Periodic, Partial Inhibition of IkB Kinase β-Mediated Signaling Yields Therapeutic Benefit in Preclinical Models of Rheumatoid Arthritis


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ABSTRACT

We have previously shown that inhibitors of IkB kinase (IKKβ), including 4(2′-aminoethyl)amino-1,8-dimethylimidazo[1,2-a]quinoxaline (BMS-345541), are efficacious against experimental arthritis in rodents. In our efforts to identify an analog as an anti-arthritis agent that provides protection to the joints in preclinical arthritis models, we have discovered the potent and highly selective IKKβ inhibitor 2-methoxy-N-((6-(1-methyl-4-(methylamino)-1,6-dihydroimidazo[4,5-d]pyrrolo[2,3-b]pyridin-7-yl)pyridin-2-yl)methyl)acetamide (BMS-066). Investigations of its pharmacology in rodent models of experimental arthritis showed that BMS-066 at doses of 5 and 10 mg/kg once daily was effective at protecting rats against adjuvant-induced arthritis, despite showing only weak inhibition at 10 mg/kg against a pharmacodynamic model of tumor necrosis factor-α production in rats challenged with lipopolysaccharide. The duration of exposure in rats indicated that just 6 to 9 h of coverage per day of the concentration necessary to inhibit IKKβ by 50% in vivo was necessary for protection against arthritis. Similar findings were observed in the mouse collagen-induced arthritis model, with efficacy observed at a dose providing only 6 h of coverage per day of the concentration necessary to inhibit IKKβ by 50%. This finding probably results from the cumulative effect on multiple cellular mechanisms that contribute to autoimmunity and joint destruction, because BMS-066 was shown to inhibit a broad spectrum of activities such as T cell proliferation, B cell function, cytokine and interleukin secretion from monocytes, TNF-α, cell function and regulation, and osteoclastogenesis. Thus, only partial and transient inhibition of IKKβ is sufficient to yield dramatic benefit in vivo, and this understanding will be important in the clinical development of IKKβ inhibitors.

The multisubunit IkB kinase (IKK) complex is essential in transducing the signal-inducible activation of the transcription factor NF-κB in response to proinflammatory stimuli. In the “canonical” pathway of NF-κB activation, the IKK complex catalyzes the phosphorylation of IkB proteins at specific serine residues, which triggers a subsequent ubiquitination and proteolysis of IkB, leaving NF-κB free to translocate to the nucleus (Ghosh and Karin, 2002). This multisubunit IKK complex is composed of two catalytic subunits, termed IKKα and IKKβ, and a regulatory subunit termed NEMO (or IKKγ), which mediates upstream signals into activation of NF-κB.

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the catalytic subunits (Schermer-Miller et al., 2006). Although either catalytic subunit is capable of catalyzing the phosphorylation of IκB proteins in vitro, the IKKβ subunit seems to play the dominant role in this canonical pathway in cells (Ghosh and Karin, 2002).

Given the importance of NF-κB in regulating immunological pathways and responses, along with the role IKKp plays in NF-κB activation, the identification of selective IKKp inhibitors as therapeutic agents in the treatment of inflammatory and autoimmune disorders has received considerable interest (for a review, see Pitts and Kempson, 2008). We have previously reported that the selective IKKp inhibitor BMS-345541 was highly efficacious against both inflammation and joint destruction in collagen-induced arthritis in mice and in a model of inflammatory bowel disease (MacMaster et al., 2003; McIntyre et al., 2003). Subsequently, other IKK inhibitors have also been reported to provide benefit in experimental arthritis models (Podolin et al., 2005; Schoep et al., 2006; Mbalaviele et al., 2009). BMS-345541 has also been shown to be effective in various tumor models and suggests that IKK inhibitors may be beneficial in the treatment of certain cancers in humans (Yang et al., 2006; Ammann et al., 2009).

In our efforts to discover more potent IKKp inhibitors with pharmacokinetic, safety, and pharmacologic properties consistent with being considered as a candidate for clinical development in autoimmune disorders such as rheumatoid arthritis, we identified BMS-066, shown in Fig. 1, ultimately derived from a medicinal chemistry effort around BMS-345541 (Beaulieu et al., 2007; Belema et al., 2007; Pattoli et al., 2005). In the present report, we provide a detailed investigation of the pharmacology of BMS-066 in rodent models of experimental arthritis. We found that only transient and partial inhibition of IKKp in vivo is sufficient for efficacy. Additional cellular characterization shows that this finding probably results from the IKKp dependence of multiple cellular mechanisms that contribute to autoimmune and joint destruction, and that a cumulative effect from partial inhibition of these mechanisms and pathways in vivo leads to significant benefit in experimental arthritis models.

Materials and Methods

All animal procedures involving animals were carried out with the approval of the Bristol-Myers Squibb Animal Care and Use Committee.

BMS-066. BMS-066 was synthesized at Bristol-Myers Squibb Co. according to the procedure reported by Pitts et al. (2006).

Enzyme Assays. IKKp assays used a 17-amino-acid peptide substrate corresponding to amino acids 26 to 42 of IκBα as described previously (Burke et al., 1998). In this assay, human recombinant IKKp was added at 30°C to solutions containing peptide, [32P]ATP (10 μM), 1 mg/ml bovine serum albumin in 10 mM MgCl2, 1 mM dithiothreitol, 10 mM Tris-HCl at pH 7.2. After 60 min, the kinase reactions were quenched by addition of EDTA to a concentration of 50 mM. High-performance liquid chromatography analysis was performed and the amount of IKKp-catalyzed incorporation of 32P into each peptide was quantitated by liquid scintillation counting. Under these conditions, the degree of phosphorylation of peptide substrate was linear with time and concentration of enzyme.

All other kinases were assayed by use of the SelectScreen Profiling Service using Z-LYTE fluorescent peptide substrate arrays and fluorescence resonance energy transfer-based detection system (Invitrogen, Carlsbad, CA). All assays were run at the Kp90 for ATP with each kinase. BMS-066 was tested at a concentration of 10 μM against 155 kinases within their panel, and select kinases were assayed in a dose response with BMS-066 to determine the IC50 values.

LPS-Induced Responses in PBMCs and Whole Blood. The potency of the compound against lipopolysaccharide (LPS)-induced TNF-α production from human peripheral blood mononuclear cells (PBMCs) was performed according to the general procedure of Kempson et al. (2009). When measuring the inhibition by BMS-066 of the phosphorylation of IκBα in human PBMCs, cells at a density of 1 × 105 per ml in RPMI 1640 medium supplemented with 10% FBS were preincubated for 30 min with various concentrations of BMS-066 at 37°C. To prevent degradation of phosphorylated IκBα upon stimulation, the proteasome inhibitor was also added to a final concentration of 1 μM during this preincubation. Cells were then stimulated for 5 min with LPS (100 ng/ml) and pelleted by rapid centrifugation; the pellets were solubilized by use of ice-cold 10× cell lysis buffer (Cell Signaling Technology Inc., Danvers, MA). A phospho-IκBα ELISA (Cell Signaling Technology Inc.) was used for quantitation per the manufacturer’s instructions. To measure effects on transcription, human PBMCs were plated on 24-well plates (Falcon; BD Biosciences Discovery Labware, Bedford, MA) at a density of 3 × 106 cells/well in RPMI 1640 medium plus 10% FBS and HEPES with varying concentrations of BMS-066. After a 1-h incubation at 37°C, LPS (Salmonella typhosa; Sigma-Aldrich, St. Louis, MO) was added to cells to a final concentration of 50 ng/ml and allowed to incubate 4 h at 37°C at 5% CO2, at which time cells were harvested and prepared for RNA isolation. Quantitative real-time polymerase chain reaction was performed for TNF-α, IL-6, the p19 subunit of IL-23, and the common p40 subunit of IL-12/IL-23 with procedures reported previously (Pattoli et al., 2005).

For human whole-blood experiments, blood from healthy volunteers was collected in collection tubes containing anticoagulant citrate dextrose solution A as an anticoagulant. Collected blood (300 μl) was pipetted into deep-well, 96-well assay plates (Corning Life Sciences, Lowell, MA) and BMS-066 in phosphate-buffered saline containing 16% (v/v) DMSO was then added to give a final DMSO concentration of 1% (v/v). After incubating 45 min at 37°C, LPS (S. typhosa, Sigma-Aldrich) was added to a final concentration of 1 μg/ml. After a 6-h incubation at 37°C, the plates were centrifuged for 10 min at 14,000 rpm and plasma was removed for TNF-α measurement by ELISA (BD Pharmingen, San Diego, CA).

For rat and mouse whole-blood experiments, blood was collected by cardiac puncture and orbital puncture, respectively, using collection tubes containing ACD-a as an anticoagulant. Three hundred microliters of pooled whole blood was incubated with BMS-066 and stimulated with LPS as above, with the exception that the final DMSO concentrations was 0.2% (v/v) and the LPS concentrations used were 5 and 10 μg/ml for rat and mouse whole blood, respectively. Stimulated plates containing compounds were incubated for 4 h at 37°C with use of slow rotation. Plates were centrifuged for 10 min at 14,000 rpm and plasma was removed for TNF-α measurement by ELISA (BioSource International, Camarillo, CA).

Osteoclastogenesis. Osteoclast precursor cells obtained from Cambrex (East Rutherford, NJ) were plated in 96-well plates at 10,000 cells/well in 0.2 ml of basal medium supplemented with the following cytokines or growth factors: 10% FBS, 25 ng/ml human interleukin-10, 10 ng/ml mouse IL-1β, 10 ng/ml mouse TNF-α, and 100 ng/ml human RANKL. BMS-066 was tested at a concentration of 1 μM against 155 kinases within their panel, and select kinases were assayed in a dose response with BMS-066 to determine the IC50 values.
macrophage–colony-stimulating factor, 25 ng/ml human RANKL, 2 mM L-glutamine containing various concentrations of BMS-066. All media and reagent supplements were supplied as a single media kit from Cambrex. The cells were replenished with BMS-066-containing media on day 5 by demedication (half of the medium withdrawn and replaced with the fresh medium). After an additional 4 days in culture, the cells were fixed with acetone/citrate solution and stained for tritiated-resistant acid phosphatase (TRAP) by use of the kit from Sigma-Aldrich according to manufacturer’s instructions. The cells were fixed in acetone/citrate solution for 30 s and rinsed with deionized water before TRAP staining. The number of TRAP-positive multinucleated cells (>2 nuclei per cell) was measured at predetermined site areas on each well of a 96-well plate, and three wells were measured in total for each condition. A mean value was calculated for each well. The average number of TRAP-positive multinucleated cells in controls was approximately 215 to 250 cells/well (96-well plate). The IC50 value was calculated from a BMS-066 dose range of 2 to 0.06 μM.

Lymphocytes Responses. Jurkat T cells with stable integration of luciferase under the control of either an NF-κB- or NFAT-driven reporter (J.-H. Wang, Bristol-Myers Squibb) were added at a density of 1 × 10^8 cells/ml in RPMI 1640 medium with glutamine containing 10% heat-inactivated FBS and 1% Pen/Strep were added to anti-CD3-coated (G19-4, 1 μg/ml) 96-well plates in the presence of varying concentrations of BMS-066. After 6 h at 37°C, the luciferase activity was assayed by use of the Steady-Glo Luciferase Assay reagent (Promega, Madison, WI).

Human peripheral blood T lymphocytes were prepared from E+ fractions of PBMCs (isolated as above) rosetted with sheep red blood cells. T cells at a density of 1.3 × 10^6 cells/well were added to anti-CD3-coated (G19-4, 0.5 μg/ml) 96-well plates in the presence of varying concentrations of BMS-066 in RPMI 1640 medium with glutamine containing 10% heat-inactivated FBS and 1% Pen/Strep. After 24 h at 37°C, supernatants were collected and analyzed by ELISA for IL-2 (BD Biosciences, Franklin Lakes, NJ), IL-17 (R&D Systems, Minneapolis, MN), and IL-1β (BD Biosciences). For proliferation assays, human T cells were incubated for 3 days and then labeled with [3H]thymidine for 6 h. Lysates were prepared and harvested onto filter plates, and the amount of [3H]thymidine incorporation measured by liquid scintillation counting.

To measure the phosphorylation of IκBα in response to B cell receptor activation, mouse splenic B cells were isolated by use of nylon fiber columns from spleens of BALB/c mice. To 96-well round-bottom plates were then added 3.6 × 10^6 cells/well in RPMI 1640 medium with glutamine containing 10% heat-inactivated FBS and 1% Pen/Strep along with the proteasome inhibitor (1 μM; Calbiochem, San Diego) and varying concentrations of BMS-066. After 1 h at 37°C, F(ab’)2 fragment goat anti-mouse IgG + IgM (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) was added to 25 μg/ml to stimulate the cells and incubated for an additional 30 min, at which time the cells were pelleted by centrifugation and lysed (no. 9803; Cell Signaling Technology Inc.) on ice for 30 min; the lysates were analyzed for phosho-IκBα by ELISA (Cell Signaling Technology Inc.). Human B cells were isolated from tonsils obtained from patients undergoing routine tonsillectomies (National Disease Research Interchange, Philadelphia, PA). Tonsillar tissue was minced, mashed through coarse screens, and filtered through 70-μm cell strainers (Falcon; BD Biosciences Discovery Labware). Mononuclear cells were obtained by density-gradient separation (Lymphocyte Separation Media; Mediatech, Herndon, VA) and rosetted with sheep red blood cells (Colorado Serum Company, Denver, CO). Purified B cells were obtained from the resulting E-rosette negative populations and were routinely >95% CD19+ as measured by fluorescence-activated cell sorting. For tonsillar B cell proliferation and immunoglobulin secretion assays, 1 × 10^6 cells/ml in RPMI 1640 medium containing 10% heat-inactivated FBS and gentamycin with varying concentrations of BMS-066 were stimulated either with anti-IgM (5 μg/ml; Southern Biotechnology Associates, Birmingham, AL) plus IL-4 (20 ng/ml; R&D Systems) or anti-CD40 (1 μg/ml; BD Pharmingen) plus IL-4 and incubated at 37°C for 72 h. After collecting aliquots of supernatants for immunoglobulin measurements, the cells were labeled with [3H]thymidine and incubated overnight at 37°C. Cells were then harvested onto filter plates and the amount of [3H]thymidine incorporation measured by liquid scintillation counting. Levels of human IgM and IgG were measured by specific ELISA kits (Bethyl Laboratories, Montgomery, TX).

**Pharmacokinetics in Mice, Rats, and Dogs.** The pharmacokinetics of BMS-066 was characterized by use of male BALB/c mice. Four groups of animals (n = 9 per group, 20–25 g) were dosed intravenously via the tail vein at a dose of 2 mg/kg or orally at 3 mg/kg in 50% PEG400 and 50% of 0.2 N HCl. Blood samples (0.2 ml) were obtained by retro-orbital bleeding at 0.05 (intravenous dose only), 0.25 (oral dose only), 0.5, 1, 3, 6, 8, and 24 h after dose. Within each group, three of the animals were bled at 0.05 (or 0.25 for oral), 1, and 6 h, three were bled at 0.5, 3, and 8 h, and the last three were bled at 24 h, which resulted in a composite pharmacokinetic profile (three mice per time point). Blood samples were allowed to coagulate and centrifuged at 4°C (1500–2000g) to obtain serum. Serum samples were stored at −20°C until analysis by LC/MS/MS.

Nonfasted male Lewis rats (275–325 g, n = 3 per group) were used in the pharmacokinetic studies of BMS-066. An intravenous dose of 2 mg/kg was given as a 10-min infusion via the jugular vein, and the oral dose was 10 mg/kg. The vehicle used was 50% PEG400/50% 0.2 N HCl. Serial blood samples (0.3 ml) were obtained by use of EDTA as an anticoagulant at predose and at 0.17 (intravenous dose only), 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 10, and 24 h after dose. Plasma samples, obtained by centrifugation at 4°C (1500–2000g), were stored at −20°C until analysis.

The pharmacokinetic profile of BMS-066 was evaluated in male beagle dogs (9–11 kg, n = 3). The intravenous and oral (solution) studies were conducted in a crossover design. There was a one-week washout period between the intravenous and oral dosing. Dogs were fasted overnight before dosing and continuously fasted for an additional 4 h after dosing. In the intravenous study, BMS-066 was infused through a chronically implanted vascular access port at 1 mg/kg over 10 min. In the oral arm of the study, BMS-066 was administered by oral gavage at 5 mg/kg (1 ml/kg in 0.01 N HCl). Serial blood samples were collected by with of EDTA as an anticoagulant by direct jugular venipuncture at 0.17 (intravenous dose only) 0.25, 0.5, 0.75, 1, 2, 4, 8, and 24 h after dose. Plasma samples were prepared immediately and stored at −20°C until analysis.

**Determination of BMS-066 Levels in Serum Samples.** Serum and/or plasma samples were treated with two volumes of acetonitrile containing 1 μg/ml of an appropriate internal standard. After centrifugation to remove precipitated proteins, a 3-μl portion of the resulting supernatant was analyzed by LC/MS/MS. Data acquisition was via selected reaction monitoring. Ions representing the (M + H) + species for both the analyte and internal standard were selected in MS1 and collisionally dissociated with argon at a pressure of 1.2 × 10⁻³ Torr to form specific product ions that were subsequently monitored by MS2. The transition monitored for BMS-066 was m/z 380→291 and m/z 364→305 for the internal standard. The retention time for BMS-066 was 1.66 min. The eight-point standard curve ranged from 1 to 8000 ng/ml and was fitted with a quadratic regression weighted by reciprocal concentration (1/x).

**Equilibrium Serum Protein Binding.** The extent of protein binding of BMS-066 was determined in mouse, rat, and human serum using a 96-well equilibrium dialysis device (HTDialysis, LLC, Gales Ferry, CT). The experiment was done in triplicate at a substrate concentration of 10 μM. The dialysis device, containing a dialysis membrane (molecular mass cutoff of 12–14 kDa) in each well to separate the serum and dialysis buffer (0.133 M phosphate buffer at pH 7.4), was shaken for 4 h in an incubator maintaining 37°C with 5% CO₂, and 85% humidity. Aliquots of buffer and serum were taken at 4 h and collected in the following manner. Buffer samples (50 μl) were collected in a 96-well plate that contained 50 μl of species-
specific serum. Serum samples (20 μl) were collected in a 96-well plate that contained a one-to-one mixed matrix solution containing buffer solution and species-specific serum (180 μl). Samples were analyzed for BMS-066 by use of a LCM/MIS/MS method. Percentage of protein binding was calculated by the following equation: %Free = 100 × drug concentration in buffer/drug concentration in serum.

**LPS-Induced Serum TNF-α and Liver IκBα Degradation in Rodents.** Male Lewis rats (175–200 g; Harlan, Indianapolis, IN) were dosed by oral gavage with vehicle (50% PEG400, 50% 0.02 N HCl) or BMS-066 in vehicle. Four hours later, rats were injected intraperitoneally with 1 ml per rat of 100 μg of LPS (Escherichia coli strain 0111:B4, Sigma-Aldrich) suspended in phosphate-buffered saline. Ninety minutes after LPS challenge, blood was collected from the retro-orbital sinus under isoflurane anesthesia. Serum was separated from clotted samples by centrifugation (5 min, 5000g, room temperature) and analyzed for the levels of TNF-α by ELISA according to the manufacturer’s instructions (BioSource International). Results are shown as mean ± S.E.M. of n = 8 rats per treatment group. In a separate experiment to measure the ability of BMS-066 to inhibit IκBα degradation in the liver of LPS-challenged rats, rats were sacrificed 40 min after LPS challenge and livers were excised. (Previous experiments determined that the optimal time point to measure IκBα degradation from rat liver was 40 min after LPS challenge.) A group of naive rats receiving only PBS (no LPS) was included. The right lobes of each liver were then cut into small 2- to 3-cm pieces immediately, flash frozen in liquid nitrogen, and stored at −80°C until use. Frozen specimens (300 mg each) were pulverized and placed into a Dounce homogenizer on ice with the addition of 2 ml of chilled lysis buffer containing 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 50 mM Tris-HCl, pH 8.0, and 1 mM phenylmethylsulfonyl fluoride. Protease inhibitors were added just before use (Protease Inhibitor cocktail set II; Calbiochem). The homogenate was kept on ice for 1 h before transfer to 1.5-ml tubes and cleared by centrifugation at maximum speed for 10 min at 4°C to collect the supernatant containing the whole cell extract. The protein content of each sample was determined by bichinonic acid protein reagent (Pierce Chemical, Rockford, IL). Lysate samples were normalized to 3000 ng/ml total protein before measurement of IκBα levels by ELISA (Total IκBα sandwich ELISA Kit, Cell Signaling Technology Inc.). ELISA procedure was followed according to the manufacturer’s instructions. Unless otherwise noted, statistical significance was determined by use of a one-way analysis of variance with a Dunnett’s post test. Values of p < 0.05 were considered significant.

With mice, female BALB/c mice (20–22 g; Harlan) were dosed by oral gavage with vehicle (50% PEG400, 50% 0.02 N HCl) or BMS-066 in vehicle. Four hours later, mice were injected intraperitoneally with 0.5 ml per mouse of 10 μg of LPS (E. coli strain 0111:B4; Sigma-Aldrich) suspended in phosphate-buffered saline. Ninety minutes after LPS challenge, blood was collected from the retro-orbital sinus under isoflurane anesthesia. Serum was separated from clotted samples by centrifugation (5 min, 5000g, room temperature) and analyzed for the levels of TNF-α by ELISA according to the manufacturer’s instructions (BioSource International).

**Adjuvant-Induced Arthritis in Rats.** Male Lewis rats (225 g; Harlan) were immunized with 0.1 ml of 1 mg/rat freshly ground Mycobacterium butyricum (Harlan) intraperitoneally on day 0 and on day 21. Mice were monitored after the second immunization for the development of paw inflammation. Each paw was individually scored as follows: 0, normal; 1, one or more swollen digits; 2, mild paw swelling; 3, moderate paw swelling; 4, fusion of digits; 2, mild paw swelling; 3, moderate paw swelling; 4, fusion of joints/ankylosis. The scores for all four paws are summed for each group. Bone with rare full-thickness resorption, grade 4, full-thickness resorption; grade 3, moderate resorption of cortical or trabecular bone with rare full-thickness resorption, grade 4, full-thickness resorption with distortion of bone architecture. Scores for each group were compared by one-way analysis of variance and a Bonferroni multiple comparison post-test.

**KLPH-Induced Serum IgM and IgG Production in Rats.** Male Lewis rats (250 g; Harlan) were immunized intraperitoneally with 200 μg/rat of keyhole limpet hemocyanin (KLH; Sigma-Aldrich) diluted in phosphate-buffered saline. BMS-066 in 0.02 N HCl was administered by oral gavage once daily beginning on the day of immunization. Blood samples were collected on day 7 and on day 14 under isoflurane anesthesia. Threefold serial dilutions of serum that had been separated from clotted samples by centrifugation (5 min, 5000g, room temperature) were added in duplicate to flat-bottomed 96-well ELISA plates precoated with KLH (10 μg/ml) overnight at 4°C and blocked with milk diluent. After a 2-h incubation at room temperature, the plates were washed, and horseradish peroxidase-conjugated goat anti-mouse IgM or anti-IgG detection antibody (Southern Biotechnology Associates) was added. After a final washing step, the plates were developed with tetramethylbenzidine substrate (Kirkegaard and Perry, Gaithersburg, MD) and read on a spectrophotometer at 450 nm. Anti-KLH titers were expressed as the reciprocal of the serum dilution that yielded an optical density of 1.0 (within the linear portion of the dilution curve).

**Collagen-Induced Arthritis in Mice.** Male DBA/1 mice (20–25 g; Harlan) were immunized with 200 μg/mouse of bovine type II collagen (Elastin Products Company, Owensville, MO) in Ribi Adjuvant System/RAS (Ribi ImmunoChem, Hamilton, MT) in 0.1 ml at the base of the tail on day 0 and on day 21. Vehicle (0.02 N HCl) or BMS-066 in vehicle was administered by oral gavage twice daily beginning on day 21. Mice were monitored after the second immunization for the development of paw inflammation. Each paw was individually scored as follows: 0, normal; 1, one or more swollen digits; 2, mild paw swelling; 3, moderate paw swelling; 4, fusion of joints/ankylosis. The scores for all four paws are summed for each mouse, and results shown as the mean of n = 15 mice per treatment group. Statistically significant among the groups was determined by use of the nonparametric Mann-Whitney U Test. Measurements of serum anticollagen IgG were performed in flat-bottomed 96-well ELISA plates precoated overnight with bovine type II collagen (1 μg/ml). After blocking the plates with milk diluent, 3-fold serial dilutions of mouse sera were added to duplicate wells and incubated for 2 h at room temperature. The plates were washed, and horseradish peroxidase-conjugated goat anti-mouse IgG detection antibody (Southern Biotechnology Associates) was added. After a final washing step, the plates were developed with tetramethylbenzidine sub-
strate (Kirkegaard and Perry) and read at 450 nm. Anticollagen titers were expressed as the reciprocal of the serum dilution that yielded an optical density of 1.0 (within the linear portion of the dilution curve).

Results

Biochemical and Cellular Potency and Selectivity of BMS-066. BMS-066 was shown to inhibit IKKβ-catalyzed phosphorylation of IκBα in vitro with an IC\textsubscript{50} of 9 nM and was more than 500-fold selective for IKKβ over the closely related IKKα. To understand the selectivity on a more comprehensive scale, BMS-066 was assayed against 155 additional kinases at 10 μM, and only six of these kinases were inhibited more than 75% (Table 1 and Supplemental Information). This indicates that BMS-066 is more than ~400-fold selective for IKKβ over more than 95% of the kinases tested. For the six kinases showing more than 75% inhibition at 10 μM, IC\textsubscript{50} values were determined in dose-response assays and BMS-066 was shown to be more than 30-fold selective against even the next most potently inhibited kinase (Brk).

To our knowledge, BMS-066 represents the most potent and selective IKKβ inhibitor reported to date.

Consistent with its biochemical potency, BMS-066 was effective in IKKβ-dependent cellular assays. As shown in Table 2, the compound inhibited LPS-stimulated cytokine production in human peripheral blood mononuclear cells, both at the protein and message level, with IC\textsubscript{50} values of approximately 200 nM against these endpoints. The compound inhibited the IKKβ-catalyzed phosphorylation of IκBα in LPS-stimulated cells with a similar IC\textsubscript{50} value, consistent with the observed cytokine inhibition being caused by the compound’s inhibition of IKKβ.

To better understand the amount of systemic exposure needed to inhibit IKKβ in vivo, the potency of BMS-066 against LPS-stimulated TNF-α production in whole blood was determined, with IC\textsubscript{50} values in the 1 to 3 μM range for human, rat, and mouse whole blood as shown in Table 3. This potency, as well as the lack of significant species difference, is consistent with the serum protein-binding determinations showing approximately 92% protein binding (8% free fraction) in all three species.

Pharmacokinetic and Pharmacodynamic Determinations in Vivo. As shown in Table 4, studies measuring the pharmacokinetic profiles of BMS-066 in rats showed the compound to have excellent oral bioavailability, low clearance, and an acceptable half-life of approximately 4 h. The clearance, oral bioavailability, and half-life in dogs were similar to the pharmacokinetic parameters in rats. Although the oral bioavailability in mice was also high, the compound showed considerably shorter half-life and higher clearance than in rats, and with a lower C\textsubscript{max}.

Because the pharmacokinetic profile favored testing in rats, we first investigated the pharmacodynamic potency by challenging rats that had received an oral dose of BMS-066 with LPS and measuring the resulting serum TNF-α levels. The results shown in Fig. 2A demonstrate that BMS-066 dose-dependently inhibited LPS-induced TNF-α production, with doses of both 10 and 30 mg/kg BMS-066 showing significant inhibition when administered 4 h before endotoxin challenge. As also shown in Fig. 2A, the serum drug levels at 30 mg/kg were well above the rat whole-blood potency of 1.7 μM, whereas the 10 mg/kg dose gave drug levels about equal to the whole-blood IC\textsubscript{50} value. This is consistent with the moderate, but significant, inhibition observed at 10 mg/kg and the robust inhibition with the 30 mg/kg dose. The 3 mg/kg dose showed no TNF-α inhibition, and exposure at this dose was below the rat whole-blood potency. Further demonstration that this suppressive effect on LPS-induced serum TNF-α is due to IKKβ comes from measurements of liver IκBα degradation. As shown in Fig. 2B, BMS-066 dose-dependently inhibited the LPS-induced degradation of IκBα (triggered by IKKβ-catalyzed phosphorylation of IκBα), with the effect correlating well with the inhibition of TNF-α production (no inhibition at 3 mg/kg, active at 10 and 30 mg/kg).

The close correlation between the plasma concentration at the 10 mg/kg dose and the IC\textsubscript{50} against LPS-stimulated TNF-α production in rat whole blood argues against an active metabolite being formed. Indeed, disposition and metabolite studies using radiolabeled BMS-066 showed no appreciable circulating metabolite in rats (results not shown). Therefore, we conclude that the pharmacodynamic activity of BMS-066 can be attributed solely to parent drug levels.

In a similar study in mice, BMS-066 was also shown to dose-dependently inhibit LPS-induced TNF-α production in vivo, with doses of 32 and 75 mg/kg, but not 12 mg/kg, showing activity (Fig. 2C). As with the rat, the activity in

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**TABLE 1**

Selectivity of BMS-066 for IKKβ versus other kinases

Of the 155 other kinases screened with 10 μM BMS-066, only six were inhibited more than 75%. A multipoint dose response was run to determine the IC\textsubscript{50} values shown. All kinase assays were run at an ATP concentration equal to the apparent Km for each kinase.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>IC\textsubscript{50} (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IKKβ</td>
<td>0.009 ± 0.002</td>
</tr>
<tr>
<td>IKKα</td>
<td>4.96 ± 0.78</td>
</tr>
<tr>
<td>PTK6 (Brk)\textsuperscript{a}</td>
<td>0.310 ± 0.034</td>
</tr>
<tr>
<td>Fli4 (VEGFR3)\textsuperscript{a}</td>
<td>0.360 ± 0.043</td>
</tr>
<tr>
<td>Ret\textsuperscript{a}</td>
<td>0.614 ± 0.079</td>
</tr>
<tr>
<td>EphA1\textsuperscript{a}</td>
<td>0.686 ± 0.083</td>
</tr>
<tr>
<td>EphB1\textsuperscript{a}</td>
<td>1.110 ± 0.156</td>
</tr>
<tr>
<td>KDR (VEGFR2)\textsuperscript{a}</td>
<td>1.49 ± 0.14</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Kinases showing >75% inhibition at 10 μM BMS-066 in broad screening by use of the Z'-LYTE kinase screening service (Invitrogen).

**TABLE 2**

Cellular activity of BMS-066 against LPS-induced responses in human peripheral blood mononuclear cells

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Detection</th>
<th>IC\textsubscript{50} (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α (protein)</td>
<td>Immunoassay</td>
<td>0.27 ± 0.17</td>
</tr>
<tr>
<td>IκBα phosphorylation</td>
<td>Immunoassay</td>
<td>0.29 ± 0.10</td>
</tr>
<tr>
<td>TNF-α (mRNA)</td>
<td>qPCR</td>
<td>0.18 ± 0.06</td>
</tr>
<tr>
<td>IL-6 (mRNA)</td>
<td>qPCR</td>
<td>0.16 ± 0.05</td>
</tr>
<tr>
<td>IL-12/p40 (mRNA)</td>
<td>qPCR</td>
<td>0.12 ± 0.04</td>
</tr>
<tr>
<td>IL-23/p19 (mRNA)</td>
<td>qPCR</td>
<td>0.19 ± 0.06</td>
</tr>
</tbody>
</table>

qPCR, quantitative real-time polymerase chain reaction.

**TABLE 3**

Whole-blood potency and serum protein binding of BMS-066

<table>
<thead>
<tr>
<th>Species</th>
<th>Whole-Blood LPS-Induced TNF-α IC\textsubscript{50} (μM)</th>
<th>Serum Protein Binding (% Bound)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>2.9 ± 0.9</td>
<td>92.6 ± 0.8</td>
</tr>
<tr>
<td>Mouse</td>
<td>1.2 ± 0.4</td>
<td>91.6 ± 0.8</td>
</tr>
<tr>
<td>Rat</td>
<td>1.7 ± 0.5</td>
<td>93.1 ± 0.5</td>
</tr>
</tbody>
</table>

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In a similar study in mice, BMS-066 was also shown to dose-dependently inhibit LPS-induced TNF-α production in vivo, with doses of 32 and 75 mg/kg, but not 12 mg/kg, showing activity (Fig. 2C). As with the rat, the activity in
Drug levels from rats administered drug on day 21 show that the drug levels at 5 and 10 mg/kg were above the rat whole-blood IC_{50} value of 1.2 μM, whereas the inactive dose gave drug levels below this value.

**Efficacy of BMS-066 in Rodent Models of Experimental Arthritis.** The efficacy of BMS-066 was investigated in a chronic disease model of adjuvant-induced arthritis in the Lewis rat. As shown in Fig. 3A, once daily oral doses of 5 and 10 mg/kg starting at the time of adjuvant injection on day 1 significantly reduced paw swelling compared with vehicle-treated rats at the end of the study (day 20), with the high dose showing nearly complete suppression. Histological evaluation of the joints in the vehicle group showed moderate to marked inflammation and pronounced edema, inflammatory infiltrates consisting of mixed neutrophils and mononuclear cells diffused in the articular and periarticular soft tissues, granulomas in the joint soft tissues and trabecula of the distal tibia and calcaneus (see Fig. 3F for representative sections). The talocrural (ankle) joint space was narrowed. The joint fluid was squeezed into adjacent soft tissues forming synovial crypts. Inflammatory exudates containing neutrophils and cell debris were also evident within the joint space. The numerous areas of marked bone resorption were present in the distal tibia, calcaneus, and tarsal bones, sometimes involving the full thickness of the bone. Fibrous ankylosis and periosteal bone proliferation were also seen in areas undergoing resorption. In contrast, the joints from animals receiving 10 mg/kg BMS-066 were normal or showed minimal changes in both inflammation and bone resorption. Clear reduction of inflammation was also evident in the low dose of BMS-066 (5 mg/kg) and the bone resorption seemed to be more focal and less severe compared with the vehicle control animals. Blinded scoring of sections from the various treatment groups showed treatment with BMS-066, in particular, the high dose, to be effective at inhibiting both bone destruction endpoints and inflammation and edema (Fig. 3C).

Consistent with the histological findings, microcomputed tomography of the hind limbs also showed that BMS-066 provided a dose-dependent protection against the pitting, loss of bone mass, woven porous bone, and fusion of the small bones evident in the rats receiving only vehicle, with the animals receiving 10 mg/kg showing nearly complete protection as evidenced by the presence of a smooth bone surface and easily recognizable small individual bones of the foot and ankle (see Fig. 3E). Bone density measurements also showed a clear dose-dependent benefit of treatment with BMS-066 as shown in Fig. 3B.
mately 3 h (6 h daily with twice daily dosing) of the mouse whole-blood IC₅₀ value against LPS-induced TNF-α. Similar exposure was measured in animals receiving compound with repetitive dosing over 3 weeks (results not shown), indicating that accumulation of drug did not account for the efficacy observed. Although measurements of cytokines within the joints or synovium of study animals were not evaluated in this study, we and others have previously shown that IKK inhibitors suppress TNF-α and IL-1β production within these tissues in experimental arthritis models (McIntyre et al., 2003; Schopf et al., 2006).

**Impact of IKKβ Inhibition on Cellular Assays Underlying Autoimmunity and Joint Destruction.** Although BMS-066 was effective against experimental arthritis at doses in both rats and mice that would be expected to provide only transient coverage of receptor-stimulated cytokine production, IKKβ-dependent NF-κB activation has been implicated in many pathways that affect autoimmunity and inflammation. Therefore, the cumulative effects of IKKβ inhibition in multiple pathways might be expected to have a more profound impact on autoimmune disease progression than one pathway alone. To investigate this more fully, we profiled the activity of BMS-066 in various cellular assays thought to be important in autoimmune disorders. With abatacept and rituxan demonstrating that T and B lymphocytes play critical roles in the progression of rheumatoid arthritis, we determined the effect of BMS-066 on lymphocyte function as shown in Table 5. In Jurkat T cells with stable integration of either an NF-κB-dependent or NFAT-dependent luciferase reporter, BMS-066 showed a submicromolar IC₅₀ value against the NF-κB-dependent luciferase expression without effect on the NFAT-driven response, consistent with the high selectivity of the inhibitor for IKKβ. In human peripheral blood T cells, α-CD3 stimulation of the TCR-induced proliferation and the production of IL-2, IL-17, and IL-1β, was also inhibited by BMS-066.

Stimulation of the BCR with α-IgM was used to induce proliferation of human tonsillar B cells, and BMS-066 was shown to inhibit proliferation with a potency in close agreement to the B cell receptor-dependent stimulation of IκBα phosphorylation in these cells and the LPS-induced endpoints in peripheral blood mononuclear cells. CD40-dependent proliferation was also inhibited in B cells with a similar potency. IgG production in response to BCR stimulation was also inhibited, but the inhibition of IgM production was less potent. A similar effect was observed in vivo, with once-daily oral dosing of BMS-066 in rats showing a considerably more potent inhibition of a neoantigen (KLH)-induced IgG response compared with inhibition of the IgM response. As shown in Fig. 5, the compound was only effective at the 15 mg/kg dose in inhibiting serum IgM in this humoral response, whereas doses as low as 2.5 mg/kg were effective against the IgG response. This in vivo humoral response not only depends on BCR stimulation in B cells, but also depends on T cell help.

Osteoclasts play an important role in the bone loss that occurs in inflammatory arthritis, and IKKβ has been shown to play a critical role in the RANKL-induced osteoclastogenesis both in vitro and in vivo (Ruocco et al., 2005). It has been postulated, therefore, that inhibitors of IKKβ would provide protection from bone erosion in inflammatory arthritis through direct suppression of RANKL-induced osteoclasto-

![Fig. 2. BMS-066 dose-dependently inhibits TNF-α production and IκBα degradation induced in rodents challenged by intraperitoneal injection of LPS. BMS-066 was administered perorally 4 h before LPS challenge, and blood was drawn subsequent to challenge for measurements of serum TNF-α and serum drug concentrations, or livers were excised to determine effects on LPS-induced IκBα degradation. A, TNF-α response in rats (90 min after LPS challenge). B, liver IκBα degradation in rats (40 min after LPS challenge). C, TNF-α response in mice (90 min after LPS challenge). n = 8 animals per group for TNF-α measurements; n = 5 per group for IκBα determinations. Data represent the means ± S.E.M. *P < 0.05 versus LPS-stimulated control without inhibitor.](image-url)
Fig. 3. Once daily administration of BMS-066 inhibits adjuvant arthritis in rats. BMS-066 was administered by oral gavage once daily beginning at the time of the adjuvant injection, at doses of 0 (vehicle control), 5, or 10 mg/kg. A, paw swelling. The x-axis denotes the number of days after the immunization with complete Freund’s adjuvant. Error bars have been omitted for clarity of presentation. B, bone density in left hind paws as measured by micro-CT analysis. C, histological evaluation of the right hind paws of rats at the end of the study. The scores are divided into measures of inflammation and edema, bone resorption, and total score. D, serum drug levels in immunized satellite animals (n = 3) after the last dose of compound on day 21. Error bars have been omitted for clarity of presentation. The dashed line represents the IC50 of BMS-066 against LPS-induced TNF-α in rat whole blood (see Table 3). E, representative micro-CT images of the left hind paws of rats at the end of the study. F, histological comparison of rats with adjuvant-induced arthritis that received either vehicle (left) or 10 mg/kg BMS-066 (right). Representative joints are shown. *, P < 0.05 versus vehicle control.
To investigate this, the effect of BMS-066 against RANKL-stimulated osteoclastogenesis was measured by use of primary osteoclast precursors isolated after limited expansion from hematopoietic CD43-positive progenitors. As shown in Fig. 6, stimulation of progenitors with RANKL for 10 days resulted in large, multinucleated osteoclasts that stain positive for TRAP. The presence of BMS-066 at 1 \( \mu M \) during the culture completely blocked the formation of these osteoclasts such that the cells resembled the mononuclear cells not receiving RANKL. BMS-066 at a concentration of 0.5 \( \mu M \) showed an intermediate response. Therefore, BMS-066 inhibited IKK-dependent, RANKL-induced osteoclastogenesis with a potency consistent with other cellular assays.

**Discussion**

Given the attractiveness of NF-\( \kappa \)B as a target in inflammatory and autoimmune disorders, many pharmaceutical companies have drug discovery efforts targeting IKK\( \beta \). Indeed, we and others have reported previously that IKK\( \beta \) inhibitors are highly efficacious in rodent models of experimental arthritis, among other preclinical models. However, because of concerns that complete and continuous blockade of the IKK\( \beta \)-NF-\( \kappa \)B pathway may prove to be associated with adverse consequences such as susceptibility to infection, it is important to understand the degree and duration of inhibition required in vivo to yield a benefit when treating autoimmune and inflammatory disorders such as rheumatoid arthritis. This will aid in the dose selection and assessment of IKK\( \beta \) inhibitors as therapeutic agents in the clinic.

The findings presented here demonstrate that the potent and highly selective IKK\( \beta \) inhibitor BMS-066 shows robust efficacy in both rat and mouse models of experimental arthritis at doses that provide incomplete inhibition for only relatively short periods of time. In rats, a 10 mg/kg p.o. dose...
given once daily provided nearly complete protection from disease even though the dose provides \( \frac{1}{2} \times 10^{2} \) inhibition of IKK for only 9 h between doses. Moreover, a 10 mg/kg dose provides only partial inhibition of the IKK-dependent production of TNF-\( \alpha \) when animals are challenged with LPS 4 h after dose. A similar effect was found in mice, with a highly efficacious dose of 20 mg/kg p.o. (twice daily) providing \( \frac{1}{2} \times 10^{2} \) inhibition for only approximately 6 h daily.

Evidence that the robust efficacy is not the result of a large tissue concentration (penetration) not reflected by serum drug levels comes from the in vivo LPS-induced TNF-\( \alpha \) production in both rats and mice. In both studies, an excellent correlation between whole-blood potency and drug levels was observed, and the TNF-\( \alpha \) in this context has been shown to be derived predominantly from the liver rather than cells in the blood (Kumins et al., 1996; Wurfel et al., 1997; Gross and Piwnica-Worms, 2005). Moreover, the apparent volume of distribution calculated from the intravenous pharmacokinetics is not large in either rats or mice.

Instead, we conclude that the robust efficacy results from a cumulative effect on multiple cell types and pathways that have been shown to be important in driving the underlying pathophysiology of disease. Indeed, BMS-066 inhibits the production of cytokines and interleukins critical in driving the inflammatory and immune mechanisms underlying rheumatoid arthritis and other autoimmune disorders. This includes effects on T cells, B cells, monocytes, and osteoclastogenesis activated through diverse receptors such as the toll-like receptors, TCR, BCR, and CD40. The IKK-\( \alpha \)-NF-\( \kappa B \) pathway has also been shown to regulate inflammatory cell recruitment through NF-\( \kappa B \)-dependent expression of cell adhesion molecules such as intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 and chemoattractants such as monocyte chemoattractant protein-1 and IL-8 (Viemann et al., 2004), although the effects of BMS-066 against these endpoints were not measured. IKK-\( \beta \) also regulates the expression of matrix metalloproteinases that degrade cartilage and bone, and this can be blocked with an IKK-\( \beta \) inhibitor (Pattoli et al., 2005).

A particularly intriguing finding is the ability of BMS-066 to inhibit components of the novel pathogenic T helper subset named TH17 that has recently emerged as a critical driver of autoimmune disorders such as rheumatoid arthritis, Crohn’s disease, multiple sclerosis, and psoriasis (Ivanov and Lindén, 2009). The production of IL-17, which can induce proinflammatory cytokines and matrix metalloproteinases important in driving the underlying pathogenesis in rheumatoid arthritis (Lubberts, 2008), from T cells stimulated through the TCR was blocked by BMS-066. Although this assay is not a bona fide TH17 cellular measurement per se, the promoter of IL-17 has been shown to contain NF-\( \kappa B \) binding sites requisite for expression (Liu et al., 2004). Moreover, the expression of both the p40 and p19 subunits of IL-23 were blocked by BMS-066, consistent with the reported NF-\( \kappa B \) dependence for the expression of these subunits (Mise-Omata et al., 2007; Ogawa et al., 2008). Because IL-23 is critical in the terminal differentiation of TH17 cells (McGeachy et al., 2009), IKK-\( \beta \) inhibitors will affect this important autoimmune pathway in multiple ways. In addition, the p40 subunit is also a component of IL-12 that drives the TH1 responses important in autoim-

**Fig. 5.** BMS-066 inhibits neoantigen-induced antibody responses in rats. BMS-066 at doses of 2.5, 5, and 15 mg/kg was administered by oral gavage once daily beginning at the time of challenge with KLH antigen. A, serum anti-KLH IgM measured 7 days after KLH immunization. B, serum anti-KLH IgG measured 14 days after KLH immunization.

**Fig. 6.** BMS-066 blocks RANKL-induced osteoclastogenesis. Primary osteoclast precursors isolated after limited expansion from hematopoietic CD43-positive progenitors were stimulated with RANKL for 10 days. Large, multinucleated osteoclasts that are stained positive for TRAP were induced under these conditions. The images are representative of wells containing either no RANKL during the 10-day culture (A), or with RANKL but containing either 0 \( \mu \)M (B), 0.5 \( \mu \)M (C), or 1 \( \mu \)M (D) BMS-066.
munity, and anti-p40 antibodies have been shown to provide significant benefit in patients with psoriasis, psoriatic arthritis, and Crohn’s disease (Krueger et al., 2007; Sandborn et al., 2008; Gottlieb et al., 2009).

The effects on B cells are also intriguing given the efficacy of B cell-targeted therapies such as rituximab against rheumatoid arthritis and severe lupus nephritis (Schuna, 2007; Melander et al., 2009). Although not particularly potent against IgM production in B cells stimulated through the BCR, BMS-066 was considerably more potent against IgG production in B cells stimulated in vivo LPS-induced TNF-α response was observed, suggesting that cell switching in B cells is especially sensitive to IKKβ inhibition. This may result from direct effects both on the BCR and on CD40 signaling which is critical in driving class switching. Although CD40 signaling is thought to signal through the “noncanonical” pathway of NF-κB x activation, with the p100 to p52 processing being IKKα-dependent (Coope et al., 2002), the expression of p100 itself depends on the canonical IKKβ-mediated pathway (Liptay et al., 1994; Li et al., 2005). Moreover, the thymus-dependent antibody response to KLH also requires T cell help that may itself be affected by IKKβ inhibition. The idea that B cells are an important target of IKKβ inhibitors is also evident from the collagen-induced arthritis model in which we have shown, presently with BMS-066 and previously with BMS-345541 (McIntyre et al., 2003), that the efficacy correlates with the inhibition of the development of anticollegen antibody titers.

In summary, the work presented here demonstrates that efficacy in experimental arthritis models requires far less than complete or continuous inhibition of IKKβ in vivo. This most likely is due to the dependence on the IKKβ-NF-κB pathway of numerous pathways and cell types important in driving autoimmunity, and these findings support the idea that therapy with IKKβ inhibitors will probably be tailored to dampen the immune system in situations with aberrant activation, rather than shutting it down completely. Efficacious doses, therefore, may not be particularly immunosuppressive, which is an important consideration in the development of IKKβ inhibitors as drug candidates. Although it is still unclear whether there will be detrimental effects with chronic use of IKKβ inhibitors, a more complete understanding of the efficacy and utility as well as the toxicities associated with IKKβ inhibition in the treatment of human disease will emerge as selective inhibitors progress into clinical trials.

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References


Podolín PL, Callahan JF, Bolognese BJ, Li YH, Carlson K, Davis TG, Mellor GW,


Address correspondence to: Dr. James R. Burke, Bristol-Myers Squibb, P.O. Box 4000, Princeton, NJ 08543. E-mail: James.Burke@bms.com