A selective small molecule agonist of the melanocortin-1 receptor inhibits lipopolysaccharide-induced cytokine accumulation and leukocyte infiltration in mice

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Abstract: It is well established that melanocortins are peptides that have potent anti-inflammatory activity. Recent research has focused on understanding which of the known melanocortin receptors mediates the anti-inflammatory actions of the melanocortins. The aim of this study was to assess the anti-inflammatory activity of a synthetic MC-1R agonist. BMS-470539 is a potent, selective, full agonist of human and murine MC-1R with EC_{50} values in a cAMP accumulation assay of 16.8 and 11.6 nM, respectively. BMS-470539 dose-dependently inhibited TNF-α-induced activation of a NF-κB transcriptional reporter in human melanoma cells, which endogenously express MC-1R. In vivo studies with BMS-470539 demonstrated that subcutaneous administration of BMS-470539 resulted in a dose-dependent inhibition of LPS-induced TNF-α production in BALB/c mice. In this model, the compound had an ED_{50} of approximately 10 μmol/kg and a pharmacodynamic half-life of ~8 h. Pharmacokinetic analysis of the compound indicated that the compound had a $t_{1/2}$ of 1.7 h. In a model of lung inflammation, administration of 15 μmol/kg BMS-470539 resulted in a 45% reduction in LPS-induced leukocyte infiltration (an infiltrate comprised primarily of neutrophils). The compound was also effective in a model of delayed-type hypersensitivity, reducing paw swelling by 59%, comparable with that seen with 5 mg/kg dexamethasone. These studies demonstrate that a selective small molecule agonist of the melanocortin-1 receptor is a potent anti-inflammatory agent in vivo and provides compelling evidence for the involvement of this receptor in the modulation of inflammation. *J. Leukoc. Biol.* 80: 897–904; 2006.

Key Words: *inflammation · MC-1R · NF-κB · GPCR · α-melanocyte-stimulating hormone*

INTRODUCTION

Melanocortins, such as α-melanocyte-stimulating hormone (α-MSH), have pleiotropic effects on physiology, ranging from effects on pigmentation and inflammation to effects on host defense and feeding behavior [1, 2]. The effects of melanocortins are mediated by a subfamily of G protein-coupled receptors, the melanocortin receptors, namely, MC-1R, MC-2R, MC-3R, MC-4R, and MC-5R [3]. α-MSH is a nonselective ligand at MC-1R, MC-3R, MC-4R, and MC-5R and like all the melanocortins, contains a highly conserved core sequence, His-Phe-Arg-Trp, which is essential for most of its physiological actions [4]. Minor variants of this sequence are contained within the superpotent melanocortin agonist Nle^4-D-Phe^7 (NDP)-MSH and the potent cyclic melanocortin peptides SHU-9119 and MT-II. A number of recent studies have shown that an additional message sequence exists at the C terminus of α-MSH. This sequence, Lys-Pro-Val (KPV), has been shown to have potent anti-inflammatory and antimicrobial activity [5, 6], but evidence is emerging that the effects of KPV are independent of the melanocortin receptors [7] or that KPV potentially acts as a weak, partial agonist [8].

Melanocortins are potent modulators of inflammation [9]. Administration of melanocortins is immunoprotective in models of systemic inflammation [10, 11], peritonitis [7], myocardial ischemia [12], renal ischemia [13], allergic airway inflammation [14], experimental heart transplantation [15], and colonic inflammation [16]. In many of these studies, administration of melanocortin peptides results in the suppression of cytokine production, inhibition of leukocyte infiltration, and preservation of tissue histology. Melanocortins have been shown to have direct anti-inflammatory actions on isolated immune cells. Incubation with α-MSH significantly inhibits the expression of the costimulatory molecule CD86 in LPS-treated monocytes [17], inhibits production of NO in the RAW

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Received December 23, 2004; revised May 19, 2006; accepted June 6, 2006; doi: 10.1189/jlb.1204748.

264.7 macrophage cell line [18], and results in the up-regulation of IL-10 in human monocytes [19]. α-MSH appears to exert its anti-inflammatory actions, at least in part, by inhibiting the activation of the inflammatory mediator NF-κB. α-MSH has been shown to inhibit TNF-α-induced activation of NF-κB in a variety of cell types [20], and administration of α-MSH reduces NF-κB activation in a murine model of experimental brain inflammation [21].

There is emerging evidence that the anti-inflammatory effects of melanocortins are mediated by more than one melanocortin receptor. MC-1R is expressed in a number of immune cells, including monocytes [17], neutrophils [22], lymphocytes [23], and dendritic cells [24]. A peptide specific for MC-1R inhibited LPS and PMA-induced TNF-α secretion from HTB-14 astrogliocytoma cells and inhibited LPS-induced TNF-α production in mice [23]. MC-3R is expressed in monocytes and macrophages [25], and there is pharmacological evidence that activation of MC-3R elicits an anti-inflammatory response. Specifically, the MC-3R/4R-selective agonist MT-II attenuates monosodium urate monohydrate crystal-induced peritonitis in mice, and this effect is blocked by the MC-3R/4R antagonist SHU-9119 but not by the MC-4R-selective antagonist HS024 [26]. Thus, MC-1R and MC-3R may be able to mediate the anti-inflammatory actions of melanocortins, although it remains to be understood if these receptors have a redundant or a distinct role in inflammation. It is clear that additional pharmacological tools are necessary to further understand the roles of the melanocortin receptors in inflammation.

The aim of this study was to assess the anti-inflammatory activity of a synthetic MC-1R-selective agonist. Our laboratory recently reported the discovery of a potent and selective MC-1R agonist BMS-470539 [27]. This current study further characterizes this compound and describes its pharmacokinetic and pharmacodynamic properties along with its effects on LPS-induced cytokine production, LPS-induced leukocyte infiltration into the lung, and delayed-type hypersensitivity (DTH).

MATERIALS AND METHODS

Cell lines and cell culture

The human melanoma cell line HBL was licensed from Professor Ghanem Ghanem (Laboratory of Oncology and Experimental Surgery, Free University of Brussels, Belgium). Mouse B16 melanoma cells were purchased from American Type Culture Collection (Manassas, VA). Human MC-1R and MC-5R were amplified from genomic DNA and subcloned into the mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, CA). The resulting constructs, hMC-1R-pcDNA3 and hMC-5R-pcDNA3, were transfected into Chinese hamster ovary (CHO) cells, and clonal lines were selected with 600 μg/ml G418 (Gibco, Grand Island, NY). High expressing clonal cell lines were screened by a whole cell-binding assay using [^{125}I]-NDP-MSH (NEN, Boston, MA). Human embryo kidney 293 cells expressing human MC-3R and MC-4R were obtained from Dr. Ira Grantz (University of Michigan, Ann Arbor). An HBL cell line stably expressing a NF-κB luciferase reporter was developed by stably transfecting HBL cells with the 12-Al NF-κB luciferase reporter (Strategene, La Jolla, CA). HBL-NF-κB-luciferase cells were grown in complete F-10 (Hams) medium (Gibco, Paisley, UK), supplemented with 5% FCS (Labtech International, UK), 5% newborn calf serum (Sigma, Poole, UK), penicillin (100 U/ml) and streptomycin (100 μg/ml; Gibco, Paisley, UK), and 375 μg/ml G418 (Promega, Madison, WI).

cAMP accumulation assay

cAMP was measured using the cAMP scintillation proximity assay (SPA) direct-screening assay system (RPA 559, Amersham Biosciences, Piscataway, NJ). Assay buffer, rabbit antisuccinyl cAMP serum, tracer, adenosine 3′,5′-cyclic phosphoric acid 2′-0-succinyl-3-[^{125}I] iodotyrosine methyl ester, and SPA antirabbit reagent SPA PVT beads were prepared as per kit instructions. Cells (2.5×10^4) in 100 μl phenol red-free F12 medium containing 1% FBS were plated into each well of a one-half area, 96-well white plate. Cells were treated with 25 μM isobutylmethylxanthine (IBMX) for 15 min prior to drug addition. Compounds dissolved in DMSO were added to the IBMX-treated cells as 100× concentrated stocks. α-MSH (50 nM) was used for the maximum response, and 1 μl DMSO was included in the negative control wells. The final concentration of DMSO was 1% in all the samples. After 30 min of stimulation at 37°C, the reaction was terminated by the aspiration of the contents of the wells followed by addition of 15 ul assay buffer containing 0.1 N HCl. Reagent mixture (15 μl; 1:1:1 of antiserum, tracer, and SPA antirabbit reagent) was then dispensed into each well. Plates were incubated at room temperature for a minimum of 5 h and subsequently counted for 6 min per well in a TopCount scintillation reader with background subtraction. Results were analyzed in relation to a cAMP standard curve.

NF-κB luciferase activity

Cells were seeded into a 96-well plate at a density of 1.25×10^4 cells/well. The cells were allowed to culture to 24 h in the well prior to treartment, reaching approaximately 60% confluence. BMS-470539 (10–1000 nM) or forskolin (10 μM) was added to the cells for an incubation period of 15 min, followed by TNF-α addition (0.5 ng/ml). Cells were incubated for a further 4 h. After this incubation, the medium was removed, and the cells were washed once with PBS. Reporter lysis buffer (100 μl per well; 1×; Promega) was added, and the cells were subjected to one freeze-thaw cycle to complete the lysis. Cell lysate (20 μg) was added to 100 μl reconstituted luciferase assay reagent (Promega) in a luminometer tube and mixed. The luminescence was then measured with a Berthold FB12 luminometer, using a regime of a 2-s delay followed by a 10-s measurement. Each of the control and test sample wells was conducted in triplicate for a single experiment. Three independent experiments were run, and data were combined to find mean values ± SEM for each sample point. Data were expressed relative to stimulated positive control samples. Thus, the maximum value was for the TNF-α alone-stimulated sample, expressed as 100% ± SEM, and all other values were expressed relative to this maximum point (as means±SEM).

Pharmacokinetics

Male BALB/c mice (Harlan Labs, Indianapolis, IN) were dosed with BMS-470539, as an i.v. bolus or s.c. injection at 33 μmol/kg (18.47 mg/kg) in a vehicle consisting of a 50% poly(ethylene glycol)/50% water mixture (100 μl/mouse). Blood samples were collected at selected times thereafter and allowed to clot on ice. The serum was then separated and stored at –20°C until assayed. Pharmacokinetic parameters were estimated using noncompartmental analysis. Kinetica® Version 4.2 (Innaphase, Philadelphia, PA) was used for this purpose. All studies using animals were reviewed and approved in advance by the Bristol-Myers Squibb Institutional Animal Care and Use Committee (Pennington, NJ).

LPS-induced TNF-α accumulation in BALB/c mice

Melanocortin receptor agonists were evaluated for anti-inflammatory activity in vivo by using an endotoxin-induced TNF-α accumulation model in BALB/c mice [28]. Female BALB/c mice, aged 6–8 weeks, were injected i.v. in the tail vein with 50 μg/kg LPS (*Escherichia coli* Strain 0111:B4, Sigma), suspended in sterile saline. Ninety minutes later, mice were sedated by CO_2:O_2 inhalation, and a blood sample was obtained. Serum was separated and analyzed for TNF-α concentrations by a commercial ELISA assay, per the manufacturer's instructions (R&D Systems, Minneapolis, MN). BMS-470539 in PBS was administered s.c. at various times before, at, or after the time of LPS injection.

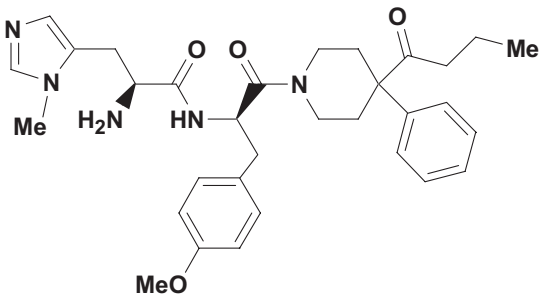

Fig. 1. Chemical structure of BMS-470539.

LPS-induced lung inflammation

Acute inflammation was induced in the lungs of BALB/c female mice by intranasal instillation of 0.5 ug LPS (*E. coli* 0111:B4, Sigma) in 50 μl sterile saline. Four hours later, mice were killed by i.p. injection of an overdose of barbiturate. The trachea was cannulated via a tracheotomy. Lungs were lavaged with 2 × 1.0 ml HBSS, and the lavage samples were pooled. Total leukocytes in the lavage sample were enumerated by an electronic cell-counter. Differential leukocyte counts (200 cells per sample) were performed on Wright's stained cytocentrifuge smears.

DTH in BALB/c mice

C57BL/6 female mice (8–10 weeks of age, Charles River Labs, Portage, MI) were immunized s.c. with 250 μg methylated BSA (mBSA; Sigma) emulsified in CFA [29]. Eight days later, the mice were anesthetized and then injected in one hind footpad with mBSA in sterile saline (100 μg in 20 μl). The contralateral footpad received saline alone. Twenty-four hours later (on Day 9), the thickness of both footpads was measured with a constant-tension, spring-loaded caliper (Mitutoyo, Japan), and the difference in thickness between the footpads was calculated. For drug-treated experiments, mice on Day 8 were treated s.c. with vehicle or BMS-470539 2 h prior to footpad challenge. Mice were again treated with vehicle or BMS-470539 approximately 6 h after the challenge. On Day 9, the thickness of the footpads was measured. As a control, some mice received a single dose of dexamethasone, 5 mg/kg by oral gavage, 2 h prior to footpad challenge. Data were analyzed by ANOVA with Dunnett's post-test for multiple comparisons (GraphPad InStat software, GraphPad, San Diego, CA).

RESULTS

An effort focused on developing small molecule agonists of MC-1R led to the identification of BMS-470539 [27], the structure of which is shown in **Figure 1**. BMS-470539 is a potent, full agonist of recombinant human MC-1R, stably expressed in CHO cells, with an EC_{50} of 16.8 nM and an intrinsic activity of 88% in a cAMP accumulation assay (**Table 1**). The effects of the compound on cAMP accumulation were dependent on MC-1R expression, as the compound has no effect on naïve, untransfected CHO cells (data not shown). BMS-470539 was a full agonist in human (HBL) and mouse (B16) melanoma cell lines (Table 1), which express MC-1R at levels similar to those observed in melanocytes [30]. The potency of BMS-470539 for stimulation of cAMP accumulation in the melanoma cells was similar to that seen in the CHO/MC-1R cells (Table 1). In all three cell lines, BMS-470539 was 300- to 1000-fold less potent than the superpotent, nonselective melanocortin, NDP-MSH. Radioligand-binding experiments confirm that BMS-470539 binds specifically to MC-1R; the compound dose-dependently inhibited the binding of [^{125}I]-NDP-MSH to HBL cells with an IC_{50} of 120 nM [27]. A similar affinity was observed in CHO cells stably expressing MC-1R. We previously had demonstrated that BMS-470539 is highly selective for MC-1R, relative to the other melanocortin receptors [27].

As melanocortins are known to inhibit activation of NF-κB, the effects of BMS-470539 on NF-κB activation were investigated (**Fig. 2**). For these studies, an HBL melanoma cell line was established that stably expresses a NF-κB luciferase reporter. In these cells, 0.5 ng/ml TNF-α induced a dose-dependent increase in NF-κB luciferase activity. Treatment of HBL-NF-κB cells with BMS-470539 elicited a dose-dependent, statistically significant reduction in TNF-α-stimulated NF-κB luciferase activity. BMS-470539 had no effect on luciferase reporter activity in the absence of TNF-α stimulation. At the highest dose of BMS-470539 (1000 nM), the inhibition is comparable with that afforded by 10 μM forskolin (data not shown). Additional experiments in nontransfected HBL cells have shown that treatment with BMS-470539 resulted in a dose-dependent inhibition of NF-κB nuclear translocation as measured by immunofluorescent detection of NF-κB and subsequent quantitation (data not shown).

Pharmacokinetic analysis of BMS-470539 indicated that the compound had a similar exposure profile when dosed s.c. or i.v. (**Fig. 3**). The compound is 100% bioavailable after s.c. administration, attains a maximum plasma concentration of 11 μM after administration of 33 μmol/kg, and has a $t_{1/2}$ of 1.7 h. These results suggested that the compound was suitable for in vivo evaluation in acute in vivo models.

BMS-470539 was tested for in vivo anti-inflammatory activity in a murine model of LPS-induced cytokine accumulation. We had previously shown that s.c. administration of BMS-470539 1 h before i.v. injection of 1 μg LPS resulted in a

TABLE 1. cAMP Accumulation Induced by BMS-470539 and NDP-MSH in Cells Expressing MC-1R

	CHO-MC-1R		HBL		B16	
	$EC_{50\ (nM)}$	IA (%)	$EC_{50\ (nM)}$	IA (%)	$EC_{50\ (nM)}$	IA (%)
BMS-470539	16.8 ± 1.6	87.5 ± 2.7	16.2 ± 3.1	94.4 ± 3.8	11.6 ± 1.5	96.2 ± 2.9
NDP-MSH	0.059 ± 0.012	100	0.046 ± 0.01	100	0.007 ± 0.002	100

CHO cells overexpressing recombinant MC-1R, HBL melanoma cells, or B16 melanoma cells were pretreated with 25 μM IBMX and then incubated with a range of concentrations of BMS-470539 or NDP-MSH. After 30 min of stimulation at 37°C, the reaction was terminated by aspiration, the cells were lysed, and accumulation of intracellular AMP was determined by SPA. Results were expressed relative to a cAMP standard curve, and the EC_{50} and intrinsic activities (IA) were determined. Average values from $n = 3$ determinations.

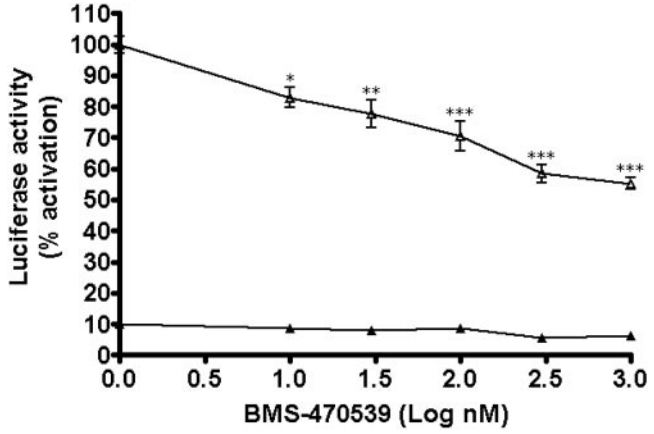

Fig. 2. Effect of BMS-470539 on TNF-α-stimulated luciferase activation in HBL cells stably transfected with the NF-κB luciferase reporter. Cells were stimulated for 15 min with BMS-470539, followed by stimulation with TNF-α at 0.5 ng/ml (△) for 4 h. The effect of BMS-470539 alone is shown (▲). Values have been normalized to express activity as a percentage of the maximum activity (100% given as the activity of the positive control sample). Significance for the 0.5 ng per ml TNF-α tests: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

dose-dependent reduction in TNF-α levels, and a maximal reduction of 92% was observed with a dose of 100 μmol/kg [27]. We were interested in determining the pharmacodynamics of the anti-inflammatory effect of BMS-470539. This was accomplished by administering 15 μmol/kg s.c. BMS-470539 at various times prior to administration of LPS (i.v.). Administration of BMS-470539 0.5–1 h before the LPS resulted in the greatest reduction in TNF-α levels (**Fig. 4A**). Administration of BMS-470539 16 h before LPS resulted in no change in plasma TNF-α levels, indicating that the effect of the compound was lost when administered this far in advance. Administration of the compound 8 h prior to LPS resulted in a 54% reduction in TNF-α levels. The optimal timing for the administration of BMS-470539 prior to LPS was from 0.5 to 1 h, with a reduction of TNF-α levels of 76–78%. From this experiment, it was determined that the pharmacodynamic half-life of the compound was ~8 h. A follow-up study was conducted to better refine the early temporal effects of administering drug prior to LPS and to determine if administration of the drug after LPS had an effect on cytokine accumulation. Administration of 15 μmol/kg BMS-470539 5 or 15 min prior to LPS resulted in a similar reduction in TNF-α levels to that observed when the compound was administered 30 min prior to LPS (range of reduction of TNF-α levels of 61–68%). Coadministration of BMS-470539 and LPS (e.g., s.c. administration of BMS-470539 followed immediately by tail-vein injection of LPS) resulted in no statistically significant change in TNF-α levels. Administration of BMS-470539 5 or 15 min after tail-vein injection of LPS had no effect on TNF-α levels. These results indicate that the optimal effect of BMS-470539 on reducing LPS-induced TNF-α levels was observed when the drug was administered between 5 and 60 min prior to LPS. The effect of BMS-470539 administration on LPS-induced accumulation of other cytokines was determined, and it was found that the drug had no effect on the LPS-induced levels of IL-1β, IL-6, or IL-10 (data not shown).

BMS-470539 was tested in a mouse model of chronic obstructive pulmonary disease (**Fig. 5**). Intranasal administration of 0.5 μg LPS resulted in a significant accumulation of leukocytes in the lung. The infiltrate at this time-point (4 h) was comprised largely of neutrophils. s.c. administration of BMS-470539 1 h prior to LPS resulted in a dose-dependent decrease in LPS-induced leukocyte accumulation. The reduction in leukocyte infiltration with BMS-470539 was statistically significant at 15 and 60 μmol/kg (43% and 71%, respectively). The effect observed upon administration of the maximum dose of BMS-470539 (60 μmol/kg) was comparable with that seen with the maximally efficacious dose of dexamethasone (5 mg/kg).

The anti-inflammatory efficacy of BMS-470539 was assessed further in a model of DTH. C57BL/6 mice, after initial immunization with mBSA, were shown to be hypersensitive 8 days later to mBSA upon injection in the hind footpad (**Fig. 6**). Two treatments of mice with BMS-470539 (2 h prior to and 6 h postinjection of mBSA) resulted in a dose-dependent reduction in paw swelling. Treatment with 20 μmol/kg resulted in a 19.6% reduction in paw swelling relative to the contralateral paw, although this difference did not attain statistical significance. Treatment with 100 μmol/kg resulted in a 59% ($P<0.01$) reduction in paw swelling, an effect comparable with that of 5 mg/kg dexamethasone.

DISCUSSION

In this study, we have further characterized a selective, small molecule agonist of MC-1R and have shown that this compound exhibits anti-inflammatory efficacy in three murine models: LPS-induced cytokine accumulation in plasma, LPS-induced infiltration of leukocytes into the lung, and DTH. BMS-470539 was designed to mimic the central His-Phe-Arg-Trp pharmacophore of the melanocortins [27] and was developed as part of an effort to develop MC-1R-selective ligands to understand the role of MC-1R in immunomodulation. The compound is a full agonist at human recombinant MC-1R and at MC-1R

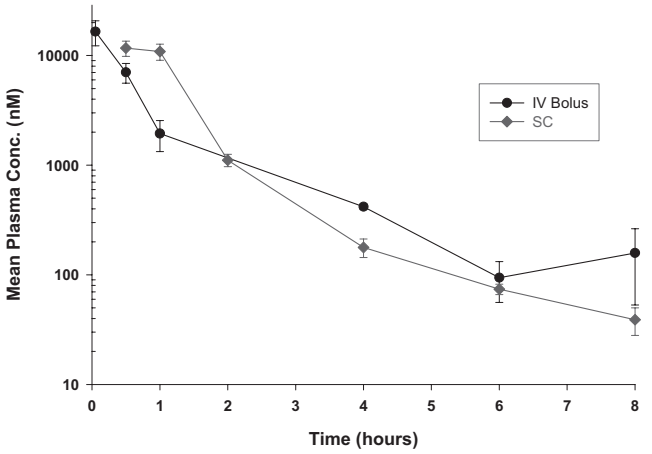

Fig. 3. Pharmacokinetic profile of BMS-470539. Male BALB/c mice were dosed with BMS-470539 as an i.v. bolus (●) or s.c. injection (◆) at 33 μmol/kg. Blood samples were collected at selected times, and the mean plasma concentration of the drug was determined.

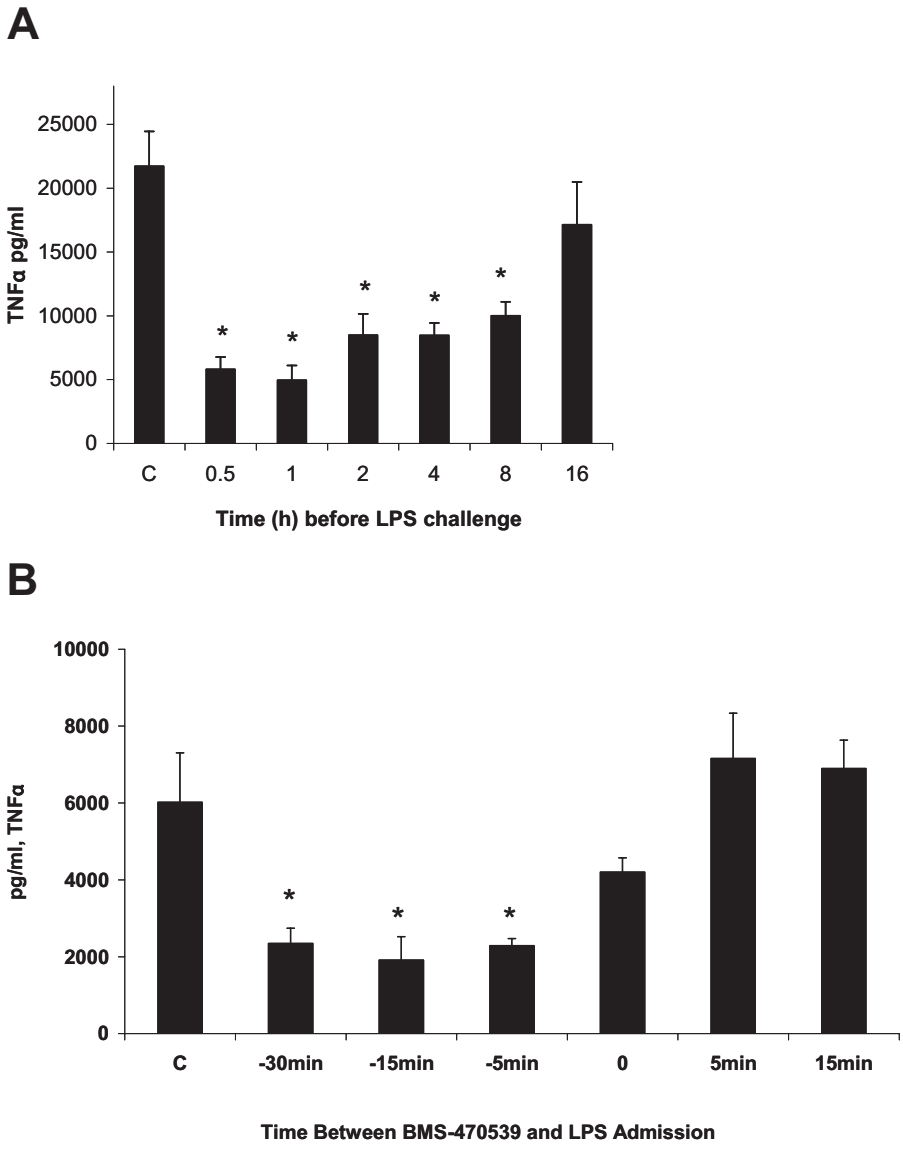

Fig. 4. Effect of BMS-470539 on LPS-induced TNF-α production in BALB/c mice. (A) BMS-470539 (15 μmol/kg) or vehicle (PBS) was administered 0.5–16 h before i.v. injection of 1 μg LPS. Plasma samples were taken 1 h after LPS administration. (B) BMS-470539 (15 umol/kg) or vehicle (PBS) was administered 5–30 min prior to i.v. injection of LPS, at the same time as LPS, or 5 or 15 min after LPS. Plasma concentrations of TNF-α are average values from five mice. *, $P < .05$, versus vehicle treatment. C, Control.

endogenously expressed in human and mouse melanoma cell lines with a potency of 12–17 nM in the cAMP accumulation assay (300- to 1000-fold less potent than the nonselective melanocortin, NDP-MSH). In a prior study, we showed that BMS-470539 is highly selective relative to the other melanocortin receptors [27], with no measurable activity at MC-3R and MC-2R and with weak, partial agonist activity at MC-4R (2.7 μM and 39% intrinsic activity) and MC-5R (4.4 μM and 15% intrinsic activity). BMS-470539 was also selective against a wider panel of G protein-coupled receptors. Data from a receptor-screening panel (data not shown) indicated the compound had no activity at adrenergic (α_1, α_2, β_1, β_2), dopaminergic (D_1 and D_2), purinergic (A_1 and A_{2A}), histaminergic (H_1, H_2), cholinergic, and opiate receptors. In addition to selectivity, BMS-470539 had favorable pharmacokinetic properties, with a prolonged half-life relative to NDP-MSH (1.7 h vs. 20 min) and 100% bioavailability after s.c. administration, making the compound well-suited for in vivo studies.

It is now well established that melanocortin peptides inhibit activation of NF-κB. Manna and Aggarwal [20] have shown that α-MSH inhibits NF-κB activation in a number of immune cell types, presumably through a cAMP-dependent mechanism. Consistent with this melanocortin-induced effect, BMS-470539 dose-dependently inhibited TNF-α-induced activation of a NF-κB-luciferase reporter stably expressed in HBL cells. Additional experiments in nontransfected HBL cells have shown that treatment with BMS-470539 resulted in a dose-dependent inhibition of NF-κB nuclear translocation, as measured by immunofluorescent detection of NF-κB (data not shown). Studies examining the ability of BMS-470539 to reduce activation of NF-κB in immune cells are ongoing.

It has long been evident that melanocortins, when administered in vivo, have robust and diverse anti-inflammatory actions: Melanocortin peptides have been shown to be immunoprotective in a wide range of acute and chronic models of inflammation [2]. What has been less evident, largely as a result of the lack of receptor-selective ligands which are suitable for in vivo studies, is the identity of the melanocortin receptors responsible for mediating the various immunomodulatory effects of the melanocortins. Several recent papers have shown that the MC-3R/MC-4R agonist, SHU-9119, and the MC-3R-selective melanocortin, γ2-MSH, inhibit monosodium

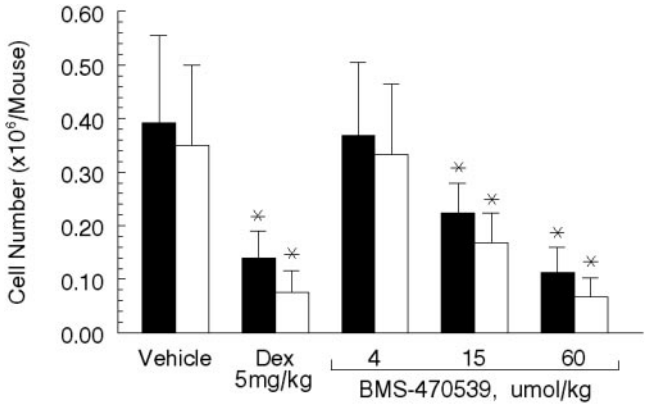

Fig. 5. Effect of BMS-470539 on LPS-induced leukocyte infiltration in murine lung. Lung lavage fluid was collected 4 h after intranasal instillation of LPS. BMS-470539, dexamethasone (Dex), or vehicle (PBS) was administered 1 h prior to LPS. Total leukocytes (solid bars) and total neutrophils (open bars) were determined. Data are representative of three individual experiments and show the mean ± SD of five mice per treatment group. *, $P < .05$, versus vehicle treatment.

urate crystal-induced neutrophil accumulation, cytokine elaboration, and resultant peritonitis in mice [25, 26]. These results provide evidence for a role of MC-3 receptors in mediating an anti-inflammatory response to a proinflammatory stimulus. Ignar et al. [23] showed that a MC-1R-selective peptide, 154N-5, inhibits LPS-induced TNF-α accumulation in mice, providing specific evidence for the role of MC-1R in modulating inflammation in vivo. They have also shown that the 154N-5 peptide inhibits LPS-induced TNF-α production in HTB-14 human astrogliocytoma cells.

Our findings confirm and extend the results of Ignar et al. [23]. In a previous study, we showed that BMS-470539 dose-dependently reduced LPS-induced TNF-α accumulation in mice, with an ED_{50} of between 3.7 and 11.1 μmol/kg and a maximal reduction of 92% [29]. BMS-470539 was effective in reducing elicited TNF-α levels after s.c. administration and was also effective when administered i.p. or i.v., but not orally (oral data not shown). In the current study, we demonstrated that BMS-470539 was effective in reducing LPS-induced TNF-α levels when it was administered between 5 min and 8 h prior to tail-vein injection of LPS. The fact that the compound can inhibit TNF-α levels when dosed up to 8 h ahead of LPS indicates that the compound has a relatively long pharmacodynamic half-life. This is considerably longer than the pharmacokinetic $t_{1/2}$ of 1.7 h and is likely a result of the agonist mechanism of action of the compound. BMS-470539, however, was ineffective when coadministered with LPS and was unable to reduce elicited TNF-α levels when administered after LPS, suggesting that the drug would not be an effective therapy for endotoxin-induced septic shock. This is in contrast to the ability of the nonselective melanocortin peptide adrenocorticotropic hormone 1–24 [ACTH-(1–24)] to reverse LPS-induced TNF-α levels when administered 15 min after LPS administration [31]. It is possible that a pan melanocortin receptor agonist, such as ACTH-(1–24), has greater anti-inflammatory potential than a MC-1R-selective agent alone, perhaps through a combined anti-inflammatory effect mediated by MC-3R and MC-1R. We also determined in this study that BMS-470539 was effective in reducing LPS-induced TNF-α levels but not the levels of other cytokines, specifically, IL-1β, IL-6, and IL-10. Previous studies with nonselective melanocortin peptides have demonstrated a reduction in several cytokines (IL-6, IL-1α, TNF-α) [11, 32]. This again may suggest that a MC-1R-selective agonist may have a more limited impact on the inflammatory response than nonselective melanocortin peptides.

In addition to inhibiting the accumulation of cytokines, we have shown that BMS-470539 can dose-dependently inhibit the accumulation of leukocytes into the lungs of mice, which were administered LPS intranasally. The effect observed upon administration of the maximum dose of BMS-470539 (60 μmol/kg) was comparable with that seen with the maximally efficacious dose of dexamethasone (1.3 μmol/kg). This supports a previous observation that α-MSH reduces leukocyte infiltration into the lungs of mice administered LPS endotracheally [33] and provides evidence that MC-1R is mediating at least part of the effect of α-MSH The infiltrate in this model is largely comprised of neutrophils, which have been shown by others to express MC-1R [22]. It is not clear whether the site of action of BMS-470539 in this study is the neutrophil or whether it is acting on other immune cells, perhaps to decrease the elaboration of neutrophil chemoattractants.

To further understand the anti-inflammatory efficacy of BMS-470539 and to test the compound in a non-LPS-based model of inflammation, the compound was tested in a model of DTH in mice. BMS-470539 was found to impair a DTH response substantially, as indicated by significantly reduced paw swelling on mBSA challenge of mice initially sensitized with mBSA. The reduction in paw swelling with BMS-470539 was comparable with that seen with a maximally efficacious dose of dexamethasone. This indicates that BMS-470539 is able to inhibit cellular immune responses in addition to its ability to modulate TNF-α secretion. α-MSH itself has also been shown

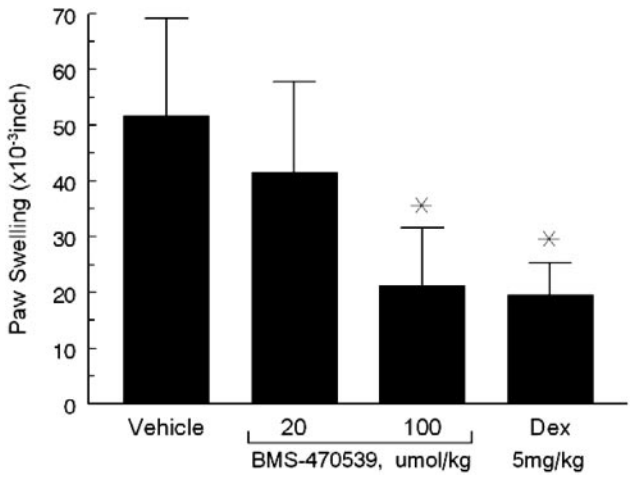

Fig. 6. Effect of BMS-470539 on mBSA-induced DTH. Paw swelling was measured 24 h after mBSA challenge (initial immunization with mBSA occurred 8 days prior to challenge). BMS-470539, dexamethasone (Dex), or vehicle (PBS) was administered 2 h prior to mBSA. BMS-470539 was administered again 6 h after mBSA challenge. Data are expressed as the mean ± SD of eight mice per treatment group. *, $P < .01$, versus vehicle treatment.

to modulate cellular immune responses, as treatment of mice with α-MSH induces trinitrochlorobenzene-specific tolerance [34]. These results suggest that nonselective melanocortin peptides as well as selective MC-1R molecules could have a role in the treatment of antigen-mediated immune responses.

Given the effects of BMS-470539 on inhibiting the activation of NF-κB and the potent anti-inflammatory effects of this compound, we propose that BMS-470539 stimulates MC-1R in immune cells and results in a reduction in the ability of inflammatory stimuli to activate NF-κB. Inhibition of NF-κB activation subsequently results in the blunting of the inflammatory response, manifested by decreased cytokine accumulation and leukocyte infiltration into the site of inflammation. What is not evident at this time, from this study or from the melanocortin literature, is which immune cells are mediating the anti-inflammatory effects of systemically administered melanocortin peptides or melanocortin receptor-selective ligands. There is ample evidence that MC-1R is expressed in cells of the immune system, such as monocytes, macrophages, neutrophils, and dendritic cells [17, 20, 22]. It cannot be ruled out, however, that melanocortins and receptor-selective agents such as BMS-470539 modulate inflammation indirectly by acting through MC-1R which is expressed on nonimmune cells. There is some data to suggest that melanocortins can modulate inflammation through neuroimmunomodulatory pathways [9].

In conclusion, BMS-470539 is a selective MC-1R small molecule agonist, which has pharmacokinetic properties that make it an attractive molecule for understanding the physiology of MC-1R. The data obtained in this study strongly suggest a role of MC-1R in mediating at least some of the immunomodulatory effects of the potent anti-inflammatory melanocortins. It will be of interest to examine the effect of BMS-470539 in other models of inflammation, particularly models of chronic inflammation. In unpublished work from our laboratory, BMS-470539 administered for 28 days by osmotic mini-pumps significantly reduced atherosclerotic lesions in low-density lipoprotein receptor-deficient mice (Carlson et al., manuscript in preparation). Based on the accumulating evidence, MC-1R-selective agonists could prove to be effective anti-inflammatory, therapeutic agents.

ACKNOWLEDGMENTS

We thank Carrie Xu and Neil Durso from Cellomics (Pittsburgh, PA) for analytical support.

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