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Biased Agonism as a Novel Strategy To Harness the Proresolving Properties of Melanocortin Receptors without Eliciting Melanogenic Effects

Trinidad Montero-Melendez,* Thomas Gobbetti,* Sadani N. Cooray,* Thomas E. N. Jonassen,† and Mauro Perretti*

There is a need for novel approaches to control pathologies with overexuberant inflammatory reactions. Targeting melanocortin (MC) receptors represents a promising therapy for obesity and chronic inflammation, but lack of selectivity and safety concerns limit development. A new way to increase selectivity of biological effects entails the identification of biased agonists. In this study, we characterize the small molecule AP1189 as a biased agonist at receptors MC1 and MC3. Although not provoking canonical cAMP generation, AP1189 addition to MC1 or MC3, but not empty vector, transfected HEK293 cells caused ERK1/2 phosphorylation, a signaling responsible for the proeroferocytic effect evoked in mouse primary macrophages. Added to macrophage cultures, AP1189 reduced cytokine release, an effect reliant on both MC1 and MC3 as evident from the use of Mc1r and Mc3r−/− macrophages. No melanogenesis was induced by AP1189 in B16-F10 melanocytes. In vivo, oral AP1189 elicited anti-inflammatory actions in peritonitis and, upon administration at the peak of inflammation, accelerated the resolution phase by ∼3-fold. Finally, given the clinical efficacy of adrenocorticotropin in joint diseases, AP1189 was tested in experimental inflammatory arthritis, where this biased agonist afforded significant reduction of macroscopic and histological parameters of joint disruption. These proof-of-concept analyses with AP1189, an active oral anti-inflammatory and resolution-promoting compound, indicate that biased agonism at MC receptors is an innovative, viable approach to yield novel anti-inflammatory molecules endowed with a more favorable safety profile. The Journal of Immunology, 2015, 194: 000–000.

Melanocortin (MC) receptors (MC1–MC5), a family of class A druggable G protein–coupled receptors (GPCRs), are attractive therapeutic targets for a number of conditions due to their wide distribution and diversity of physiological processes they regulate (1). MC1 regulates UV light–induced skin tanning and other immune responses because of its expression on leukocytes. MC2 regulates cortisol production on the adrenal glands, whereas MC3 plays a role on exocrine glands secretions. MC3 and MC4 exert nonredundant functions on energy homeostasis in addition to specific anti-inflammatory roles: whereas MC3 activation is particularly protective for arthritides (2), MC4 provides neuroprotection in brain inflammation (3). Accordingly, the array of pathological situations that could be targeted with MC drugs includes skin conditions (vitiligo, melanoma, erythropoietic protoporphyria), cardiovascular pathologies, joint inflammation (gout, rheumatoid arthritis [RA]), obesity, cachexia, or sebaceous glands disorders such as acne vulgaris (4).

Peripheral MC1 and MC3 can be pharmacologically activated to induce anti-inflammation. The endogenous agonist α-melanocyte–stimulating hormone (αMSH), like other protective mediators, is released by immune cells to counterbalance proinflammatory signals, thus preventing excessive tissue damage (5, 6). In line with the resolution of inflammation concept, therapeutics targeting MC1 and MC3 will then be acting by mimicking the body’s own protective resources (7, 8) and might be characterized by a lighter burden of side effects.

Shown to be effective in RA rheumatic diseases since the early 1950s (9), the use of corticosterone or adrenocorticosterone hormone (ACTH) declined when synthetic glucocorticoids became available. However, the discovery of an alternative anti-inflammatory mechanism for ACTH involving activation of peripheral MC receptors on immune cells (10) has revived the interest in developing novel ACTH-like molecules with no steroidogenic effects for the treatment of joint diseases such as gout or RA (11, 12). However, the limitation in the translational delivery of novel MC drugs besides the marketed ACTH formulations (H.P. Acthar Gel, Synacthen Depot, or Cortrosyn) is imposed by the lack of receptor selectivity achieved so far.

Innovative approaches in G protein–coupled receptor drug discovery might help to overcome this limitation. Allosteric modulation consists in the ability of a molecule to enhance (positive modulation) or reduce (negative modulation) the effect of the endogenous ligand by binding to a distinct site of the receptor protein, termed allosteric site (13). A higher degree of selectivity is expected as allosteric regions are less conserved among the five MCRs, and indeed, allosteric modulators at MC4 are currently under development for the treatment of obesity (14).

Abbreviations used in this article: ACTH, adrenocorticotropin hormone; AGRP, agouti-related protein; EIA, enzyme immuno assay; MC, melanocortin; αMSH, α-melanocyte–stimulating hormone; NDP-αMSH, [Nle4,D-Phc7]-αMSH; RA, rheumatoid arthritis; WT, wild-type.

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Another emerging concept of significant therapeutic interest is the one of biased agonism. The obsolete notion that receptors could exist in two unique conformations, the active one and the inactive one, has been replaced with the conception that multiple active conformations can exist, each one creating a distinct signal yielding to multiple functional outcomes (15, 16). Receptor activation, rather than linear and static, is emerging as a highly dynamic and multidimensional process in which a diversity of active conformations may be induced by different molecules leading to distinct effects.

We reasoned that this new pharmacological approach would have a positive impact on MC-based drug development because it sets the focus on “pathway selectivity” rather than “receptor selectivity,” given the proven difficulty of the latter strategy. In addition, fine-tuned molecules could be designed to engage only the therapeutically relevant pathway and not those leading to side effects. In this article, we present the first, to our knowledge, biased dual agonist at MC1r and MC3r, and describe its anti-inflammatory properties together with its lack of effect on melanogenesis.

Materials and Methods

MC drugs and inhibitors

αMSH and [Nle4,D-Phe7]αMSH (NDP-αMSH) were purchased from Tocris Bioscience (Abingdon, U.K.). (E)-N-[125I]NDP-MC3 and empty vector pCMV6 were purchased from Origene (Rockville, MD). Serum-starved cells were transfected with Lipofectamine 2000 (Invitrogen, Paisley, U.K.) according to manufacturer’s instructions and used 24 h after transfection.

Radioligand binding assay

Determination of binding affinities was performed by 125I-NDP-αMSH radioligand binding. Cells were suspended in HEPES buffer and incubated with 0.05 nM 125I-NDP-αMSH. AP1189 dissolved in DMSO was added, and cells were incubated for 90 min at 22˚C (MC1 cells -B16-F10-) or for 60 min at 37˚C (MC3 cells -CHO stable transfected cells-). After incubation, the amount of bound radioligand was determined and expressed in percentage of total binding in the absence of AP1189.

ELISA and enzyme immunoassay

IL-1β, IL-6, and TNF-α were measured in cell supernatants by ELISA using Ready-SET-Go ELISA kits from eBioscience (Hatfield, U.K.). cAMP was measured using the cAMP enzyme immunoassay (EIA) kit from Cayman Chemical (Cambridge, U.K.).

Western blotting

HEK293A cells were lysed using radioimmunoprecipitation assay buffer (Thermo Scientific, Waltham, MA) containing protease inhibitors mixture (Calbiochem, Hertfordshire, U.K.). Samples were subjected to standard SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Merck Millipore). These were incubated with rabbit anti-phospho-ERK1/2 (42/44 kDa, 1:1000; Cell Signaling, Leiden, the Netherlands) overnight and hors eradicator anti-rabbit IgG for 1.5 h (1:2000; Dako, Stockport, U.K.). Membranes were rebotted with anti-α-Tubulin (55 kDa, 1:5000; Sigma-Aldrich, Dorset, U.K.) for 1 h and horseradisher anti-mouse IgG for 1 h (1:2000; Dako, Stockport, U.K.). Proteins were detected with Luminata Forte Western HRP substrate (Merck Millipore, Hertfordshire, U.K.) and visualized on the FluorChem E Digital Room (ProteinSimple, East Sussex, U.K.).

Ca2+ mobilization assay

Twenty-four hours after transient transfection, HEK293 cells were incubated with 2 μM Fura 2-AM (Molecular Probes, Paisley, U.K.) in HBSS without Ca2+ (Sigma-Aldrich) at 37˚C for 45 min in the dark. Subsequently, cells were washed three times in HBSS. HBSS containing 0.185 mM CaCl2 was then added before stimulation with agonists at the indicated concentrations. Ionomycin (1 μM) was used as a positive control. Mobilization of intracellular calcium was measured by recording the ratio of fluorescence emission at 510 nm after sequential excitation at 340 and 380 nm using the NOVostar microplate reader (BMG LABTECH, Aylesbury, U.K.) during 70 s after drug addition. Then data corresponding to time 25 s after drug stimulation were selected to generate concentration-response curves.

Determination of melanin accumulation

B16-F10 cells were cultured in αMEM containing 10% FCS and 1% penicillin/streptomycin, and kept at 37˚C with 5% CO2. For melanin determination, cells were plated in 96-well plates using phenol red free media. Melanin content was determined spectrophotometrically at 405 nM in supernatants 72 h after drug stimulation (17).

Isolation of primary peritoneal macrophages

Mice were injected i.p. with 1 ml of 2% Biogel (Bio-Rad, Hemel Hempstead, U.K.). Four days later, peritoneal cells were collected by lavage using 4 ml of 3 mM EDTA in PBS and plated in 24-well plates at a density of 0.5 × 10^6 cells/well, in RPMI 1640 containing 10% FCS and 50 mg/ml gentamicin. After 2 h of incubation, nonadherent cells were removed. Compounds or vehicle were added 30 min before stimulation with 25 μg/ml zymosan A (Sigma-Aldrich). Supernatants were collected after 5 h.

Isolation of human primary neutrophils

Experiments using healthy volunteers were approved by the local research ethics committee (P/000/029 East London and The City Local Research Ethics Committee 1). Informed written consent was provided, according to the Declaration of Helsinki. Blood was collected into 3.2% sodium citrate and diluted 1:1 in RPMI 1640 before separation through a double-density gradient using Histopaque 10771 and 11191 (Sigma-Aldrich). Contaminating erythrocytes were removed by hypotonic lysis. Polymorphonuclear cells were incubated in 10% FCS overnight at 37˚C, 5% CO2 to let neutrophils undergo spontaneous apoptosis.

Phagocytosis and efferocytosis

Primary peritoneal macrophages were stimulated with compounds/vehicle for 30 min before the addition of zymosan A at 1:10 (macrophage/zymosan) ratio for 15 min, or apoptotic neutrophils (1:2, macrophage/neutrophil) for 1 h. Cells were fixed and neutrophils stained using the myeloperoxidase assay by adding 0.1 mg/ml dimethoxybenzidine (Sigma-Aldrich) and 0.03% (v/v) hydrogen peroxide for 1 h (18). Cells were analyzed by light microscopy with three random fields being acquired per well (n = 3 wells/treatment). More than 400 cells were blindly counted per treatment point.

Animals

All animal studies were approved by and performed under the guidelines of the Ethical Committee for the Use of Animals, Barts and The London School of Medicine and Home Office regulations (Guidance on the Operation of Animals, Scientific Procedures Act, 1986). Male (7–8 wk old) C57BL/6J wild-type (WT) and BALB/c mice were purchased from Charles River Laboratories. Breeding pairs of the McIr mutant (McIr−/−; recessive yellow e/e) and Mc3r−/− colonies were originally obtained from Jackson Laboratories or donated by Dr. H.Y. Chen (Merck), respectively.

Zymosan-induced peritonitis model

Peritonitis was induced by the injection of 1 mg zymosan A (Sigma-Aldrich) i.p. in 0.5 ml sterile PBS. At the indicated times, mice were sacrificed by CO2 exposure, and peritoneal cavities were washed with 4 ml ice-cold PBS containing 3 mM EDTA. Cells were stained with Turk’s solution (0.01% crystal violet in 3% acetic acid) and counted using a Neubauer hemocytometer or were stained with FITC-conjugated mAb for Ly-6G/Gr1, F4/80, and corresponding isotype controls (eBioscience, Hatfield, U.K.), and subjected to flow-cytometry analysis using a BD FACSCalibur platform (BD Biosciences, Oxford, U.K.).

KB/BxN serum-induced arthritis model

Arthritis was induced with two i.p. injections of 100 μl KB/BxN serum on days 0 and 2. Disease was monitored daily until day 8 by assessing the paw volume using a plethysmometer (Ugo Basile, Comerio, Italy), disease incidence, and clinical score (score per paw: 0 = no signs of inflammation, 1 = subtle inflammation, localized, 2 = easily identified inflammation but localized, 3 = evident inflammation, not localized; maximum score = 12).
per mouse). Pharmacological treatments were administered orally once daily from day 2 until the end of the experiment.

Histological analysis

Tissues (ankles) were fixed with 4% neutral-buffered formalin, decalcified with 10% formic acid, and paraffin embedded. Sections (4 μm) were stained with H&E (Sigma-Aldrich) or fast green and safranin O (Sigma-Aldrich). Sections were graded from 0 (no disease) to 3 (severe) based on the degree of synovitis and cartilage degradation.

Statistical analysis

Experiments were repeated from two to five independent times. Data were analyzed by Student t test, one- or two-way ANOVA, followed by Dunnnett’s or Bonferroni’s multiple comparison test when appropriate. Nonlinear regression models were used to generate dose–response curves for cAMP and ERK phosphorylation. In all cases, data are presented as mean ± SEM of n independent observations and were considered statistically significant when p < 0.05.

Results

API1189 is a biased agonist at the MC receptors MC1 and MC3

API1189 structure was designed on phenyl-pyrrrole-aminoguanidine derivatives, shown to activate MC receptors (patent no. WO200714143A1). However, when API1189 was tested on HEK293A cells transfected with mouse receptors for activation of the canonical cAMP pathway, no signal was observed with MC3 cells and a response on MC2 cells only at high concentrations (>10 μM; Fig. 1A). In addition, no response was obtained in B16-F10 murine melanocytes (Supplemental Fig. 1A). cAMP accumulation for controls (empty vector pCMV6 transfected cells) are shown in Supplemental Fig. 2. These results are in agreement with binding assays where API1189 displayed the tracer NDP-αMSH at high concentrations for MC1 with no effect at MC3 (Supplemental Fig. 1B).

In contrast, API1189 addition to mouse MC1 and MC3 transfected HEK293 cells afforded consistent and robust activation of ERK1/2 phosphorylation in Fig. 1B). This response was not restricted to the murine receptors and was confirmed in human MC1 and MC3 transfected cells (Supplemental Fig. 3). In addition, API1189 induced Ca2+ mobilization, another melanocortin signaling response (Fig. 1C). To further explore this ligand bias observed with API1189, we generated “biased plots” (15) using equimolar concentration–response curves for two signaling pathways (cAMP versus ERK1/2, as well as cAMP versus Ca2+) plotted as a function of each other (Fig. 2). These plots were constructed using the concentration–response curves for the three pathways shown in Fig. 1, and expressed as percentage maximal response of the reference αMSH. Considering the natural ligand αMSH as the reference standard, API1189 showed preferential response to ERK1/2 phosphorylation and Ca2+ mobilization over cAMP, an effect observed for both MC1 and MC3 receptors (Fig. 2).

Collectively, these results demonstrate that API1189 is a biased agonist. Next, we questioned whether such a selective activation of MC receptors yielded biological activity.

API1189 exerts anti-inflammatory and proresolving actions in vitro

Because mouse macrophages express both MC1 and MC3 receptors (18), we began the investigation of the functional properties of API1189, using peritoneal macrophages from WT, Mc1r<sup>−/−</sup>, and Mc3r<sup>−/−</sup> mice. Added to cells at nanomolar concentrations, API1189 significantly reduced IL-1β, IL-6, and TNF-α in WT macrophages, whereas no effect was observed in Mc3r<sup>−/−</sup> cells; the inhibitory effect was retained for IL-1β and IL-6, but not TNF-α, on Mc1r<sup>−/−</sup> cells (Fig. 3A–C).

Whereas cytokine inhibition is a typical anti-inflammatory effect, proresolving molecules must activate processes like phagocytosis and effecrocytosis (19), effects we have reported for MC agonists (18). API1189 promoted phagocytosis of zymosan particles by increasing both the proportion of phagocytic macrophages and the number of particles internalized per single cell (Fig. 3D). In addition, API1189 promoted phagocytosis of apoptotic neutrophils (effecrocytosis), a crucial event in resolution and for restoration of tissue homeostasis after an inflammatory event. API1189 (optimal concentration of 1 nM) augmented effecrocytosis of apoptotic neutrophils by ~60% in WT macrophages (Fig. 3E). This effect was not evident in cells lacking either MC1 or MC3, suggesting a contribution of both receptors in the prophagocytic abilities of API1189.

To functionally associate biased agonism to biological outcome, we established the relevance of ERK1/2 phosphorylation in effecrocytosis: addition of the ERK1/2 inhibitor FR180204 (1 μM) abrogated the proeffecrocytic actions of API1189 (Fig. 3F).

Melanogenesis is not induced by API1189

Active in vitro concentrations of 0.1–1 nM API1189 were tested on B16-F10 cells for potential promelanogenic properties. Incubated with this mouse melanocyte cell line, API1189 did not induce formation of dendrites (at 24 h; Fig. 4A) or production of eumelanin, a response evident at day 3 on pelleted cells treated with the pan-agonists αMSH and NDP-αMSH (Fig. 4A, insets), as well as by the quantification of melanin in supernatants (Fig. 4B). These data, not unexpected because melanogenesis depends on the cAMP pathway, provide further functional confirmation to the biased properties of API1189.

API1189 promotes resolution of acute inflammation in vivo

The efficacy of API1189 on cytokine release and phagocytosis justified follow-up studies to establish its ability to affect leukocyte migration in acute peritonitis using either a prophylactic (administration 30 min before zymosan) or a therapeutic (administration 2 h after the inflammatory insult) design. In addition, distinct administration routes were evaluated including i.p., i.v., and oral. Results, presented in Fig. 5A–D, report significant protective effects of API1189 in vivo with ~30–70% inhibition of neutrophil infiltration alongside both administration protocols and regardless of the administration route. Monocyte infiltration was also reduced (Supplemental Fig. 4A).

In vivo proresolving effects were investigated by treating animals with API1189 at the peak of inflammation (12 h postzymosan), where neutrophil numbers are the highest, and measuring the resolution indices as described previously (20). API1189 accelerated resolution by reducing the time when peak values are reduced by 50% from 38 to 21 h (Fig. 5E). The resolution index (time interval from maximal response to time when peak values are reduced by 50%) was almost three times faster in the group of animals with AP1189 at the peak of inflammation (12 h postzymosan), as compared with vehicle-treated mice (9 and 26 h, respectively). In addition, total cell (Fig. 5F) and monocyte/macrophage (Fig. 5G) infiltration were significantly reduced by API1189. We also observed an increase in efferocytosis at 22 h afforded by API1189 (Supplemental Fig. 4B), from 1.8 to 3.60% phagocytic cells, although this difference did not reach statistical significance (p = 0.34). API1189 did not alter the F4/80<sup>high</sup> Cd11b<sup>low</sup> population (12.1 and 15.12% for vehicle and API1189 treated, respectively, p = 0.14, t test).

These data indicate that tissues were more effectively cleared from recruited immune cells when treated with API1189, effects conducive to resolution of inflammation and restoration of a physiological environment.
Orally active AP1189 reduces arthritis in mice

MC compounds are well-known for their antiarthritic actions in both preclinical (11, 21) and clinical settings, with ACTH reported to be clinically effective for the treatment of RA and gout (22, 23). We used the K/BxN serum transfer model, characterized by rapid-onset severe inflammatory arthritis. Mice were challenged with two doses of arthritogenic serum (days 0 and 2), and AP1189 was administered once daily, 25 or 50 mg/kg orally, from day 2, when the arthritis begins to be macroscopically detectable. At 50 mg/kg, AP1189 reduced all signs of arthritis measured: clinical score (−42%), paw swelling (−87%), proportion of animals with all four paws affected (−50%), and the severity of the inflammation (−70%; Fig. 6A–E).

Histological analyses (shown in Fig. 6F, 6G) revealed a significant reduction of synovitis, evident by the lower extent of leukocyte infiltration (monitored after H&E staining), whereas no evident effects were observed on cartilage protection (safranin O staining).

![Figure 1](http://www.jimmunol.org/Downloadedfrom)

**FIGURE 1.** Signaling profile of AP1189 at MC receptors. cAMP production, ERK1/2 phosphorylation, and intracellular Ca^{2+} mobilization were studied in HEK293A cells transiently transfected with mouse MC1 and MC3. (A) For cAMP assay, cells were stimulated with compounds for 15 min and samples analyzed by EIA. Forskolin (3 μM) was used as positive control. (B) For ERK1/2 phosphorylation analyses, cells were stimulated with AP1189 for 8 min and with αMSH for 5 min. ERK1/2 phosphorylation was analyzed by Western blot using α-Tubulin as loading control. Representative blots of three to four experiments are shown. Bands were quantified using ImageJ64 and the ratio of phospho-ERK1/2 and α-Tubulin calculated. Data were then expressed as the percentage of αMSH response. (C) Intracellular Ca^{2+} mobilization was measured using Fura-2 AM–labeled cells in the NOVOstar microplate reader. Ionomycin (1 μM) was used as positive control. The ratio of fluorescence emission at 510 nm after sequential excitation at 340 and 380 nm was recorded. Concentration–response curves were generated using the Ca^{2+} response 25 s after drug stimulation. Experiments were repeated two to five times and analyzed by nonlinear regression.

![Figure 2](http://www.jimmunol.org/Downloadedfrom)

**FIGURE 2.** Biased agonism at MC1 and MC3 reduces inflammation by guest on April 23, 2017 http://www.jimmunol.org/Downloadedfrom

Biased plots were generated using the cAMP concentration–response curves (EIA assays) and those generated from ERK1/2 Western blots densitometric analyses (A) or Ca^{2+} flux assays (B); see Fig. 1 for original data. All data for cAMP, ERK1/2, and Ca^{2+} are expressed as percentage maximal response of αMSH.
Discussion

We report in this article that biased agonism at MC receptors can be a viable, and innovative, approach to exploit a large wealth of MC biology for therapeutic treatment of pathological inflammation. We performed an integrated series of experiments weaved on a rationale that started from the discovery of unorthodox agonism to the assessment of in vivo and in vitro biological properties of AP1189. Our results provide proof-of-concept data to effect therapeutic innovation beyond classical orthosteric ligands to deliver translational MC drugs. Biased agonists at MC receptors offer new opportunities for the development of therapeutics with improved profile by activating pathways that are therapeutically relevant and evading those associated with side effects (15, 16).

The appreciation that therapeutic outcomes might derive from distinct downstream signaling events indicates that not all signaling pathways ascribed to a specific receptor need to be activated by a candidate drug molecule. Indeed, biased opiate analgesics devoid of respiratory depressive effects are under development (24). The concept of ligand bias, relatively new for therapeutic approaches, calls for: 1) a better dissection of signaling pathways required for therapeutic efficacy, 2) the identification of pathways associated to side effects, and 3) a careful design of drug screening and lead candidates optimization according to the desired pathways. In our attempts to establish new biology that supports approaches for novel anti-inflammatory/proresolving therapeutics, we focus on the MC receptor subtypes MC1 and MC3, both expressed and playing tonic and nonredundant protective roles in immune cells and synovial tissue, among others (2, 18, 21, 25).

The MC peptide ACTH has long been known to be effective for the treatment of gout and RA (26). Subsequently, efficacy in inflammatory bowel disease, multiple sclerosis exacerbations, or nephrotic syndrome has been reported, but overall the use of ACTH is by and large limited for side effects associated with increased cortisol production through MC2 activation (23, 27, 28). MC1 represents an appealing anti-inflammatory target: its selective activation inhibits leukocyte adhesion and emigration in a model of ischemia-reperfusion injury (29) and evokes protection against lung injury as well as in a model of delayed-type hypersensitivity (30). However, its promelanogenic actions impose a considerable limitation to MC1 as a safe target candidate when chronic treatment is predicted. Selective targeting of MC3 may result in improved safety profile and particular efficacy for joint inflammation. This receptor mediates the antiarthritic actions of the synthetic polypeptide DTrp8-ß-MSH in the K/BxN model, as well as crystal-induced inflammation (21, 31). The
major obstacle in this study derives from the absence of selective MC3 agonists devoid of off-target effects associated particularly with MC1 and MC4 activation.

In this article, we characterize the orally active small molecule AP1189 as a biased agonist with the additional advantage of targeting both tissue-protective MC1 and MC3 receptors. AP1189 activation of MC receptors did not induce cAMP accumulation, the canonical pathway ascribed to MC agonists and used for drug screening programs (e.g., see Pantel et al. [14]). AP1189 interaction with MC1 and MC3 activated a second pathway centered on ERK1/2 phosphorylation, as well as intracellular Ca2+ mobilization. Although somewhat neglected, these two signaling pathways have been described by downstream activation of most MC receptors (32). The immediate aspect we addressed was the analysis of the consequences of this unusual bent toward the currently considered noncanonical pathways.

Eumelanogenesis is unquestionably dependent on cAMP induction by MC1 (33) whereas ERK1/2 phosphorylation seems to mediate the development of skin coloration (34). AP1189 activation was exploited in the current study to investigate whether MC3 agonists could enhance this development.

**FIGURE 5.** In vivo anti-inflammatory actions of AP1189. Acute peritonitis was induced with one single injection of 1 mg zymosan, and leukocyte infiltration was analyzed by flow cytometry. (A–C) Prophylactic experimental design: AP1189 was administered 30 min before zymosan injection and cells were analyzed at the 4-h time point. (D) Therapeutic design: AP1189 was administered 2 h after zymosan, and cells were analyzed at the 6-h time point. (E) AP1189 promotes resolution. AP1189 (1 mg/kg) or vehicle were injected i.p. at 12 h postzymosan, and cells were analyzed at the 22- or 44-h time point. Neutrophils infiltration and resolution indices are shown: WM = neutrophils infiltration; Tm = time when maximal neutrophils infiltration is achieved; T50 = time when maximal neutrophils infiltration is reduced by 50%; Rv = time from maximal response to T50. (F and G) Total cells numbers and monocytes/macrophages values from the experiment in (E). AP1189 was administered i.p. (A and E–G), i.v. (B and C), or orally [p.o., (D)]. C57BL/6 mice were used in (A) and (E–G). BALB/c mice were used in (B and D). Data are the mean ± SEM of n = 5–6 per group. *p < 0.05 versus control (vehicle).

**FIGURE 6.** Antiarthritic actions of AP1189 in the K/BxN serum transfer model. Arthritis was induced on C57BL/6 mice by two i.p. injections of arthritogenic serum on days 0 and 2. From day 2, AP1189 or vehicle (PBS) were administered orally once daily. Clinical score (A), paw swelling (B), disease incidence (C), number of mice with all paws affected (D), and the number of paws per mouse severely affected (E) were recorded over 8 d. (F and G) Paraffin tissue sections were stained with H&E and fast green and safranin O. Sections were graded from 0 (no disease) to 3 (severe) based on the degree of synovitis (purple staining in the H&E sections) and cartilage degradation (loss of red coloration in the safranin O sections, arrows). Representative images are shown (original magnification ×4). Data are the mean ± SEM of n = 6 per group. *p < 0.05 versus control (vehicle). B, bone; S, synovitis.
mediate the anorexigenic effects of MC₄ (34); but whether ERK1/2 phosphorylation occurs downstream of cAMP is controversial. Inhibition of NF-κB activation upon MC activation also has been linked to the cAMP pathway (35). However, anti-inflammation via ERK1/2 activation, in conjunction or not with cAMP induction, has also been suggested (36, 37). In this respect, we show that AP1189 reduced cytokines release by zymosan-stimulated macrophages and promoted both phagocytosis and efferocytosis by this cell type. These effects were produced with distinct profiles when using Mc1r⁻/⁻ and Mc3r⁻/⁻ cells, suggesting nonredundant engagement for both receptors by AP1189. The protective actions of AP1189 were absent in Mc3r⁻/⁻ cells for IL-1β, IL-6, and TNF-α release and efferocytosis. With Mc1r⁻/⁻ cells, only the effects on TNF-α release and efferocytosis were prevented. In addition, promotion of efferocytosis by AP1189 was entirely dependent on ERK1/2 activation, as observed by using a selective inhibitor. As predicted, the biased signaling dissociated from cAMP also prevented the unwanted pigmenatory effects on melanocytes, including eumelanin production and formation of dendrites. These findings, together with the fact that the in vivo experiments were performed in BALB/c mice (Fig. 5B–D), exclude any involvement of melanogenesis and melanin, a molecule with protective antioxidant properties (38), in the actions of AP1189.

We have pioneered the concept that therapeutic innovation for inflammatory diseases could derive from appreciation and exploitation of the mechanisms operative during the resolution phase of inflammation (39, 40). Such an approach, we propose, would yield therapeutics that would not be resolution toxic by repressing or delaying tissue repair processes and the ensuing restoration of homeostasis (40). To this end, we took advantage of the resolution indices, introduced by Serhan’s group, to quantify the impact of novel drugs in the resolution phase of inflammation (20). When administered at the peak of neutrophil infiltration, which is, right before the beginning of resolution, AP1189 promoted resolution. Analysis of the resolution indices indicated that in AP1189-treated mice, recovery was achieved three times faster than in vehicle-treated animals. Monocyte/macrophage counts were also reduced by AP1189. This effect could be because of reduced infiltration, because we observed that AP1189 at 10 mg/kg afforded a 37% reduction of peritoneal infiltration of this cell type after zymosan (Supplemental Fig. 4A). However, increased efflux of macrophages after increased efferocytosis could also contribute to the lower counts quantified at a given time point (Supplemental Fig. 4B). Exciting results were also obtained with the oral treatment with AP1189 in the serum transfer model of arthritis. This arthritogenic serum contains anti-glucose-6-phosphate-isomerase Abs that, when passively transferred to naive mice, form immunocomplexes on the cartilage and synovial surface, which attract and activate neutrophils and mast cells initiating joint inflammation (41). In line with data produced with classical MC agonists such as AP214 (18) or DTrip⁻γMSH (21, 31), oral administration of AP1189 reduced clinical score, paw swelling, incidence of severe disease incidence, and the overall disease severity. Microscopically, AP1189 significantly decreased leukocyte infiltration and synovitis, although no significant changes were observed on cartilage degradation. These findings, together with the well-known protective effects of ACTH in human joint diseases, present the MC AP1189 as a promising oral candidate for pathologies of the joint including RA.

MC receptors are products of genes that are very polymorphic, thus making it necessary to determine whether gene variants could impact on the pharmacological profile. In this vein, Herrera et al. (42) found that red hair variants of MC₁ presented reduced cAMP response to NDP-αMSH, but normal ERK1/2 response. Moreover, Doyle et al. (37) showed that red hair variants do not impact on the anti-inflammatory properties, measured as inhibition of NF-κB activation, of the MC₁ selective small molecule BMS-470539. These studies indicate to us that although MC receptor polymorphism must be considered and tested for each MC candidate agonist, fully active molecules on MC₁ variants are plausible, hence the low activity elicited by the endogenous ligand does not necessarily predict low activity for synthetic molecules. Indeed, a similar situation occurs for MC₄ Haslach et al. (43) recently identified a number of peptides with full agonistic activity at loss-of-function obesity-related MC₄ variants.

Another matter of interest derived from the work presented in this article will be the identification of molecular or chemical determinants that lead to biased agonism. AP1189 was not able to displace NDP-αMSH from binding to MC₂ but was able to induce ERK1/2 phosphorylation and Ca²⁺ mobilization. The same was essentially true also for MC₁, where apparent competition occurred at concentrations >1,000-fold higher than those active for ERK1/2 phosphorylation. Altogether these studies suggest that AP1189 binds to an allosteric site, hence it acts as a biased allosteric agonist, explaining the differential activation profile observed when compared with classical “orthosteric” agonists. Elucidation of these determinants might guide a rational design of a new class of drugs combining allosterism and ligand bias. We note how Ca²⁺ mobilization, albeit not novel for MC receptor agonists, is yet to be functionally linked to the anti-inflammatory actions of MC-based compounds.

Our data on the biased agonist AP1189 also suggest the urgency for a redesign of current MC-based drug discovery approaches (by including noncanonical pathways) and possibly even reassessment of known compounds. In fact, AP1189 could have been mistakenly described as a weak antagonist at MC₁, active at micromolar concentrations, if cAMP signaling and binding properties were solely studied. Similar conclusions have been suggested for the endogenous antagonist, or more correctly inverse agonist, agouti-related protein (AGRP). AGRP can actually initiate Gₛ₀ protein–induced signaling (44), as well as internalization of MC₁ and MC₃, a receptor turn-off mechanism attributed to agonistic activity (45). More recently, AGRP has been identified as a biased agonist at MC₃ causing, indeed, activation of the ERK1/2 pathway (46).

In summary, we report in this article a novel strategy to harness the tissue-protective properties of MC receptors in inflammatory settings. Our data indicate that, in addition to a redefinition of efficacy (quality/quantity), drug selectivity needs to be reconsidered in terms of “receptor” or “pathway” selectivity, given the impact this may have on the development and characterization of novel therapeutics and subsequent medicinal chemistry programs. We also show that ERK1/2 phosphorylation downstream MC₁ and MC₃ activation leads to proresolving and anti-inflammatory actions, despite absence of cAMP signaling. New knowledge from basic research needs to be better translated into industry and incorporated into the drug-discovery process: despite that the existence of ligand bias has been known for >20 y, it is only now that its relevance is being appreciated. We propose that in the MC receptor discovery arena, biased agonism is a novel approach that can lead to improved therapeutics, perhaps like AP1189 acting on two nonredundant anti-inflammatory targets at once, yet likely devoid of side effects associated with cAMP activation.

Disclosures
T.E.N.J. is shareholder in SynAct Pharma and owns intellectual property on AP1189.