Corrections

PHARMACOLOGY

The authors note that an additional affiliation should be listed for Emanuela Galliera. This author’s affiliations should appear as “Department of Biomedical, Surgical and Dental Sciences, University of Milan, I-20133 Milan, Italy; and Istituto di Ricerca e Cura a Carattere Scientifico (IRCCS) Galeazzi Orthopaedic Institute, I-20161 Milan, Italy.” The corrected author and affiliation lines appear below. The online version has been corrected.

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NEUROSCIENCE

The authors note that the author name Benoit Lebonté should instead appear as Benoit Labonté. The corrected author line appears below. The online version has been corrected.

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APPLIED PHYSICAL SCIENCES

The authors note that on page 19269, right column, fifth full paragraph, line 4, “200 ms” should instead appear as “200 μs.”

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Targeting the minor pocket of C5aR for the rational design of an oral allosteric inhibitor for inflammatory and neuropathic pain relief

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Chronic pain resulting from inflammatory and neuropathic disorders causes considerable economic and social burden. Pharmacological therapies currently available for certain types of pain are only partially effective and may cause severe adverse side effects. The C5a anaphylatoxin acting on its cognate G protein-coupled receptor (GPCR), C5aR, is a potent proinflammatory mediator in several models of inflammatory and neuropathic pain. Although there has long been interest in the identification of C5aR inhibitors, their development has been complicated, as for many peptidomimetic drugs, mostly by poor drug-like properties. Herein, we report the de novo design of a potent and selective C5aR noncompetitive allosteric inhibitor, DF2593A, guided by the hypothesis that an allosteric site, the "minor pocket," previously characterized in CXC chemokine receptors-1 and -2, is functionally conserved in the GPCR class. In vitro, DF2593A potently inhibited C5a-induced migration of human and rodent neutrophils. In vivo, oral administration of DF2593A effectively reduced mechanical hyperalgesia in several models of acute and chronic inflammatory and neuropathic pain, without any apparent side effects. Mechanical hyperalgesia after spared nerve injury was also reduced in C5aR−/− mice compared with WT mice. Furthermore, treatment of C5aR−/− mice with DF2593A did not produce any further antinociceptive effect compared with C5aR−/− mice treated with vehicle. The successful medicinal chemistry strategy confirms that a conserved minor pocket is amenable for the rational design of selective inhibitors and the pharmacological results support that the allosteric blockade of the C5aR represents a highly promising therapeutic approach to control chronic inflammatory and neuropathic pain.

C5a | inflammatory pain | neuropathic pain | allosteric antagonism | GPCR

Inflammatory and neuropathic pain are the most prevalent types of pathological pain and represent important health problems. Whereas inflammatory pain is one of the classic symptoms of the inflammatory process, neuropathic pain arises from any of multiple nerve lesions or diseases, with symptoms including hyperalgesia or allodynia (1, 2). Some of the most powerful painkillers, including opioids and nonsteroidal anti-inflammatory drugs, are only partially effective and prolonged exposure can cause unwanted effects (3, 4). As a result, there is continuous effort to identify novel therapeutics for pain control with alternative biological mechanisms and that elicit fewer side effects. Inflammatory mediators, including cytokines/chemokines, play a critical role in the pathogenesis of inflammatory and neuropathic pain (5, 6). Emerging evidences suggest that C5a, the anaphylatoxin produced by complement activation, has potent nociceptive activity in several models of inflammatory and neuropathic pain by interacting with its selective receptor C5aR (7, 8). C5aR belongs to the class A subfamily of the seven-transmembrane (TM) G protein-coupled receptors (GPCR) (9) and is widely expressed in immune cells, including neutrophils (polymorphonuclear cells, PMN), monocytes, microglia, and in nonimmune cells, including neurons in the CNS and dorsal root ganglia (10, 11). Evidence for a role of C5a in nociception sensitization has been obtained in several models of inflammatory pain. For example, C5a was produced at the inflammatory sites and elicited mechanical hyperalgesia by activating the C5aR on infiltrated PMN (7). Direct intraplantar injection of C5a in mice elicited both heat and mechanical hyperalgesia by sensitizing primary afferent C-nociceptors (12, 13). Local activation of C5aR has been also implicated in the pathogenesis of postsurgical pain, a model of postoperative pain (13). Finally, local administration of PMX-53, a C5aR antagonist, attenuated mechanical hyperalgesia induced by carrageenan, zymosan, or lipopolysaccharide (7). In addition to the peripheral role of C5a/C5aR in inflammatory pain, up-regulated levels of C5 and C5aR have been...
found in spinal cord microglia in animals subjected to spared nerve injury (SNI), a model of neuropathic pain (8). Indeed, C5-null mice or the infusion of PMX-53 into the intrathecal space reduced neuropathic pain hypersensitivity in the SNI model (8). Collectively, these data suggest that a neuroimmune interaction TM2 in the periphery and spinal cord through activation of the complement cascade and the production of C5a contributes to the genesis of both inflammatory and neuropathic pain.

As for other peptidic GPCRs, the efforts to identify small molecular weight C5aR antagonists have led to a limited number of molecules, mostly lacking adequate potency and selectivity (14). The most promising candidate so far described, PMX-53, is a cyclic peptidomimetic antagonist designed to mimic the C-terminal portion of C5a (15). Despite the encouraging results obtained in preclinical studies, as for many peptide drugs, the development of PMX-53 has been limited by its short half-life and unfavorable bioavailability (16). In the present study, we report the successful design of a nonpeptidic C5a allosteric small molecular weight inhibitor driven by the structural information on a minor pocket spanning between TM1, -2, -3, -6, and -7 that is highly conserved across the GPCR family and that has been recently proposed as a key motif for the intracellular activation process. Reparixin was previously reported as a neutral allosteric inhibitor of CXCR1 and CXCR2 that binds the TM in a region that features a deep minor pocket (17, 18). Combining the information from independent sources on structural and functional features of allosteric sites in homologous chemokine receptors, this paper intends to provide what is, to our knowledge, the first example of de novo design of a new class of allosteric small molecular weight inhibitors of a GPCR not belonging to the chemokine receptor family, C5aR. The preclinical candidate, DF2593A, is a potent and orally active C5a noncompetitive allosteric inhibitor with significant antinociceptive effects in a wide range of inflammatory and neuropathic pain models.

Results

Binding Mode Characterization of DF2593A to C5aR. The human C5aR (hC5aR) homology model was originally built using the human CXCR1-reparixin complex (19) as a template, and subsequently refined and compared with the C5aR model built starting from the human C-C chemokine receptor type 5 (hCCR5) crystal structure (PDB ID code 4MBS), in which CCR5 is bound with the marketed HIV allosteric drug maraviroc (20, 21). Sequence identity between hCCR5 and hC5aR is 21.3%, whereas sequence similarity is 52.4%. Despite a low sequence identity, the key structural features defining the minor pocket, the proline kink in TM2-3 and the water-mediated hydrogen bond network between the inner cellular segments of TM1, -2, -3, -6, and -7 is highly conserved between chemokine receptors and C5aR. The preclinical candidate, DF2593A, is a potent and orally active C5a noncompetitive al- losteric inhibitor with significant antinociceptive effects in a wide range of inflammatory and neuropathic pain models.

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DF2593A is a selective noncompetitive allosteric inhibitor of C5aR. (A) Two-dimensional structure of DF2593A. (B) Two-dimensional representation of DF2593A binding mode inside C5aR. Pink, green/blue and orange lines represent, respectively: charge-charge interaction, H-bond/halogen-bond interaction, π- and π interactions. (C) Three-dimensional representation of DF2593A in the TM region of C5aR (violet). Interacting residues are shown using the following color scheme: C (green), N (blue), O (red), S (yellow), F (cyan), and H (white); labeling is based on the Ballesteros-Weinstein numbering scheme. (D) Effect of DF2593A on the C5a-induced migration of WT (●), L41A (▲), F93A (▼), I96A (▲), L278A (▲), N119A (▼), and Y258A (▲) C5aR/L1.2 transfectants preincubated with indicated DF2593A concentrations and then stimulated with 10 nM of C5a for 4 h. All mutants show a significant (P < 0.05) resistance to DF2593A at all doses tested compared with WT C5aR transfectants. (E) Effect of DF2593A on the C5a-induced migration of human PMN preincubated for 15 min with indicated DF2593A concentrations and then stimulated with 10 nM C5a (●), 1 μM C5a-desarg (○), 1 nM CXCL1 (▲), 1 nM CXCL8 (△), 10 nM CXCL12 (▲), 30 nM CCL3 (△), and 10 nM fMLP (▼) for 1 h. (F) Effect of DF2593A on the C5a-induced migration of human (●), mouse (○), and rat (▲) PMN preincubated with indicated DF2593A concentrations and then stimulated with 10 nM C5a of corresponding origin. All leukocytes were significantly inhibited at doses above 10^{-9} M and were equally sensitive. (G) Human PMN incubation with vehicle (●) or 1 μM of DF2593A (○) and then aliquots of 0.2 nM of [35S]-C5a and serial dilution of cold C5a were added to 10^6 cells in 100 mL of binding medium. There was no significant difference between vehicle and DF2593A. (H) Human PMN were directly exposed to vehicle or different concentrations of DF2593A (○) and then aliquots of 0.2 nM of [35S]-C5a and vehicle (DF2593A-treated PMN) or serial dilution of unlabeled C5a (vehicle-treated PMN) (●) were added to 10^6 cells in 100 mL of binding medium. Significant difference were observed at doses above 10^{-9} M. In D to F, data are expressed as percent of migrated cells observed in the absence of DF2593A (mean ± SD of three independent experiments). P < 0.01 versus cell migration in the absence of DF2593A by Mann-Whitney U test. In G and H, data are reported as mean ± SD of three independent experiments.

In Vitro Characterization of DF2593A. Pharmacological characterization showed that DF2593A did not inhibit spontaneous cell migration per se and was >1,000-fold selective versus other chemokine receptors, including CXCL8 and CXCL1 (IC_{50} > 10 μM) (Fig. 1E). DF2593A effectively inhibited C5a-induced human PMN migration (IC_{50} = 5.0 nM) and cross-reacted with rat and mouse orthologs (IC_{50} = 60 nM and IC_{50} = 1.0 nM, respectively) (Fig. 1F). DF2593A (tested at 10 μM) was selective on a panel of different GPCRs (Table S3) and ion channels (Table S4) and was also completely inactive (percent inhibition = 10 ± 3; mean ± SD; three experiments performed in duplicate) in a prostaglandin E_{2} (PGE_{2}) production assay (28).

Binding experiments carried out on PMN membranes with radiolabeled C5a showed that DF2593A did not compete with binding of C5a. Pretreatment of PMN with DF2593A (1 μM) did not change C5a affinity for C5aR (K_{d} = 1.8 ± 1 nM and 1.0 ± 0.4 nM in vehicle and DF2593A-treated groups, respectively; n = 3 per group). In addition, DF2593A did not affect the number of C5aR molecules expressed on the cell membrane (127,000 ± 13,000 and 133,000 ± 21,000 binding sites per cell in vehicle and DF2593A-treated groups, respectively) and did not alter C5a binding to C5aR in displacement experiments (Fig. 1G and H).

Pharmacokinetic Profile of DF2593A. The pharmacokinetics of DF2593A was investigated in mice after intravenous and oral administration. Following oral administration (1 mg/kg) in mice, DF2593A was well absorbed (F = 83%) with a C_{max} of 0.1 μM, a t_{max} of 1.2 h, and plasma samples harvested over a period of 12 h generated free plasma drug concentrations in the range 0.016–0.004 μM, about 4- to 16-fold greater than its in vitro IC_{50}. For
brain permeation studies, DF2593A was dosed intravenously at 10 mg/kg and the levels of DF2593A were 913 ng/g tissue and 673 ng/mL in brain and plasma, respectively, at 5 min postdose. At 8 h postdose, significant levels of DF2593A were still present in the brain (114 ng/g) and also in the plasma (56 ng/mL). The brain-to-plasma ratios of DF2593A ranged from 1.36 (at 5 min postdose) to 2.04 at 8 h postdose. In parallel, the distribution coefficient, logBB, increased from 0.13 (at 5 min) to 0.3 (at 8 h), suggesting good permeation of the molecule into the brain.

**Antinociceptive Effects of DF2593A in Several Models of Inflammatory Pain.** C5a/C5aR interactions mediate carrageenan-induced mechanical hyperalgesia in rodents, suggesting it was a reasonable model to evaluate the potential analgesic effect of DF2593A (7). Corroborating this finding, carrageenan-induced inflammatory hyperalgesia is reduced in C5aR−/− mice compared with WT mice (Fig. S4). Consistent with its in vitro potency, oral administration of DF2593A inhibited carrageenan-induced mechanical hyperalgesia in a dose-dependent manner, but did not alter mechanical threshold in naive mice (Fig. 2A). C5a-induced hyperalgesia was also blocked by DF2593A, supporting the concept that DF2593A blocks hyperalgesia by inhibiting the C5aR (Fig. 2B). Neither DF2593A nor C5aR−/− deletion affected mechanical hyperalgesia induced by PGE2 injected in the mice paws (Fig. 2C and Fig. S4). Furthermore, DF2593A did not alter carrageenan-induced hyperalgesia in the mice paws (Fig. 2C). In an attempt to test the effect of DF2593A against chemical nociception, a writhing test induced by zymosan was studied (29). Oral treatment of mice with DF2593A significantly reduced zymosan-induced writhing response (Fig. 2D). DF2593A had no opioid-like effect in the hot-plate test and did not cause any motor impairment that could have accounted for the analgesic effects observed (Fig. 2 E and F). Treatment with DF2593A or genetic deletion of C5aR (C5aR−/− mice) did not alter carrageenan-induced PMN accumulation (Fig. S5), in agreement with our previous findings (7).

The analgesic effect of DF2593A was then tested in the complete Freund’s adjuvant (CFA)-induced chronic inflammatory pain model. Oral pretreatment with DF2593A inhibited CFA-induced mechanical hyperalgesia (Fig. 3A). Consistent with its plasma levels, the effect of DF2593A was detected at least 6 h after CFA injection, but not at 24 h (Fig. 3A). Next, we probed whether the posttreatment with DF2593A could modify mechanical CFA-induced hyperalgesia and whether the analgesic effect was maintained. As reported in Fig. 3 B and C, delayed treatment with DF2593A (24 h after CFA injection) reduced the CFA-induced mechanical and thermal inflammatory hyperalgesia. In a therapeutic setting, treatment with DF2593A starting at 24 h after CFA injection and then twice a day for 1 wk reduced mechanical hyperalgesia (Fig. 3D). Interestingly, the effect of DF2593A was maintained and when DF2593A treatment was stopped, mechanical hyperalgesia resumed to basal levels (Fig. 3D). There is evidence to suggest that C5a contributes to the pathogenesis of rheumatoid arthritis, including arthritic pain (7, 30). Therefore, in the next step we evaluated the effect of DF2593A in the genesis of articular hyperalgesia in two models of arthritis in mice, antigen- and zymosan-induced arthritis. Oral pretreatment with DF2593A effectively inhibited articular hyperalgesia in both models (Fig. 3 E and F).

**Antinociceptive Effects of DF2593A in the SNI Model of Neuropathic Pain.** Because activation of the complement system at the site of nerve injury and in the spinal cord contributes to induction and establishment of neuropathic pain (8, 31, 32), the effect of DF2593A in the SNI-induced neuropathic pain model in mice was evaluated. Oral treatment with DF2593A 7 d after surgery clearly reduced mechanical hypersensitivity induced by SNI (Fig. 4A). The effects of DF2593A persisted at least 6 h after treatment and, coherently with the pharmacokinetic profile, a progressive loss of antinociceptive effect was observed after 24 h (Fig. 4A). Confirming the involvement of C5aR in the genesis of neuropathic pain, mechanical hypersensitivity 7 d after SNI was also reduced in C5aR−/− mice compared with WT mice (Fig. 4B). Furthermore, treatment of C5aR−/− mice with DF2593A did not produce any further antinociceptive effect compared with C5aR−/− mice treated with vehicle (Fig. 4B). The mechanical nociceptive threshold of C5aR−/− mice did not differ from WT mice (mechanical nociceptive threshold of naive WT mice: 7.2 ± 0.6 g and naive C5aR−/− mice: 6.8 ± 0.1 g).

In a therapeutic setting, DF2593A was given twice a day from day 7 to day 14 after surgery and mechanical hypersensitivity measured 6 h after the first daily dose. As seen in Fig. 4C, the effects of DF2593A were immediate and maintained over the observation period. Mechanical hypersensitivity returned to the same level of SNI control group 2 d after the suspension of the DF2593A treatment (Fig. 4C).

**Discussion**

This paper reports the molecular conception, synthesis, and characterization of the preclinical candidate DF2593A that shows potent and selective inhibitory effect on the C5a-induced PMN migration and optimal pharmacokinetic and pharmacological profile in a panel of relevant inflammatory and neuropathic pain experimental models.

In recent years, allosteric modulation of GPCRs has been presented as a promising new paradigm for the design of potent and selective drugs with improved drug-like properties, finely modulating the receptor function. There is emerging evidence to suggest that, despite marked differences between the natural ligands, GPCRs share common activation mechanisms involving specific microswitches that regulate interhelical movements, which offer unprecedented opportunities for the rational drug design of
novel allosteric modulators. In this context, modeling and crystallographic studies of the GPCRs have identified in the TM region of GPCRs, a major pocket and a minor pocket, which has been proposed as a "triggering domain" not involved in natural ligand binding but crucial for the fine tuning of the global receptor activation process (17). Previous studies elucidated that reparixin, an allosteric inhibitor of CXCL8 receptors, binds CXCRI within the minor pocket, locking the receptor in the inactive state by interhelical polar interactions with residues of TM1, -3, -6, and -7 (19).

Guided by the hypothesis that this minor pocket may represent a functionally conserved site across the GPCR family, homology modeling studies and molecular dynamics simulations of C5aR were carried out. Despite the low overall identity between C5aR and chemokine receptors, sequence analysis revealed that the three key residues identified for reparixin and CXCRI are conserved in C5aR (22). Taking advantage of the structural diversity of other residues surrounding the minor pocket in C5aR and CXCRI, rational design was addressed by targeting a specific pattern of interactions to retain full selectivity to C5aR. Extensive site-directed mutagenesis studies in C5aR confirmed that this region is not directly involved in ligand receptor binding or critical for receptor activation and function. This finding nicely fits with the most accredited model of C5a/C5aR interaction (33), according to which the flexible C-terminal of C5a is implicated in the recognition of a domain distinct from the allosteric pocket and delineated mainly by Arg206, TM4, and the second extracellular loop. Nevertheless, our studies clearly demonstrated that specific ligands at this minor but conserved site are potent inhibitors of the C5a-induced chemotaxis, acting as neutral noncompetitive allosteric inhibitors. Interestingly, as the key residues involved are well conserved in both rat and mouse orthologs, the above results pave the way for the rational design of allosteric C5aR inhibitors with most favorable cross-species reactivity characteristics.

Previous studies have characterized the role of the C5a/C5aR signaling in the genesis of inflammatory and neuropathic pain (7, 8). Neuropathic pain is an important and relatively common clinical condition and available therapies are only partially effective. Among the events implicated in the genesis of neuropathic pain, neuroimmune interactions, including spinal activation of glial cells and the production of proinflammatory mediators, seem to be important for pain amplification. It was shown that expression of common genes, including the complement system genes, occurred in three different models of peripheral neuropathy (8). These findings are in agreement with the inhibition of complement activation by the intrathecal administration of a soluble human complement receptor type 1, which prevented the activation of C3 and C5 convertases and reduced mechanical allodynia in various neuropathic pain models (31). Additionally, C6-deficient rats still presented nerve injury-induced mechanical allodynia, suggesting that the membrane attack complex assembly is not necessary for the induction of neuropathic pain states (8). Accordingly, the nociceptive activity of the complement system appears to be strongly dependent on the C5a/C5aR interaction. Indeed, after nerve injury, expression of C5aR and C5 in the microglial cells of the spinal cord increased, and C5-deficient mice or intrathecal treatment with the classic antagonist PMX-53 ameliorated nerve injury-induced allodynia (8). Taken together, this preclinical evidence strongly supports the hypothesis that C5aR is an interesting target for neuropathic pain control.

The precise cellular site of action of DF2593A in the nociceptive system is not fully understood. Considering the aforementioned evidence of a role of C5a in the CNS, the efficacy of DF2593A in the SNI model may arise in part from its permeability to the blood–brain barrier. However, activation of the complement system in the periphery, at the level of nerve injury and dorsal root ganglia, could also contribute to the genesis of neuropathic pain (32, 34). In the periphery, DF2593A could disrupt the activation and the recruitment of leukocytes, thus attenuating the direct sensitization of the primary nociceptive neurons expressing C5aR (35). Importantly, DF2593A was effective even when given 1 wk after SNI, and nociceptive hypersensitivity returned to basal values when the DF2593A treatment was halted. These results clearly confirm the therapeutic potential of DF2593A and show that continuous activation of the C5aR is relevant for induction and maintenance of nociceptive hypersensitivity in the SNI model.
In the inflammatory pain models, the actions of CSa appeared to be sequential to PMN activation and are recruited into the inflammatory site in response to chemokines and lipid mediators (7). Recent evidence showed that CSaR is expressed in primary nociceptors and that CSa could directly sensitize these fibers (12, 13). Our studies showed that DF2593A blocked carrageenan and CSa-dependent mechanical hyperalgesia in vivo without sedative or central opioid-like effects. Interestingly, DF2593A was also effective in a model of chronic inflammatory pain and in two models of inflammatory arthritic disease, even when administered 24 h after the induction of inflammation, sustaining the therapeutic potential of DF2593A and suggesting that CSa is continuously produced and participates in the events leading to maintenance of chronic inflammatory pain. On the other hand, DF2593A did not affect mechanical hyperalgesia induced by PGE2 or epinephrine, which directly cause sensitization of primary nociceptive neurons (6). Therefore, the analgesic effects of DF2593A cannot be ascribed to a nonspecific action and is related to its ability to block CSa/CSaR signaling, presumably on both PMN and nociceptive neurons. Finally, it is important to point out that the results obtained with CSaR−/− mice strongly provide target validation/specificity for the effects of DF2593A.

Our findings underline the functional relevance of the minor pocket in GPCR that, although not directly involved in natural ligand binding, cooperates with the fine-tuning of the receptor activation process and represents an attractive structural determinant for rational design of innovative therapeutic compounds. Our pharmacological results not only provide further support to the role of CSa/CSaR signaling in the generation and maintenance of neuropathic pain, but also demonstrate that DF2593A represents an innovative noncompetitive allosteric drug candidate to control pain in multiple therapeutic indications.

Materials and Methods
See SI Materials and Methods for a full discussion of methods used.

Molecular Modeling. The TM domains of CXCR1, CXCR2, and CSaR were identified by sequence alignments with rhodopsin structure by using the MUSCLE software. The CSaR model was refined and compared with CCR5 (PDB ID code 4M8S) and further optimized by verifying the GPCR TM-fingerprint alignment (20, 21). The CSaR TM bundle was assembled and refined as described in SI Materials and Methods and the final CSaR structure was used to dock DF2593A using LiGen.

Cells and Migration Assay. PMN and L1.2 cells migration was evaluated using a 48-well microchemotaxis chamber, as previously described (19).

Mechanical Nocturnal Paw Test in Mice. Mechanical hyperalgesia was tested in C57BL/6, BALB/c mice and in CSaR-deficient mice (CSaR−/−) using the electronic von Frey test, as previously reported (6).

SNI-Induced Neuropathic Pain-Like Behavior. The SNI procedure comprised an axotomy and ligation of the tibial and common peroneal nerves, leaving the sural nerve intact (36).

Data Analyses and Statistics. For in vivo experiments, results are presented as mean ± SEM. The differences among the groups were compared by ANOVA (one-way) followed by Bonferroni’s post hoc test. The level of significance was set at P < 0.05.

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10. Molecular Modeling.
15. Rosenkilde MM, Benned-Jensen T, Frimurer TM, Schwartz TW (2010) The minor pocket in GPCR that, although not directly involved in natural ligand binding, cooperates with the fine-tuning of the receptor activation process and represents an attractive structural determinant for rational design of innovative therapeutic compounds. Our pharmacological results not only provide further support to the role of CSa/CSaR signaling in the generation and maintenance of neuropathic pain, but also demonstrate that DF2593A represents an innovative noncompetitive allosteric drug candidate to control pain in multiple therapeutic indications.

Materials and Methods
See SI Materials and Methods for a full discussion of methods used.

Molecular Modeling. The TM domains of CXCR1, CXCR2, and CSaR were identified by sequence alignments with rhodopsin structure by using the MUSCLE software. The CSaR model was refined and compared with CCR5 (PDB ID code 4M8S) and further optimized by verifying the GPCR TM-fingerprint alignment (20, 21). The CSaR TM bundle was assembled and refined as described in SI Materials and Methods and the final CSaR structure was used to dock DF2593A using LiGen.

Cells and Migration Assay. PMN and L1.2 cells migration was evaluated using a 48-well microchemotaxis chamber, as previously described (19).

Mechanical Nocturnal Paw Test in Mice. Mechanical hyperalgesia was tested in C57BL/6, BALB/c mice and in CSaR-deficient mice (CSaR−/−) using the electronic von Frey test, as previously reported (6).

SNI-Induced Neuropathic Pain-Like Behavior. The SNI procedure comprised an axotomy and ligation of the tibial and common peroneal nerves, leaving the sural nerve intact (36).

Data Analyses and Statistics. For in vivo experiments, results are presented as mean ± SEM. The differences among the groups were compared by ANOVA (one-way) followed by Bonferroni’s post hoc test. The level of significance was set at P < 0.05.

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