in which the person who performed the surgery was not same one who made the behavioral assessment. Food and water were available ad libitum.

2.11.1. Experimental protocol

DF2755A (3–30 mg/kg) was solubilized in water and administered 60 min before the inflammatory stimuli by oral gavage. The following stimuli were used: carrageenan (100 µg/paw in saline–25 µl), CXCL1/KC (10 ng/paw in saline –25 µl), and LPS (1 µg/paw in saline –25 µl). The relevant vehicle (saline) was used as control in all experiments. Hyperalgesia and neutrophil influx were determined 3 h after the i.pl. injection of carrageenan. Antinociceptive effect of systemically (30 mg/kg, p.o) or intrathecally (30 and 90 µg/site) treatments with DF2755A was also tested in a post-treatment schedule 24 h after intraplantar injection of CFA (10 µl/paw). Previous studies have determined these parameters to optimal for evaluation of inflammatory hyperalgesia [11,34]. The effect of DF2755A in incisional nociception was investigated using the following protocol: mice were treated orally twice a day with DF2755A (30 mg/kg) starting 60 min before and 12 and 24 h after surgery. Mechanical hyperalgesia was evaluated up to 30 h subsequent to paw incision, followed by removal of the paw tissue for evaluation of MPO activity.

2.11.2. Mechanical nociceptive paw test

Mechanical hyperalgesia was tested using an electronic version of the von Frey test in mice as reported previously [35]. Mice were placed in acrylic cages (12 × 10 × 17 cm) with wire grid floors in a quiet room 15–30 min before the start of testing. The test consisted of evoking a hindpaw flexion reflex with a handheld force transducer (electronic esthesiometer, IITC Life Science, Woodland Hills, CA, USA) adapted with a 0.5-mm² polypropylene tip. The investigator (blinded to group allocation) was trained to apply the tip perpendicularly to the central area of the plantar hindpaw with a gradual increase in pressure. The gradual increase in pressure was manually performed in blinded experiments. The upper limit pressure was 15 g. The end-point was characterized by the removal of the paw followed by clear flinching movements. The intensity of the pressure that elicited paw withdrawal was automatically recorded, and the final value for the response was obtained by averaging three measurements. The animals were tested before and after treatments. The results are expressed as the nociceptive withdrawal threshold (in g mean of three measurements) before and at the indicated times after drug or solvent (control) injections. The nociceptive withdrawal threshold of naïve mice was 8.2 ± 0.4 g (mean ± S.E.M.; n = 32) before injection of the solvent or hyperalgesic agents.

2.11.3. Hot-plate test

As a means to investigate whether DF2755A presents opioid-like antinociceptive activity, the effect of DF2755A was evaluated in the hot-plate test. The hot-plate test consists of exposing mice to a 10 cm wide, 51 °C metal plate in a glass cylinder. The temperature threshold is reached when the animal suddenly withdraws or licks its paws and is based on time in seconds of exposure to the heated plate. For this test, the thermal nociceptive threshold was recorded for mice treated with vehicle, DF2755A (30 mg/kg) or morphine (8 mg/kg). At indicated time points after drug injection, thermal thresholds were recorded again. The results are expressed as thermal threshold in seconds. All experiments were performed blind so that the experimenter that administered the drugs was not the same person who assessed the nociceptive behavior.

2.11.4. Measurement of motor performance

In order to discard possible non-specific muscle relaxant or sedative effects of DF2755A, mice motor performance was evaluated on the rota-rod test [36]. The apparatus consisted of a bar with a diameter of 2.5 cm, subdivided into six compartments by disks 25 cm in diameter (Ugo Basile, Model 7600). The bar rotated at a constant speed of 22 rotations per minute. The animals were selected 24 h previously by eliminating those mice that did not remain on the bar for two consecutive periods of 120 s. Animals were treated orally with vehicle or DF2755A (30 mg/kg). Diazepam (5 mg/kg/i.p.) was used as a positive control. The animal's latency to fall off was measured automatically by a mechanical sensor located in the floor of the apparatus. The cut-off time used was 120 s.

2.11.5. Mouse model of post-surgical pain

Mouse paw incisions were performed as described previously [37]. Briefly, mice were anesthetized with 1.5–2.0% isoflurane delivered through a nose cone, and the right hindpaw of the mouse was sterilized with 10% povidone-iodine. Following sterile preparation of the hindpaw, a 0.5-cm longitudinal incision was made through the skin and fascia of the plantar surface of the foot with a number 11 scalpel blade. The incision started 0.2 cm from the proximal edge of the heel and extended distally. The underlying muscle was elevated with curved forceps, leaving the muscle origin and insertion intact. After wound hemostasis, the skin was closed with two subcutaneous mattress sutures using 6–0 nylon and covered with an antibiotic ointment. In some experiments, control mice underwent a sham procedure that consisted of anesthesia, antiseptic preparation, and application of the antibiotic ointment without an incision.

2.11.6. Determination of neutrophil accumulation in the inflammatory site

Myeloperoxidase (MPO) activity was used as an index of neutrophil accumulation in the mice's plantar tissues, based on a kinetic-colorimetric assay as described previously [38]. Mice were pretreated with DF2755A or vehicle followed by i.pl injection of carrageenan (100 µg/paw), LPS (1 µg/paw) or KC/CXCL1 (20 ng/paw). Plantar tissue was harvested from the injected and control paws (saline). Samples were collected in 50 mM K₂HPO₄ buffer (pH 6.0) containing 0.5% hexadecyl trimethylammonium bromide and kept at –80 °C. Just before the assay, the tissue was homogenized using a Polytron (PT3100) and centrifuged at 13,000 g for 4 min. In our experimental condition (low pH), the MPO assay did not detect the activity of this enzyme in mononuclear cells. To prepare the solution for the analysis, 5 µL of the supernatant was mixed with 200 µL of phosphate buffer (50 mM, pH 6.0), containing 0.167 mg/mL O-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. The solution was analyzed by spectrophotometry for MPO activity determination at 450 nm (Spectra max). The MPO activity was compared with a standard curve of neutrophils obtained from mice blood. The results were presented as number of neutrophils × 10⁴/mg tissue.

2.11.7. Intrathecal (i.t.) injection of DF2755A

Under isoflurane anesthesia (1.5% in oxygen), using insulin 30 units syringe (BD Ultra Fine™ II), drugs were administered (6 µL) between L4 and L5 vertebrae. Animal's tail reflex was expected to confirm correct application of the method [39,40].

2.12. Drugs and reagents

The following materials were obtained from the indicated sources: Chemokines were purchased from PeproTech (London, UK). Chemicals and Complete Adjuvant of Freund was purchased from Sigma Chemical Co. (St. Louis, USA). Bacterial endotoxin from

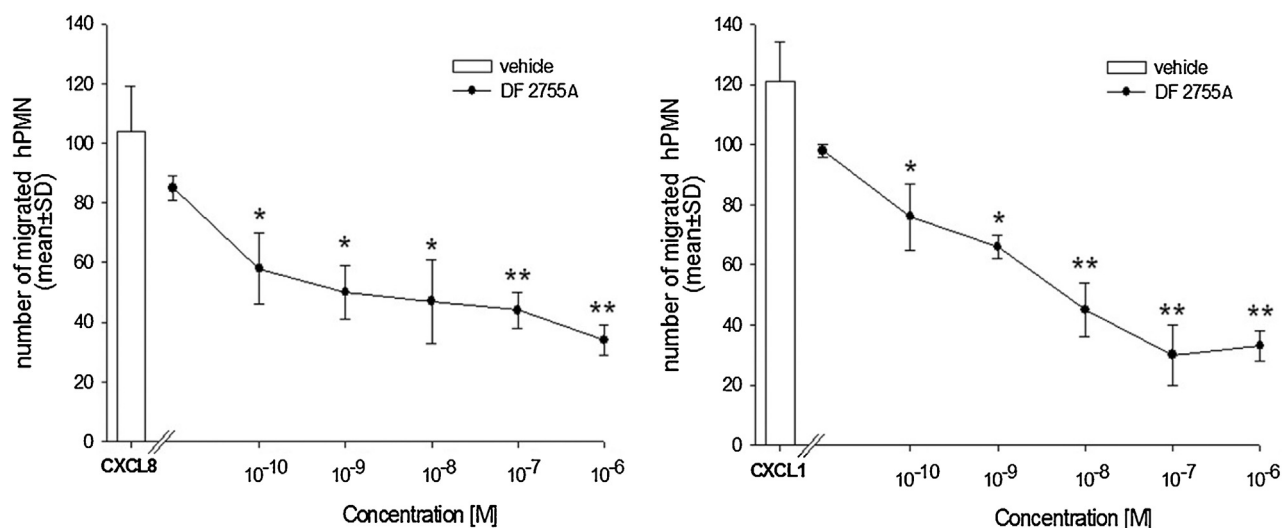


Fig. 2. Effects of DF2755A on human PMN migration in response to CXCL8 and CXCL1. hPMNs were preincubated at 37 °C for 15 min with vehicle or increasing concentration of DF 2755A and then tested for their ability to migrate in response to CXCL8 (1 nM; panel A) or CXCL1 (10 nM; panel B). hPMN migration was determined as described. CXCL8 and CXCL1-mediated migration was considered as 100%. Data are expressed as the mean \pm SD of 6 and 5 independent experiments for CXCL8 and CXCL1, respectively. Spontaneous hPMN migration was 7 ± 11 . Statistical analysis was performed by Student's *t* test and Mann–Whitney *U* test. Significance threshold was set at $P < 0.05$. * $P < 0.05$; ** $P < 0.01$ CXCL1 or CXCL8 alone (pretreated vehicle) group by Student's *t* test and Mann–Whitney *U* test.

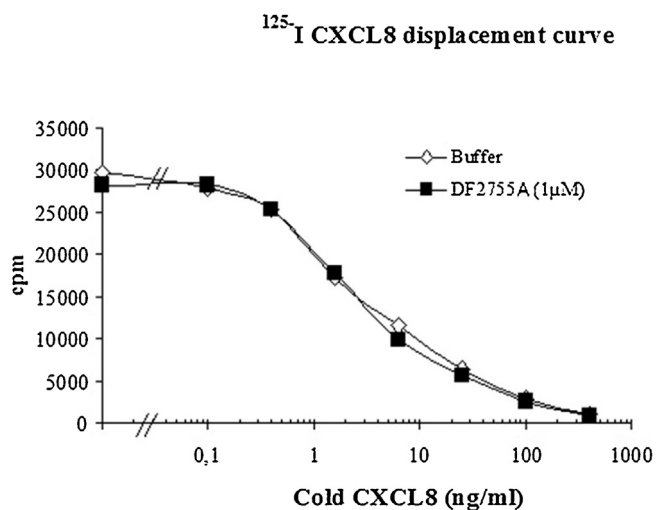


Fig. 3. Effect of DF2755A on IL-8 binding to cellular receptors. Human PMNs were pre-incubated for 15 min at 37 °C with vehicle or 1 mM DF 2755A. After incubation, aliquots of 0.2 nM of [¹²⁵I]-IL-8 and serial dilution of unlabeled IL-8 were added to 106 cells in 100 mL of binding medium and incubated at room temperature for 1 h under gentle agitation. Data are from one experiment representative of three performed.

E. coli referred to here as lipopolysaccharide (LPS-Difco Laboratories Ltd, West Molesey, Surrey, U.K.). Carrageenan was obtained from FMC (Philadelphia, USA), indomethacin was obtained from Prodome Química e Farmacêutica (São Paulo, Brazil) and Morphine was from Cristalia (Campinas Brazil). DF2755A was produced by the chemical department of Dompe farmaceutici S.p.A, L'Aquila, Italy. Diff-Quik was from Dade Behring (Milan, Italy). Polycarbonate filters and micro Boyden chambers were from Neuroprobe Inc (Pleasanton, CA). Transwell filters were from Costar (Costar, Cambridge, MA). pcDNA3 was from Invitrogen. Cellulose nitrate membrane filters were from Whatman International (Kent, CT). Cell culture reagents were from Life Technologies (Grand Island, New York). Culture plates were from Nunc (Nalge Europe; Neerijse, Belgium). [¹²⁵I]CXCL8 [0.2–0.02 nM, specific activity 2,200 Ci/mmol

(1Ci = 37 GBq) and PGE₂ EIA kit were from GE Healthcare (Bucks, UK).

2.12.1. Statistical analysis

For *in vivo* experiments data are reported as the means \pm SEM. The letter n in the legends refers to the number of mice used in the experimental group of each experiment. Two-way analysis of variance (ANOVA) was used to compare the groups at all times (curves) when the hyperalgesic responses were measured at different times after the stimulus injection. The factors analyzed were treatment, time and time by treatment interaction followed by Bonferroni's test for each time point. Alternatively, when the hyperalgesic responses or MPO activity were measured once after the stimulus injection or surgery, the differences between responses were evaluated by one-way ANOVA followed by Bonferroni's *t* test. Differences were considered to be statistically significant at $P < 0.05$.

For *in vitro* chemotaxis assays data were expressed as percentage of inhibition related to the control group (mean \pm SD of three independent experiments) whereas PGE₂ levels (pg/well) were present as mean \pm SD of three independent experiments. Differences between experimental groups were compared by Student's *t* test and Mann–Whitney *U* test. The level of significance was set at $P < 0.05$.

3. Results

3.1. DF2755A *in vitro* characterization

3.1.1. Effect of DF2755A on CXCL8 and CXCL1-induced hPMN migration

The effect of DF2755A in inhibiting human PMN (hPMN) chemotaxis induced by CXCL8 and CXCL1 was assessed over a wide range of concentrations (10^{-11} – 10^{-6} M). As reported in the Fig. 2A and B, the pre-treatment of hPMNs with DF2755A inhibited PMN migration induced by an optimal concentration of CXCL8 (1 nM) or CXCL1 (10 nM). DF2755A was equally efficacious in inhibiting CXCL8- and CXCL1-induced hPMN chemotaxis. This inhibition was concentration-dependent, being almost complete for both agonists induced-PMN migration at 1 μ M (IC₅₀ values: 4.2×10^{-9} M and 2.1×10^{-9} M for CXCL8 and CXCL1, respectively). In the absence of

Table 1
Selectivity of DF 2755A action.

| Cells | Agonist | DF 2755A [M] | % Inhibition (mean ± SD) |
|-----------|---------|------------------|--------------------------|
| hPMN | fMLP | 10 ⁻⁶ | 3 ± 11 |
| hPMN | C5a | 10 ⁻⁶ | 29 ± 11 |
| hMonocyte | CCL2 | 10 ⁻⁶ | 3 ± 9 |

Note: hPMN and monocytes were preincubated for 15 min at 37 °C with DF 2755A. hPMNs were then tested for their ability to migrate in response to fMLP (10 nM) or C5a (1 nM). Monocytes were tested for their ability to migrate in response to CCL2 (25 ng/ml). Data (subtracted of spontaneous migration) are presented as % of inhibition and were calculated using this equation: % inhibition = 100 – (the number of cells migrating in the presence of agonist + DF 2755A ÷ the number of cells migrating in the presence of agonist) × 100. Data are shown as mean ± S.D. of two independent experiments.

chemokine stimulation, DF2755A was unable to modify the PMN spontaneous migration (data not shown).

3.1.2. Effect of DF 2755A on CXCL8 binding

The effect of DF2755A on CXCL8 binding was investigated. PMN pre-incubation with DF 2755A (1 μM) at 37 °C for 15 min did not show a significant change in CXCR1/2 receptor number (210,179 ± 54,803, 180,954 ± 47,048 in vehicle, DF 2755A pre-treated PMN, respectively; Mean ± SD) or receptor affinity (Kd 2.24 ± 1.70 × 10⁻⁹ M; 1.91 ± 0.94 × 10⁻⁹ M, respectively) (Fig. 3).

3.1.3. Selectivity of DF 2755A inhibitory activity

DF2755A is a potent inhibitor of CXCR2 and CXCR1-triggered hPMN activity. With the aim to assess the selectivity of the compound, the inhibitory effect of DF2755A was tested on L1.2 cells expressing wt CXCR2. As depicted in the Fig. 4, wt CXCR2/L1.2 transfectant migration induced by CXCL8 (10 nM) was significantly inhibited by DF 2755A. The reduction of wild-type CXCR2/L1.2 transfectant migration was concentration-dependent, starting at 1 nM (28% inhibition) and reaching the maximal inhibitory effect (73%) at 100 nM (Fig. 4).

To verify the binding site hypothesis of DF2755A on CXCR2, two amino acids (D293 and E300) supposed to be directly involved in the binding were selected for alanine-replacement mutagenesis experiments. This double alanine-replacement mutant (D293A/E300A) was transiently expressed in L1.2 cells. The obtained mutant still supports CXCL8-induced chemotaxis but with abrogated sensitiveness to DF 2755A. As expected, CXCL8-induced D293A/E300A CXCR2 transfectant migration was completely resistant to DF 2755A action (IC50 > 10⁻⁶ M) (Fig. 4).

3.1.4. Effect of DF 2755A on C5a- and fMLP-induced hPMN migration and on CCL2-induced human monocyte migration

To investigate whether the inhibition of hPMN migration could be restricted to CXCL8 and CXCL1, the effect of DF2755A (tested at 1 μM) on C5a and fMLP-induced chemotaxis was tested. DF2755A did not affect hPMN migration induced by C5a and fMLP (Table 1). Furthermore, the effect on CCL2 induced monocyte migration was tested. DF2755A did not affect monocyte migration induced by CCL2, a chemokine belonging to the CC chemokine family (Table 1).

3.1.5. Effect of DF2755A on LPS-induced PGE₂ production in mouse peritoneal macrophages

Allosteric antagonists of CXCR1/2 developed by our group originally are derived from cyclooxygenase inhibitors [23]. To verify that DF 2755A biological activities in animal models are not dependent on inhibition of cyclooxygenase, the effect of DF2755A on PGE₂ production was assessed. Total PGE₂ production was measured 24 h after LPS stimulation. Results showed that the pre-incubation of murine macrophages with DF2755A (10⁻⁵ M) had no effect on

Table 2
Cross species assay.

| Treatment [M] | Rodent PMN chemotaxis % of inhibition (mean ± SD) | | | |
|-------------------|---|----------|------------|-----------|
| | Rat | Mouse | Guinea-pig | |
| | Agonist-induced PMN chemotaxis | | | |
| | MIP-2 | KC | CXCL1 | CXCL8 |
| 10 ⁻¹¹ | n.d. | n.d. | 20 ± 7 | 26 ± 9 |
| 10 ⁻¹⁰ | n.d. | n.d. | 40 ± 5** | 42 ± 12** |
| 10 ⁻⁹ | 51 ± 11** | n.d. | 50 ± 4** | 52 ± 7** |
| 10 ⁻⁸ | 55 ± 10** | 63 ± 5** | 59 ± 4** | 58 ± 11** |
| 10 ⁻⁷ | n.d. | n.d. | 68 ± 6** | 68 ± 5** |
| 10 ⁻⁶ | n.d. | n.d. | 74 ± 5** | 77 ± 6** |

Note: Rodent PMNs were preincubated at 37 °C for 15 min. with vehicle or compound. Rodent PMNs were then tested for their ability to migrate in response to CXCL2 (25 ng/ml). PMN migration was determined as described [25]. Spontaneous mouse, rat, guinea-pig PMN migration was 13 ± 8, 14 ± 15 and 10 ± 5, respectively. Statistical analysis was performed by Student's *t* test and Mann-Whitney *U* test. Significance threshold was set at *p* < 0.05.

** *p* < 0.01 agonist alone (pre-treated vehicle) group by Student's *t* test and Mann-Whitney *U* test.

Table 3
Physicochemical properties of DF2755A.

| DF2755A | |
|------------------------------------|----------------|
| Formula Weight | 356.29 |
| pK _a | 4.17 |
| logP | 3.91 |
| logD _{7.4} | 0.713 |
| Thermodynamic solubility at pH 7.4 | 44.0 mg/mL |
| Plasma protein binding | 97.48% (human) |
| | 96.12% (rat) |

the production of PGE₂ induced by LPS (1 μg/ml); % inhibition (mean ± SD) = 2 ± 29.

3.2. Cross species assay

3.2.1. Inhibition of CXCL2-induced rodent PMN migration

In this cross-reactivity assay, the inhibitory effect of DF 2755A on CXCL2-induced rodent (rat, mouse and guinea-pig) PMN chemotaxis was investigated. Data indicated that the pre-treatment of rodent PMNs with DF 2755A (10 nM) significantly reduced PMN migration induced by an optimal concentration of MIP-2, KC, CXCL1 and CXCL8 (Table 2).

3.3. Physico-chemical studies

The main physicochemical properties of DF2755A are listed in the Table 3

3.4. Bidirectional permeation of DF2755A across Caco-2 cell line

DF2755A demonstrated high permeation in both directions (from apical to basolateral and from basolateral to apical side) Papp(A-B) being about 34.5 × 10⁻⁶ cm/s and Papp(B-A) being about 35.6 × 10⁻⁶ cm/s. P-gp-inhibitor (50 μM verapamil) and BCRP-inhibitor (1 μM Ko143) had practically no effect on the permeation of DF2755A through the Caco-2 cell-line, the observed Papp-values in both directions being about 28–38 × 10⁻⁶ cm/s in each case, suggesting together with the efflux-ratio of 1, that DF2755A is not a substrate for either P-gp or BCRP and has no active efflux across Caco-2 cell-line.

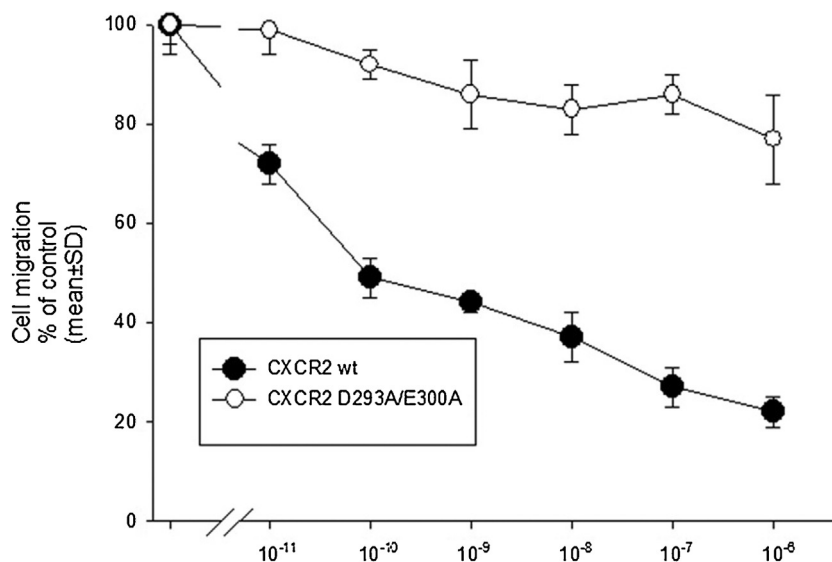


Fig. 4. DF2755A molecular mechanism of action. Cell migration was induced on L1.2 transfectants expressing wild-type CXCR2 or mutant CXCR2 (D293A/E300A) by CXCL8 (10 nM) in the presence or absence of increasing concentrations of DF 2755A. Results are expressed as percentage of migration in the absence of DF 2755A. Values are means \pm SD of three independent experiments.

3.5. DF2755A pharmacokinetic profile

3.5.1. Pharmacokinetics after single oral administration in the mouse

Pharmacokinetics of DF2755A after p.o. (14.5 mg/kg) administration was studied in 18 h-fasted male Balb/C mice (Table 4). DF2755A was dissolved in saline for p.o. dosing (10 mL/kg). Blood samples from 4 mice/time were collected at 10 min, 1, 2, 4, 8 and 24 h after p.o. administration of DF2755A. The acid derivative DF2755Y was detected in plasma (HPLC).

The pharmacokinetic profile showed a rapid absorption of the compound. After oral administration of DF2755A, the maximum concentration (C_{max}) in plasma (29.4 μ g/mL) was reached at the first collection time (10 min post administration). Overall systemic exposure (AUC) was 113.97 h μ g/mL and the $t_{1/2}$ was 2.56 h. Intravenous administration of DF2755A showed low systemic clearance (112.2 mL/h/kg). The volume of distribution at steady state was low with respect to total body water (0.42 L/kg) and the $t_{1/2}$ was 2.77 h after dosing. The bioavailability of DF2755A was calculated as 88.2%. In conclusion, DF2755A is well absorbed by the oral route, poorly distributed in the tissues and gradually eliminated from mouse plasma (Table 4).

3.6. Pre-clinical pharmacology of DF2755A

3.6.1. DF2755A reduced inflammatory and post-operative hyperalgesia in several mouse models

CXCLs/CXCR1/2 pathway mediates carrageenan-induced inflammatory hyperalgesia in mice [11,41]. Thus, initially carrageenan-induced inflammatory hyperalgesia was used to define the potential antinociceptive effect of DF2755A. In agreement with its *in vitro* efficacy (Fig. 2 and Table 2), oral treatment of mice with DF2755A reduced carrageenan-induced inflammatory hyperalgesia in a dose-dependent (3–30 mg/kg) manner (Fig. 5A). There was greater than 55% inhibition of carrageenan-induced mechanical hyperalgesia at 30 mg/kg of DF2755A. The effect of DF2755A was associated with a reduction in neutrophil recruitment to the inflamed tissue by 60% (Fig. 5B). Moreover, DF2755A also reduced carrageenan-induced the *in vivo* production/release of IL-1 β but had no effect on TNF, IL-6 or CXCL1/KC (Fig. 5C). The dose of 30 mg/kg of DF2755A was used in all further exper-

Table 4
DF2755A pharmacokinetic profile.

| Parameter | Unit | p.o. |
|----------------------|--------------|--------|
| C_0 | μ g/mL | – |
| C_{max} | μ g/mL | 29.4 |
| t_{max} | h | 0.167 |
| AUC _{clast} | h μ g/mL | 92.29 |
| AUC _{tot} | h μ g/mL | 113.97 |
| $t_{1/2}$ | h | 2.77 |
| CL | mL/h/kg | 112.2 |
| V_{ss} | L/kg | 0.42 |
| F% | | 88.2 |

Note: Main pharmacokinetic parameters in male mice after single p.o. administration of DF 2755A (14.5 mg/kg).

iments. The compound was also able to reduce both LPS- and CXCL1/KC-induced mechanical hyperalgesia (Fig. 5D).

We also evaluated the possible antinociceptive effect of DF2755A upon post-surgical pain. As shown in Fig. 6A, the pre-treatment with DF2755A was able to reduce paw incision-induced mechanical hyperalgesia at 2 and 4 h after surgery. Additional treatments (12 \times 12 h) maintained the antinociceptive effect of DF2755A in latter time points (24 and 30 h after surgery). DF2755A treatment also produced a significant reduction in neutrophil accumulation in the incised mouse paw (Fig. 6B).

The analgesic effect of DF2755A was then tested in the CFA-induced a more persistent inflammatory pain model [42]. We probed whether the post-treatment with DF2755A could modify CFA-induced mechanical inflammatory hyperalgesia and whether the analgesic effect was maintained. As seen in Fig. 7A, delayed treatment with DF2755A (24 h after CFA injection) reduced persistent inflammatory hyperalgesia. The effect of DF2755A peaked between 3 and 5 h after treatment (Fig. 7A). Interestingly, the effect of DF2755A was significant until 7 h after CFA injection but mechanical hyperalgesia returned to the vehicle control group 24 h after treatment (Fig. 7A).

In attempt to verify whether DF2755A would have antinociceptive effect at the level of the spinal cord, i.e. injection of DF2755A was performed in CFA-induced inflammatory hyperalgesia. Noteworthy, i.t. injection of DF2755A 24 h after paw injection of CFA reduced mechanical hyperalgesia in a time-dependent manner. The

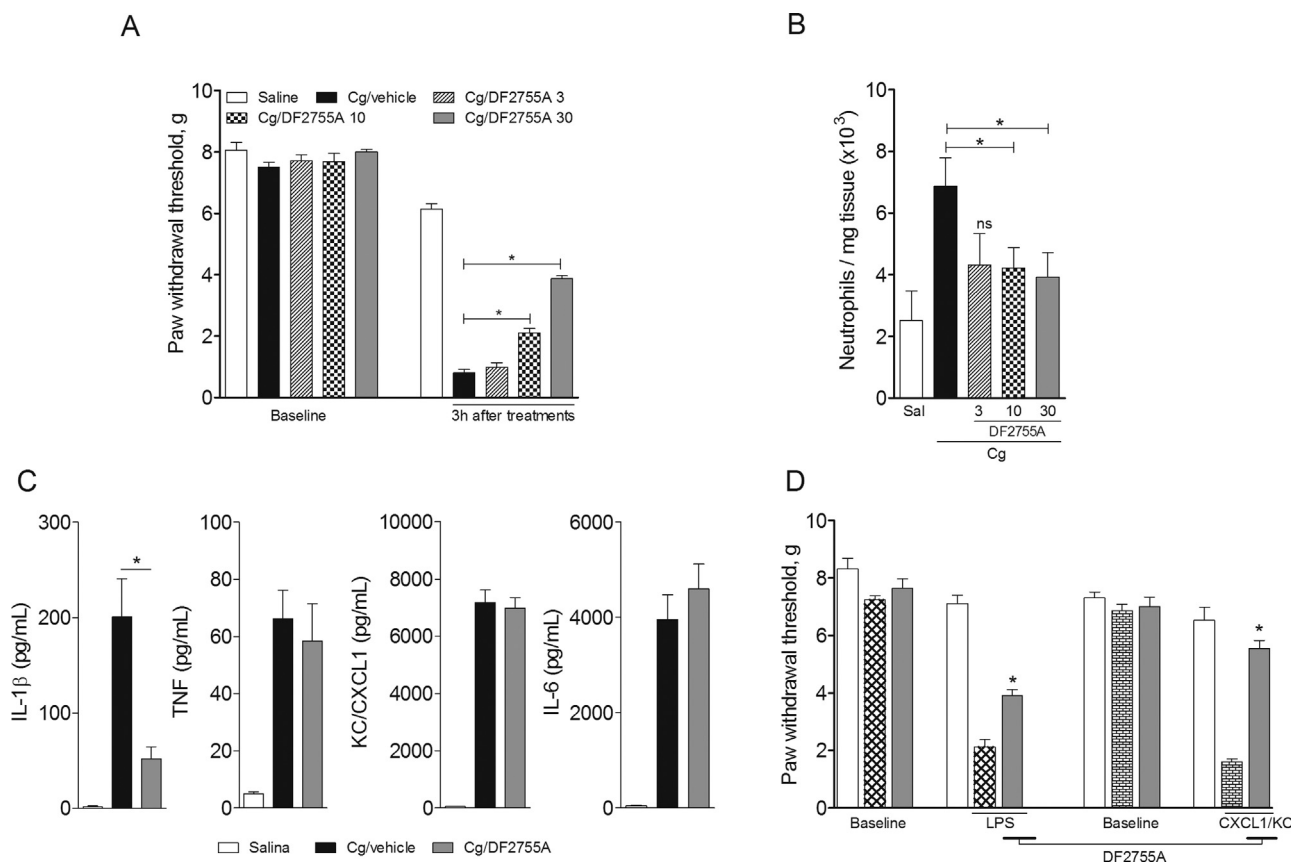


Fig. 5. DF2755A treatment reduces inflammatory hyperalgesia, neutrophil infiltration and IL-1 β production. Mechanical nociceptive threshold was measured (baseline, BL) followed by oral treatment with DF2755A (3, 10, and 30 mg/Kg, p.o) or vehicle (Veh). At 60 min latter mice received an intraplantar injection of carrageenan (Cg-100 μ g/paw) or vehicle (Sal-saline). Hyperalgesic responses were evaluated 3 h after carrageenan and hind-paws were then collected for MPO activity and cytokines levels analysis (Panel A–C respectively). Mice were also treated with DF2755A (indicated arrow; 30 mg/Kg, p.o.) or vehicle (Vh) 60 min before LPS (1 μ g/paw) or CXCL1/KC (10 ng/paw) injections. Hyperalgesic responses were evaluated 3 h later (Panel C). Data are shown as the mean \pm S.E.M. ($n = 5$ per group). * $P < 0.05$ when compared to vehicle-treated group.

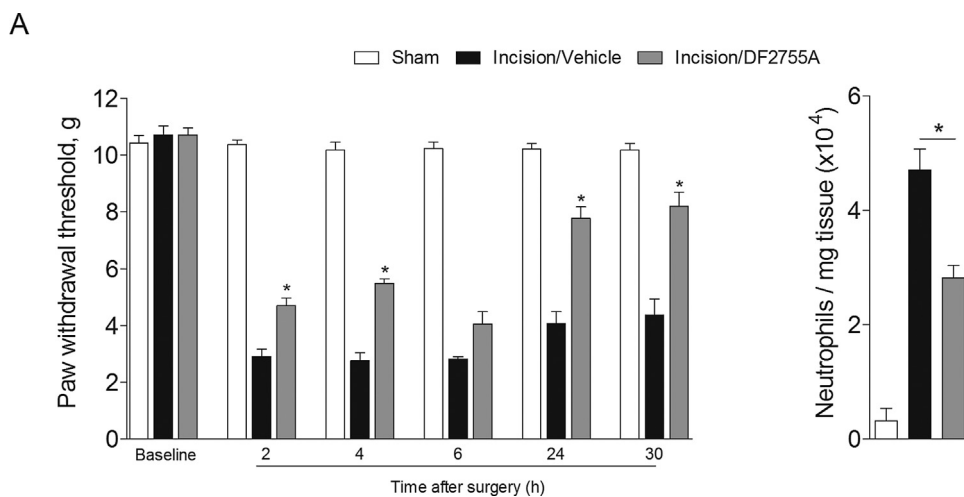


Fig. 6. Evaluation of the antinociceptive effect of DF2755A on post-operative pain model. Mechanical nociceptive threshold was measured (baseline, BL) followed by with DF2755A (30 mg/Kg, v.o) or vehicle (Veh). At 60 min latter mice were subjected to paw incision. Mechanical hyperalgesia was evaluated up to 30 h after surgery and hind-paws were then collected for MPO analysis (Panel A and B, respectively). DF2755A treatment was repeated 12 and 24 h after surgery. Data are shown as the mean \pm S.E.M. ($n = 6$ per group). * $P < 0.05$ as compared with vehicle-treated animals.

spinal effect of DF2755A peaked between 3 and 7 h after treatment returning to the control level 24 h after treatment (Fig. 7B).

Importantly, DF2755A did not show any opioid-like effect in the hot-plate test and did not cause any motor impairment that could have accounted for its analgesic effect (Fig. 8A and B).

4. Discussion

Inflammatory and postoperative pain are adequately treated with NSAIDs in the early phase. Nevertheless, this class of drugs used as the first choice presents several undesired side effects

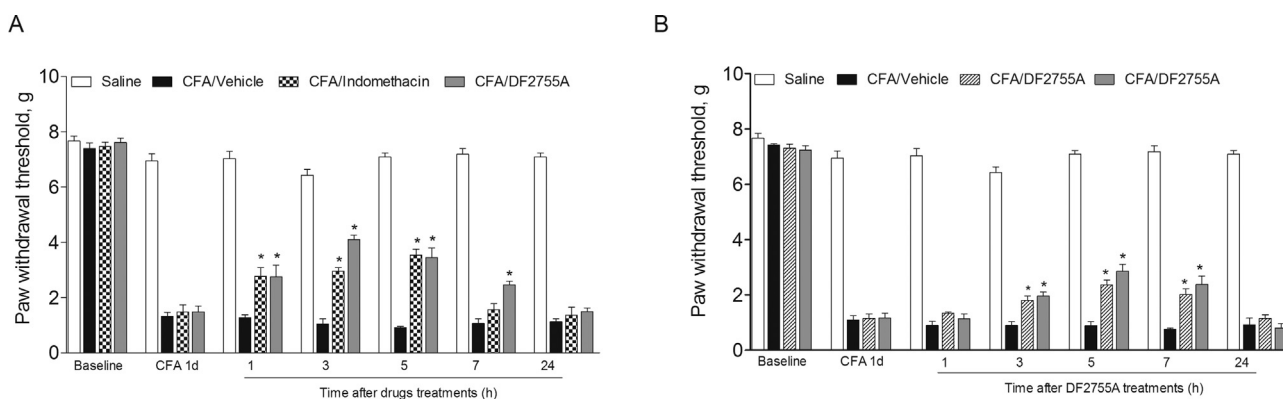


Fig. 7. Evaluation of the antinociceptive effect of DF2755A on chronic inflammatory pain model. Panel A—mechanical nociceptive threshold was measured before (baseline, BL) and 24 h after CFA (10 μ l/paw) intraplantar injection followed by treatment with vehicle, DF2755A (30 mg/Kg, v.o) or indomethacin (5 mg/kg s.c). Mechanical hyperalgesia was evaluated up to 24 h after DF2755A treatment. Panel B—mechanical nociceptive threshold was measured before (baseline, BL) and 24 h after CFA (10 μ l/paw) intraplantar injection followed by intrathecal treatment with vehicle or DF2755A (30 and 90 μ g/mice i.t.). Mechanical hyperalgesia was evaluated at 1–24 h after DF2755A treatment. Data are shown as the mean \pm S.E.M. ($n=6$ per group). * $P < 0.05$ as compared with vehicle-treated animals.

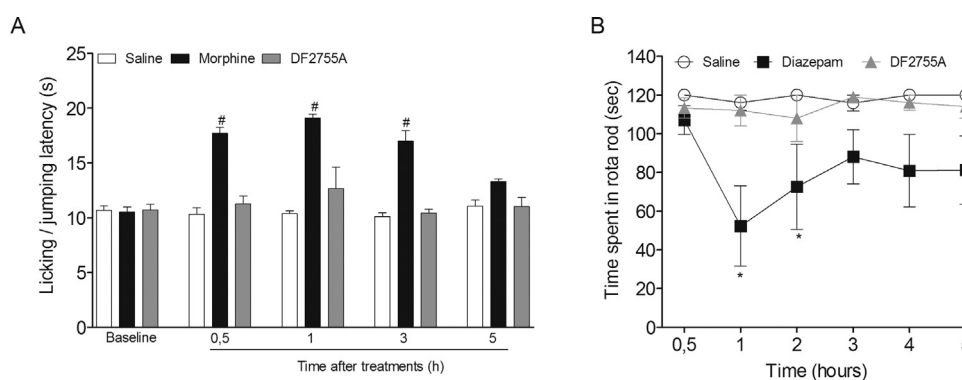


Fig. 8. Evaluation of non-specific effects of DF2755A. Panel A—the effect of DF2755A on thermal nociceptive threshold in naïve mice was evaluated after DF2755A treatment (30 mg/kg, p.o) using hot-plate test. Morphine (8 mg/kg s.c.) was used as a positive control. Effect of DF2755A on rota rod test in naïve mice. Mice were treated orally with vehicle or DF2755A (30 mg/Kg). Diazepam (5 mg/kg, s.c) was used as a positive control. Data are shown as the mean \pm S.E.M. ($n=6$ per group). * $P < 0.05$ when compared to vehicle-treated group.

that limit their use including ulcers lesions, kidney failure, and cardiovascular events. In this context, the developments of novel drugs that present more specificity to control these types of the inflammatory symptoms with less undesired effects are required. The CXCR1/2 and its ligands KC/CXCL1 have been implicated in the pathophysiology of inflammatory and post-operative pain [13,17,18,41] suggesting this pathway as a promising target for the development of novel pain-relieving drugs. In the present study, we have shown a novel orally acting allosteric antagonist of CXCR1/2, the DF2755A that was characterized and validated in *in vitro* and *in vivo* studies of inflammatory and post-incisional pain.

The biological relevance of CXCR1/2 chemokines ligands in the setting of inflammatory conditions including inflammatory pain has been extensively suggested [11–13]. For instance, antinociceptive effect of CXCR1/2 inhibitors has been previously reported [12,15,16]. In previous studies, we have shown that the blockade of CXCR1/2 reduced inflammatory and post-operative pain in several models in mice and rats [18,23,30]. These studies were further confirmed by other research groups [43,44]. Based on this concept, along the last decade an extensive medicinal chemistry program allowed the characterization of a novel class of SMW inhibitors of the chemokine receptors CXCR1/2 [23] acting as noncompetitive allosteric modulators of the receptors. The molecular mechanism of action and binding mode of the inhibitors were thoroughly investigated, and point mutagenesis studies led to the identification

of an allosteric pocket in the transmembrane region of CXCR1/2 [23,30,33].

With the aim to identify an ideal candidate able to counteract nociceptive effects associated with CXCR1/2 activation, the key characteristics regulating the penetration of drugs through the blood-cerebrospinal fluid barrier were considered. As a general rule, the ideal compound to is small, moderately lipophilic (lipid-water partition coefficient at pH 7.4 of around 1–10), with relatively low level of plasma protein binding and a volume of distribution of around 1 L/kg with weak binding affinity at P-gp or other efflux pump located at the blood-CSF barrier. Specifically, a quantitative structure-activity relationship study conducted on a series of aryl-propionic acids highlighted a significant parabolic relationship between lipophilicity expressed as the chromatographic capacity factor ($\log K_{IAM}$) and the capacity of diffusion in the CSF [45].

Taken together these evidences, we designed in the lead Optimization process a compound partially ionized at physiological pH being optimal for CSF penetration, ($\log D_{7.4}=0.3-1.7$). Within the class of dual CXCR1/CXCR2 inhibitors, DF2755A was selected as an optimized candidate for the treatment of inflammatory pain. The results reported here show that DF2755A inhibits both CXCL receptor subtypes, CXCR1 ($IC_{50}=4.2$ nM) and CXCR2 ($IC_{50}=2.1$ nM) without affecting significantly human leukocyte migration triggered by C5a, fMLP, and CCL2. As previously reported for reparixin, DF2755A was confirmed to act as a noncompetitive allosteric modulator of CXCR1/2, binding the receptor in a pocket

spanning between the transmembrane helices 1,2,3,7. CXCR2 alanine-replacement mutagenesis experiments were conducted to support the binding mode hypothesis the obtained results proved that the biological activity of DF2755A depends on the integrity of the allosteric binding pocket confirming D293 and E300 as the essential residues for receptor binding.

Molecular weight and lipophilicity of DF2755A are in the ideal range that was previously found compatible with distribution in the CSF. Based on acid/basic dissociation reactions, DF2755A shows a distribution coefficient of 0.713 at pH 7.4 (Log D = 0.714 pH 7.4) and the relatively weak P-gp affinity are in line with the characteristics required for blood-CSF barrier penetration. From a pharmacokinetic point of view, DF2755A exhibited good oral bioavailability (F% 88.2), a half-life of 2.88 h and distribution volume of 0.42 L/kg. Taken together, the excellent *in vitro* profile made DF2755A a promising compound suitable for further *in vivo* investigations on experimental models where inflammatory pain is a dominant feature.

DF2755A presented antinociceptive effect in several models of inflammatory and postoperative pain in mice. In fact, oral pretreatment with DF2755A can reduced inflammatory and post-operative pain and CXCL1/KC-induced mechanical hyperalgesia without significant sedative or central opioid-like analgesic action. The blockage of CXCR1/2 by DF2755A might counteract inflammatory and post-operative pain in several ways. In the inflammatory and post-operative pain models, the participation of CXCLs/CXCR1/2 pathway seems to be ascribed to the importance in neutrophils recruitment and activation [18,41,43]. In addition, we have shown that CXCLs/CXCR1/2 pathway triggers IL-1 β production that in turn mediates inflammatory pain in a prostaglandin dependent-manner [11]. Corroborating this hypothesis, DF2755A antinociceptive effect was associated with a reduction in the recruitment of neutrophils after paw inflammation and incision/surgery. Furthermore, DF2755A treatment was also able to reduced IL-1 β without any effect on TNF, CXCL1/KC or IL-6. Therefore, in the local of inflammation/surgery, DF2755A could disrupt the recruitment/activation of leukocytes and IL-1 β production, thus indirectly attenuating the sensitization of the primary nociceptive neurons.

Noteworthy, besides the local of inflammation, there is evidence in the literature showing that CXCLs chemokines are upregulated in the dorsal root ganglion after peripheral inflammation [46,47]. In the dorsal root ganglion, they could directly enhance primary nociceptive neurons excitability, which express CXCR2, though the modulation of TRPV1 and Na⁺ currents [48,49]. CXCL1 is also able to stimulate CGRP release and elevate intracellular calcium in DRG neurons [50]. Thus, part of the antinociceptive effect of DF2755A might be due to its ability to directly block peripheral sensitization of CXCR1/2-expressing nociceptors.

Interestingly, DF2755A was also effective to reduce inflammatory pain caused by CFA injection into the mice paw, similarly to a non-steroidal anti-inflammatory drug (indomethacin), even in a post-treatment schedule, thus confirming the therapeutic potential of the treatment and implying that CXCR1/2 is constantly stimulated and involved in the maintenance of persistent inflammatory pain.

In the CFA model, the hypothesis that antinociceptive effect of DF2755A might be at least in part due to its ability to block central sensitization was investigated. In fact, recent studies have shown that the expression of CXCLs and CXCR2 increase in the spinal cord after peripheral paw inflammation and incision [21,51]. Whereas CXCL1 seems to be produced by astrocytes, it may act on CXCR2-positive neurons of the spinal cord acts on CXCR2-expressing spinal neurons to increase ERK activation and NMDA transmission [21]. Functionally, intrathecal blockage of CXCR2 or neutralization of CXCL1 activity reduced both inflammatory

and post-operative pain in mice [21,51]. Our data extend these results showing that DF2755A is also effective when administered intrathecally. These findings confirm that the effect of systemically administered DF2755A might be also caused by its direct effect on spinal neurons.

In summary, this study characterized a novel orally acting allosteric molecule that selectively blocks human, rat and mouse CXCR1/2 signaling. The physicochemical properties of the new lead compound make DF2755A and an ideal candidate to study the potential effects of CXCR1/2 inhibition in the spinal cord. DF2755A showed antinociceptive effects in several inflammatory and post-operative pain conditions, which is coherent with its ability to inhibit CXCLs/CXCR1/2 pathway. The potent antinociceptive effect of intrathecal administration of DF2755A in the CFA-induced inflammatory pain model supports the hypothesis that drug penetration into CSF and direct action on CXCR2-expressing neurons significantly contribute to the observed pain-relieving outcome. In conclusion, this study suggests that DF2755A might be a candidate of a novel therapeutic option to control inflammatory and post-operative pain.

Conflict of interest

Marcello Allegretti, Laura Brandolini, Andrea Aramini and Gianluca Bianchini are employees of Dompé farmaceutici s.p.a., Italy. The company has interests in the development of CXCR2 allosteric modulators for the treatment of inflammatory diseases.

Acknowledgements

The authors gratefully acknowledge the technical assistance of Ieda R. dos Santos and Sergio R. Rosa. The research leading to these results has received funding from the European Union Seventh Framework Programme [FP7-2007-2013] under grant agreement n° HEALTH-F4-2011-281608 (TIMER), from São Paulo Research Foundation (FAPESP) under grant agreements n° 2011/19670-0 (Thematic project) and 2013/08216-2 (Center for Research in Inflammatory Disease-CRID) and from University of São Paulo NAP-DIN under grant agreement n° 11.1.21625.01.0.

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