Effects of the cFMS Kinase Inhibitor 5-(3-Methoxy-4-((4-methoxybenzyl)oxy)benzyl)pyrimidine-2,4-diamine (GW2580) in Normal and Arthritic Rats

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ABSTRACT

The cFMS (cellular homolog of the V-FMS oncogene product of the Susan McDonough strain of feline sarcoma virus) (Proc Natl Acad Sci U S A 83:3331–3335, 1986) kinase inhibitor 5-(3-methoxy-4-((4-methoxybenzyl)oxy)benzyl)pyrimidine-2,4-diamine (GW2580) inhibits colony-stimulating factor (CSF)-1-induced monocyte growth and bone degradation in vitro and inhibits CSF-1 signaling through cFMS kinase in 4-day models in mice (Proc Natl Acad Sci U S A 102:16078, 2005). In the present study, the kinase selectivity of GW2580 was further characterized, and the effects of chronic treatment were evaluated in normal and arthritic rats. GW2580 selectively inhibited cFMS kinase compared with 186 other kinases in vitro and completely inhibited CSF-1-induced growth of rat monocytes, with an IC50 value of 0.2 μM. GW2580 dosed orally at 25 and 75 mg/kg 1 and 5 h before the injection of lipopolysaccharide inhibited tumor necrosis factor-α production by 60 to 85%, indicating a duration of action of at least 5 h. In a 21-day adjuvant arthritis model, GW2580 dosed twice a day (b.i.d.) from days 0 to 21, 7 to 21, or 14 to 21 inhibited joint connective tissue and bone destruction as assessed by radiology, histology and bone mineral content measurements. In contrast, GW2580 did not affect ankle swelling in the adjuvant model nor did it affect ankle swelling in a model where local arthritis is reactivated by peptidoglycan polysaccharide polymers. GW2580 administered to normal rats for 21 days showed no effects on tissue histology and only modest changes in serum clinical chemistry and blood hematological parameters. In conclusion, GW2580 was effective in preserving joint integrity in the adjuvant arthritis model while showing minimal effects in normal rats.

CSF-1 promotes the survival, proliferation, and differentiation of mononuclear phagocyte lineages and regulates the response of these lineages to inflammatory challenge. CSF-1 binding to its cell surface receptor triggers autophosphorylation by receptor cFMS (cellular homolog of the V-FMS oncogene product of the Susan McDonough strain of feline sarcoma virus) (Sacca et al., 1986) kinase and a cascade of cell signaling. cFMS kinase is the cellular homolog of the v-FMS oncogene product of the Susan McDonough strain of the feline sarcoma virus (Sacca et al., 1986). The ability of CSF-1 to promote the survival, proliferation, and differentiation of bone progenitor cells, monocytes, macrophages, and osteoclasts in vitro correlates with the expression of CSF-1 receptor in these cells (for review, see Chitu and Stanley, 2006). Mice without CSF-1 (Wiktor-Jedrzejczak et al., 1990, 1992b) or its receptor (Dai et al., 2002) are osteopetrotic, deficient in several macrophage populations, show decreased TNF production by 60 to 85%, indicating a duration of action of at least 5 h. In a 21-day adjuvant arthritis model, GW2580 dosed twice a day (b.i.d.) from days 0 to 21, 7 to 21, or 14 to 21 inhibited joint connective tissue and bone destruction as assessed by radiology, histology and bone mineral content measurements. In contrast, GW2580 did not affect ankle swelling in the adjuvant model nor did it affect ankle swelling in a model where local arthritis is reactivated by peptidoglycan polysaccharide polymers. GW2580 administered to normal rats for 21 days showed no effects on tissue histology and only modest changes in serum clinical chemistry and blood hematological parameters. In conclusion, GW2580 was effective in preserving joint integrity in the adjuvant arthritis model while showing minimal effects in normal rats.

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duction after LPS challenge (Wiktor-Jedrzejczak et al., 1992a), and have a diminished response to bacterial infection (Wiktor-Jedrzejczak et al., 1996, Guleria and Pollard, 2001). Administration of exogenous CSF-1 increases cytokine production after LPS challenge (Conway et al., 2005) and increases the production of monocyte lineages in vivo (Bock et al., 1991).

Besides its homeostatic role in normal animals the CSF-1-cFMS kinase pathway could play a role in pathologies such as arthritis that involve chronic activation of tissue macrophage populations. CSF-1 expression is increased in the synovium (Cupp et al., 2007), and CSF-1 is elevated in the synovial fluid of rheumatoid arthritis patients (Kawaji et al., 1995). Synovial fibroblasts from rheumatoid arthritis patients produce high levels of CSF-1 ex vivo (Seitz et al., 1994). CSF-1 promotes osteoclast development and bone degradation in vitro (Sarma and Flanagan, 1996; Weir et al., 1996, Tanaka et al., 1993) and thus could contribute to joint destruction in arthritis. Administration of exogenous CSF-1 exacerbated arthritis in mice (Bischof et al., 2000; Campbell et al., 2000) and rats (Abd et al., 1991). Antibodies to CSF-1 (Campbell et al., 2000) and antibodies to the CSF-1 receptor (Kitaura et al., 2005) inhibited collagen-induced arthritis in mice, further implicating CSF-1 signaling in arthritis.

To help investigate the role of the CSF-1-CSF-1 receptor pathway in normal and disease states, we characterized the potency, selectivity, and bioavailability of GW2580 (Conway et al., 2005), a competitive inhibitor of ATP binding to cFMS kinase (Shewchuk et al., 2004). GW2580 inhibited CSF-1-induced monocyte growth, CSF-1-, and receptor activator of nuclear factor B ligand-induced osteoclast differentiation and degradative activity and parathyroid hormone-induced degradation of bone explants in vitro. A single oral dose of GW2580 inhibited LPS-induced TNF production and CSF-1 priming of LPS-induced interleukin-6 production in mice. In short-term 4-day models, GW2580 partially inhibited thiglycolate-induced cell influx into the peritoneal cavity and completely blocked the growth of CSF-1-dependent tumor cells in mice. In the present report, we further investigated the kinase selectivity of GW2580, determined its effect on cytokine production in rats, and characterized its effects on normal and arthritic rats after 21 days of dosing.

Materials and Methods

Animals and Compound Dosing. Male Lewis rats (Charles River Laboratories, Raleigh, NC) weighing approximately 220 g were used. Animals were free of pathogenic viruses as determined by a standard viral titer screen (Microbiological Associates, Bethesda, MD). The research complied with national legislation and with company policy on the care and use of animals and with related codes of practice.

GW2580 and prednisolone, the steroid positive control, were suspended in 0.5% hydroxy propyl methylcellulose and 0.1% Tween 80 using multiple strokes with a Teflon glass homogenizer. Compound was dosed orally at 1 ml/100 g b.w.

Kinase Selectivity Assays. GW2580 was tested at 10 μM in ATP site-dependent competition binding assays for 180 kinases by contract with Ambit Biosciences (San Diego, CA) (Fabian et al., 2005).

Effects on CSF-1-Induced Growth of Rat Monocytes. Rats were euthanized with CO2 or anesthetized with isoflurane and heparinized blood collected from the inferior vena cava. Peripheral blood mononuclear cells were isolated from heparinized blood by centrifugation through cell separation media (Accurate Chemical & Scientific, Westbury, NY), and 50,000 cells were added to each of 96 wells in 150 μl of RPMI 1640 media containing 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, UT) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA). To measure the effects of human and murine CSF-1 on monocyte growth, the media were replaced at 4 h, and 0.5 h later, 20 μl of media or 20 μl of media containing different concentrations of human or murine CSF-1 (R&D Systems, Minneapolis, MN) was added. To measure the effects of GW2580 on CSF-1-induced growth, GW2580 at 20 nM in dimethyl sulfoxide was diluted to 10 μM and 0.05% dimethyl sulfoxide in media and diluted serially to yield a 10-point concentration curve. After the 4-h incubation to allow monocyte adherence the media were replaced with the media containing GW2580, and 0.5 h later 20 μl of human or murine CSF-1 (R&D Systems) was added to each well for a final concentration of 40 ng/ml. On day 5, 10 μl of WST-1 reagent (Roche Diagnostics, Indianapolis, IN) was added to each well, and the absorbance at 440 nm was measured at 3 h. Wells with and without growth stimuli were used to calculate growth. IC50 values were estimated using the equation $Y = V_{\text{max}} \times (1 - (X/IC_{50} + X)))$, where $Y$ is the growth in the presence of inhibitor, $V_{\text{max}}$ is the growth in the absence of an inhibitor, and $X$ is the inhibitor concentration.

TNF Production in Rats in Vivo. LPS (Sigma-Aldrich, St. Louis, MO) was dissolved in phosphate-buffered saline at 80 μg/ml, and a dose of 40 μg/rat was given intravenously in 0.5 ml. After 90 min, the rats were sacrificed, and plasma was prepared from inferior vena cava blood, and TNF was measured by a rat specific enzyme-linked immunosorbent assay (BioSource International, Camarillo, CA). GW2580 was dosed orally either 1 or 5 h before the LPS injection.

Effects in Normal Male Rats. Rats were dosed b.i.d. for 21 days with vehicle, 7.5, 37.5, or 75 mg/kg GW2580 at n = 6 per group. Body weights were taken every 2 days. On day 1 and day 21, plasma was taken from three rats before the morning dose and from three rats 1 to 2 h after the morning dose for determination of GW2580 concentrations. At sacrifice, the livers and spleens were weighed, the stomach was inspected for lesions, and the following tissues were assessed histologically: skin, mammary gland, heart, thymus, lungs, liver, trachea, thyroid and parathyroid glands, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, pancreas, mesenteric and submandibular lymph nodes, salivary glands, pancreas, kidneys, spleen, testes, epididymides, sternum, femur, skeletal muscle, sciatic nerve, stifle joint, ankle joint, and foot at the level of the proximal phalangeal joint.

PGPS Arthritis. Rats were primed with an intra-articular injection of 10 μl of PGPS at 0.5 mg/ml rhonmosine in the right ankle (Schwab et al., 1993). After 2 weeks, the ankle diameters were measured with calipers, and rats were assigned to groups of n=6 to get a similar distribution of initial joint diameters. Rats then received their first dose of GW2580 followed 1 h later by an intravenous injection of 0.5 ml of PGPS (0.4 mg/ml rhonmosine) in the tail vein. GW2580 was dosed b.i.d., and ankle diameter and body weights were measured for 3 days.

Adjuvant Arthritis. Freund’s complete adjuvant was injected intradermally in the base of the tail (Conway et al., 2001) on the morning of day 0. GW2580 was given orally the afternoon of day 0 and b.i.d. from days 1 to 21, days 7 to 21, or days 14 to 21. Body weight and the diameter of the both ankles were measured every 2 to 3 days. On the morning of day 21, three rats from each group were sacrificed, and plasma was prepared to determine GW2580 levels 16 h after the last dose. The remaining three rats were dosed again and sacrificed 1 to 2 h later for measurements of GW2580 in the plasma. At necropsy, both ankles were fixed in 10% buffered formalin, and bone mineral content was measured. Microradiographs were then taken, followed by sectioning and staining with hematoxylin and eosin for histological analysis.

The bone mineral content of the ankles was measured using dual-energy X-ray absorptiometry with subregional high-resolution soft-
Inhibition of Arthritis with GW2580

Results

Activities in Vitro. We previously showed that GW2580 inhibited cFMS kinase activity in vitro at an IC\textsubscript{50} value of 0.03 \(\mu\text{M}\), with no effect on 26 other kinases assayed by ATP-dependent phosphorylation of substrates (Conway et al., 2005). To further investigate selectivity, an assay of competition for the kinase ATP site was used (Fabian et al., 2005). GW2580 was tested against 160 new kinases along with 19 that had been assayed previously (Supplemental Table 1). GW2580 at 10 \(\mu\text{M}\) inhibited cFMS activity by 100\% and TRKA activity by 80\%, but it was inactive against the other 178 kinases. Dose titration showed that GW2580 inhibited TRKA at an IC\textsubscript{50} value of 0.88 \(\mu\text{M}\).

Human CSF-1 and mouse CSF-1 increased the growth of rat monocytes isolated from rats sacrificed with carbon dioxide in dose response, with the highest concentration of 40 ng/ml increasing growth by approximately 10-fold (data not shown). In the same monocyte preparation, GW2580 completely inhibited the growth induced by 40 ng/ml human CSF-1 and mouse CSF-1 at IC\textsubscript{50} values of 0.22 and 0.15 \(\mu\text{M}\), respectively. In a separate study, GW2580 completely inhibited the cell growth induced by 40 ng/ml human CSF-1 in monocytes recovered from rats sacrificed with carbon dioxide or after isoflurane anesthesia at IC\textsubscript{50} values of 0.21 and 0.14 \(\mu\text{M}\), respectively.

Cytokine Production in Vivo. GW2580 inhibited LPS-induced production of TNF in mice in a dose-related manner (Conway et al., 2005). In rats, GW2580 administered 1 h before LPS at 25 and 75 mg/kg inhibited TNF production by 72 \(\pm\) 16\%*** and 71 \(\pm\) 4\%**, respectively, and GW2580 administered 5 h before LPS at 25 and 75 mg/kg inhibited TNF production by 59 \(\pm\) 7\%*** and 86 \(\pm\) 2\%***, respectively (Fig. 1). These data suggest that GW2580 has at least a 5-h duration of action against LPS-induced TNF production.

Effect of GW2580 in Normal Male Rats. GW2580 was dosed to normal rats b.i.d. for 21 days. Measurement of GW2580 levels in plasma 1 to 2 h and 16 h after dosing on day 1 and 21 (Table 1) showed a similar dose-related increase in GW2580 exposure on both days, indicating consistent GW2580 exposure over the 21 days. At 3 \(\mu\text{M}\) in vitro, 24 and 95\% GW2580 was bound to protein in 10\% fetal bovine serum in RPMI 1640 media and rat plasma, respectively (Conway et al., 2005). Subtracting the fraction of compound bound to plasma proteins gives maximal plasma concentrations of unbound GW2580 of 0.3 and 0.55 \(\mu\text{M}\) after dosing of 37.5 and 75 mg/kg GW2580, respectively, on day 1. These concentrations are greater than the concentrations of unbound GW2580 needed for half-maximal inhibition of CSF-1-induced growth of rat monocytes in vitro (approximately 0.14 \(\mu\text{M}\)).

Oral administration of vehicle and GW2580 b.i.d. for 21 days at 7.5, 37.5, and 75 mg/kg caused a small dose-related increase in percentage of body weight gain from day 7 onward (Supplemental Table 2). On day 21, the percentage of body weight gain in rats treated with vehicle, 7.5, 37.5, and 75 mg/kg GW2580 was 21.4 \(\pm\) 0.6, 23.8 \(\pm\) 1.3, 27.3 \(\pm\) 1.7\*, and 31.0 \(\pm\) 2.3**, respectively. Treatment showed no effect on spleen or liver weights (data not shown) or the histology of the 32 tissues examined (see tissue list under Materials and Methods). Serum clinical chemistries and blood hematology were measured at sacrifice (Supplemental Tables 3 and 4). GW2580 produced dose-related changes in several serum clinical chemistry endpoints, with the highest dose of 75 mg/kg increasing alanine aminotransferase (ALT) by 70 \(\pm\) 6\%***, increasing aspartate aminotransferase (AST) by 39 \(\pm\) 3\%*, increasing total protein by 12 \(\pm\) 1\%***, and decreasing inorganic phosphate by 18 \(\pm\) 2\%***. Platelet, neutrophil, and lymphocyte counts all changed in a dose-related manner, with the highest dose of 75 mg/kg increasing platelets by 16 \(\pm\) 3\%**, increasing neutrophils by 105 \(\pm\) 37\%*, and de-

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* Conc., concentration.

Data represent mean \(\pm\) S.E.M. from \(n\) = 3 per group.

Histological scoring endpoints were compared with vehicle-treated groups using Dunnett’s multiple comparison test. In a separate study, GW2580 administered at 25 and 75 mg/kg 1 h before LPS inhibited TNF production by 62\%*** and 83\%***, respectively (data not shown).
creased lymphocytes by 15 ± 4%. The largest change in hematology was a dose-related increase in monocyte counts, with 7.5, 37.5, and 75 mg/kg GW2580 increasing counts by 66 ± 22, 223 ± 60%, and 316 ± 61%, respectively.

**Activity in the Rat PGPS Arthritis Model.** In the PGPS reactivation model, an intravenous reactivation dose of PGPS causes massive T-cell activation and infiltration into the previously primed joint, resulting in a peak of ankle swelling at 3 days followed by chronic synovitis, pannus formation, and marginal erosion of cartilage and bone by 30 to 40 days. This swelling response over the first 3 days is inhibited by steroids (Schwab et al., 1993), anti-TNF antibodies (Schwab et al., 1993), TNF convertase inhibitors (Conway et al., 2001), and p38 kinase inhibitors (data not shown). GW2580 dosed b.i.d. at 60 mg/kg starting 1 h before the PGPS reactivation dose on day 0 caused no effect on ankle swelling, whereas prednisolone, the steroid positive control, strongly inhibited ankle swelling on days 1, 2, and 3 (data not shown).

**Activity in the Rat Adjuvant Arthritis Model.** In the rat adjuvant arthritis model, the ankles swell from approximately day 10 to day 21 after adjuvant administration, with bone and cartilage damage occurring between days 16 and 21. To assess the effect of GW2580 on the various phases of the adjuvant model, five studies were conducted where GW2580 was dosed either from days 0 to 21, 7 to 21, or 14 to 21 (dose groups in Fig. 2). GW2580 showed no consistent affect on ankle swelling, whereas the steroid positive control prednisolone showed the expected strong inhibition in all studies (data not shown). Radiological assessment of all ankles showed no effect of GW2580 on soft tissue swelling (edema and joint effusion), with prednisolone showing strong inhibition (Fig. 2). In contrast, both GW2580 and prednisolone showed strong inhibition of bone demineralization (Fig. 3), bone erosion (Fig. 4), abnormal bone growth (Fig. 5), and joint space narrowing (Fig. 6). Consistent with the radiology results, densitometry measurements of bone mineral content showed that both GW2580 and prednisolone increased bone mineral content in a dose-related manner (Fig. 7).

Adjuvant induced arthritis in rats chiefly affects the ankle joints and the histopathological changes are similar to those seen in human disorders such as rheumatoid arthritis and Reiter’s disease (Pearson and Wood, 1963). It starts as a synovitis (characterized by synovial effusion and synovial proliferation), accompanied by extensive periarticular edema and acute inflammatory cell infiltration, which accounts for the joint swelling observed early in the development of the lesion. Later on, pannus develops within the joints, and extensive granulation tissue forms, which invades and destroys subchondral bone and articular cartilage. At the same time, there is a great deal of osteoclast and fibroblast activity in and around the joints, along with profound new bone formation both within the medullary cavity and around the periphery of the damaged tarsal bones of the ankle (Chang et al., 1980; Owen, 1980). Histological assessment of the most severely affected ankle (left or right) was used to score the degree of inflammation and connective tissue damage. Several parameters were scored for inflammation, which included synovial effusion (characterized by the accumulation of fluid within the synovial space), synovitis (infiltration of inflammatory cells into the synovium, sometimes accompanied by synovial degeneration), and pannus (a mass of synovium and synovial stroma consisting of inflammatory cells, granulation tissue and fibroblasts overlying the articular cartilage). Scores for periarticular acute inflammatory foci (foci of edema, fibrin deposition and acute inflammatory cells, including neutrophils and lymphocytes, in the connective tissue around the joint) and suppurrative inflammatory foci (predominantly neutrophilic aggregates of inflammatory cells within the joint structures) were averaged in each rat and expressed as an acute inflammation score. Parameters scored for connective tissue damage were bone destruction, osteoclast activity (activated osteoclasts at bone surfaces degrading bone), medullary granulation tissue with osteoclasts...
(characterized by the presence of large numbers of macrophages, monocytes, and osteoclasts within the marrow cavity, replacing the normal bone marrow), medullary new bone (formation of woven bone within the medullary cavity), cortical new bone (formation of periosteal woven bone on the outer surface of the cortical bone), and cartilage destruction. Graphs of all the ankle histological scores are in Supplemental Figs. 1 to 10. Photomicrographs of hematoxylin/eosin sections illustrate the effects of the disease and compound treatment on joint histology (Fig. 8). GW2580 had no effect on most of the indices of inflammation such as synovial effusion, pannus, and synovitis and acute inflammation, whereas steroid treatment consistently decreased these endpoints. However, GW2580 sharply decreased bone destruction, osteoclast activity, medullary new bone formation, with higher doses of GW2580 (75–100 mg/kg b.i.d.) showing the same efficacy as the steroid treatment. In contrast, GW2580 showed only modest efficacy against cartilage destruction with no effect on cortical new bone formation, whereas the steroid treatment consistently affected both these endpoints.

Adjuvant arthritis causes a decrease in body weight gain and an increase in spleen weight due to granulomatous inflammation. GW2580 showed no effect on body weight gain in arthritic rats (data not shown), but it did cause a time- and dose-related decrease in the spleen weight (data not shown), the spleen/body weight ratio (Supplemental Fig. 11), and granulomatous splenitis (Supplemental Fig. 12). The high dose of 75 mg/kg over days 0 to 21 caused almost complete inhibition of granulomatous splenitis. The effects on the spleen weight and granulomatous inflammation were most pronounced with treatment over days 0 to 21, with progressively less effect with treatment over days 7 to 21 and 14 to 21.

Serum clinical chemistries and blood hematology were measured in arthritic rats that had received vehicle or 75 mg/kg GW2580 b.i.d. for 21 days (Supplemental Tables 5 and 6). As in normal rats, the largest changes in clinical chemistries with GW2580 were increases in serum ALT, AST, and total protein of 126 ± 7%***, 236 ± 6%***, and 16 ± 1%***, respectively. Histological assessment did not reveal any liver pathology that could account for the small increases in ALT and AST. It is possible that changes in muscle or other tissues that were not examined by histology in the arthritic rats could be associated with this small increase in AST.
Adjuvant arthritis produced approximately a 7-fold increase in blood neutrophils and about a doubling in monocytes. In contrast to its statistically significant increases in neutrophils (105 ± 37% ±) and monocytes (316 ± 61% ±) in normal rats, GW2580 decreased neutrophil and monocyte counts by 7 ± 7 and 39 ± 4%, respectively, in arthritic rats.

Adjuvant arthritis could change the absorption or clearance of GW2580. To evaluate this possibility, GW2580 was measured in the plasma 16 h after the last afternoon dose and 2 h after the morning dose on day 21. The concentrations of GW2580 seen in arthritic rats (Table 2) were similar to those seen in normal rats (Table 1).

**Discussion**

**Activity in Vitro.** GW2580 is highly selective for cFMS kinase in vitro, with an IC_{50} value of 0.03 μM, and it has no effect on 186 other kinases (Supplemental Table 1) (Conway et al., 2005). GW2580 inhibited the ability of CSF-1 to induce the growth of murine M-NFS-60 myeloid tumor cells, human monocytes, and rat monocytes at IC_{50} values of 0.33, 0.47,

and 0.2 μM respectively (see Results) (Conway et al., 2005), showing that GW2580 is active across three species.

**Comparison with Other Kinase Inhibitors.** Several multitarget kinase inhibitors inhibit cFMS kinase, CSF-1-mediated cellular activities and show activity after oral administration in vivo. SU11248 inhibits cFMS, VEGFR, KIT, and FLT3 kinases and inhibits tumor-induced bone destruction (Murray et al., 2003). Ki20227 inhibits cFMS, VEGFR, PDGFR and KIT kinases and inhibits tumor-induced bone destruction (Ohno et al., 2006). ABT-869 inhibits cFMS, VEGFR, and PDGFR kinases and inhibits tumor growth (Albert et al., 2006). Imatinib inhibits cFMS, ABL, KIT, and PDGFR kinases and inhibits joint swelling, inflammation, and joint destruction in collagen-induced arthritis in mice (Paniagua et al., 2006). In contrast, in collagen-induced arthritis in rats, imatinib inhibits joint destruction, with no effect on joint swelling and inflammation (Ando et al., 2006). It is possible that a more selective cFMS kinase inhibitor such as GW2580 could show a different efficacy and side effect profile than multitarget inhibitors.
Acute inhibition of LPS-induced TNF production in vivo is not response to LPS. However, the mechanism by which GW2580 involved in maintaining the ability to produce TNF in response to LPS challenge, suggesting that the CSF-1-CSF-1 receptor system is less serum TNF after LPS challenge, indicating that the mechanism of inhibition in vivo involves cFMS kinase inhibition and not some effect particular to GW2580.

**Effects in Normal Rats.** To investigate the effect of GW2580 in normal rats, GW2580 was administered for 21 days to duplicate the doses and maximum length of treatment used in the subsequent adjuvant arthritis studies. GW2580 caused a small dose-related increase in body weight in normal rats (Supplemental Table 2), and the mechanism of this weight gain is unknown. It is recognized that 2 to 4-fold increases in serum ALT and AST may be of clinical concern and can be indicative of liver and/or muscle pathology at the histological level (Boone et al., 2005); however, GW2580 showed no histological evidence of liver or muscle pathology, suggesting that the small increases in serum ALT (70% **p<0.1**) and AST (39%*) seen with the highest dose of 75 mg/kg GW2580 are not adverse. Life-long CSF-1 or CSF-1 receptor deficiency in mice decreases blood monocyte counts by 80 to 90% (Wiktor-Jedrzejczak et al., 1992b; Dai et al., 2002), severely hampers bone development and diminishes the ability of the mice to combat bacterial infection (Wiktor-Jedrzejczak et al., 1996; Guleria and Pollard, 2001). In contrast, 21 days of GW2580 administration caused an unexpected dose-related increase in blood monocytes, with the 75-mg/kg dose increasing monocyte counts by 316% (**p<0.01**). We have no comparator data with other cFMS kinase inhibitors in normal rats, so this effect could either be due to inhibition of cFMS kinase or something particular to GW2580. Cell labeling studies to determine the rate of monocyte entry and egress from the blood compartment may help reveal the mechanism of this increase in blood monocytes.

CSF-1 may affect many aspects of the macrophage life cycle ranging from early effects on progenitor cells in the bone marrow, to the migration of cells into the blood and subsequently into tissues, as well as the differentiation, survival, and function of long-lived tissue macrophages. It is possible that the complete life cycle of a macrophage is longer than 21 days in rats; thus, dosing for 21 days with GW2580 may not capture the full impact of cFMS kinase inhibition. Longer term dosing and quantitative assessments of bone turnover and response to bacterial infection will be needed to more completely characterize the effect of GW2580 on normal rats.

**Activity in Arthritis Models.** In the PGPS arthritis reactivation model, an intravenous reactivation dose of PGPS causes massive T-cell activation and infiltration into the previously primed joint, resulting in a peak of ankle swelling at 3 days. The lack of effect of GW2580 on ankle swelling in this model is consistent with the observation that mice with a life-long deficiency in CSF-1 mount normal T-cell-dependent immune responses (Wiktor-Jedrzejczak et al., 1992a; Chang et al., 1995; Guleria and Pollard, 2001).

In the adjuvant arthritis model, GW2580 did not inhibit joint swelling measured by calipers (data not shown) or soft

**Cytokine Production in Vivo.** GW2580 administration 1 or 5 h before LPS injection decreased TNF production in rats by 60 to 85% (Fig. 1), showing that rats and mice (Conway et al., 2005) respond similarly to GW2580 and that GW2580 has at least a 5-h duration of action against LPS-induced TNF production in vivo.

Compared with littermates, mice with a lifetime deficiency in CSF-1 produce 65% (Nishioji et al., 1999) to 80% (Wiktor-Jedrzejczak et al., 1992a) less serum TNF after LPS challenge, suggesting that the CSF-1-CSF-1 receptor system is involved in maintaining the ability to produce TNF in response to LPS. However, the mechanism by which GW2580 acutely inhibits LPS-induced TNF production in vivo is not yet clear, given that GW2580 had no effect on LPS-induced TNF production in freshly isolated mouse peritoneal macrophages, human peripheral blood mononuclear cells, human monocytes, or macrophages in vitro (Conway et al., 2005). It is possible that the in vitro cellular assays do not replicate the regulation of TNF production in tissue-specific macrophage populations in vivo. cFMS kinase inhibitors with different structures also inhibit TNF production in rats and mice in vivo (data not shown), indicating that the mechanism of inhibition in vivo involves cFMS kinase inhibition and not some effect particular to GW2580.

![Graph](image-url)  
**Fig. 7.** Effect of GW2580 on bone mineral content in the ankles of adjuvant arthritis rats. These are same rats and treatments as explained in legend of Fig. 2. The mineral content of the ankles was measured by bone densitometry. In studies 1, 2, and 5, only the left ankle was measured. In study 3, values from the left and right ankle were averaged. Study 4 was not measured (N.D.). *p < 0.05; **p < 0.01; and ***p < 0.001 compared with vehicle-treated rats using Dunnett’s multiple comparison test.
Fig. 8. A to F, effect of GW2580 on adjuvant arthritis joint histology. These are representative sections through the ankle joints of rats. Sections are cut sagittally, stained with hematoxylin and eosin, and photographed at magnifications of 7.5x and 50x. A and B, normal rat. Note the tibio-tarsal articulation of the tibia (T), the synovial space around the joint (S), articular cartilage (AC), the depth of the subchondral bone (SCB), and the marrow cavity (MC) containing bone marrow. C and D, vehicle-treated adjuvant arthritis rat. Note the extensive periarticular inflammation (PI), the expanded synovial space (S) filled with synovial effusion, and the cortical new bone (CNB). In addition, the tibial marrow cavity has become filled with medullary granulation tissue (MG) and medullary new bone (MBN). At higher power, there is pannus (P) extending over the articular surface of the joint and into the medullary cavity, with bone and cartilage destruction (BD/CD), marked thinning of the subchondral bone and intense osteoclast activity (OC). E and F, GW2580-treated adjuvant arthritis rat. Note that although the periarticular inflammation (PI) and synovial effusion (S) are as extensive as in the vehicle-treated rat, there is no bone destruction, osteoclast activation or filling of the marrow cavity with granulation tissue or new bone. Likewise, pannus (P) is present, but also to a lesser extent. GW2580 was dosed at 75 mg/kg b.i.d. on days 7 to 21 after adjuvant administration.
In summary, GW2580 showed no adverse effects in normal rats and minimal activity on the joint inflammation endpoints in the PGPS and adjuvant arthritis models. In contrast, GW2580 showed strong inhibition of granuloma formation in the spleen and strong protection against bone destruction in adjuvant arthritis rats. This profile is different from a recent report that oral administration of GW2580 blocks the progression of paw swelling, erythema, and joint rigidity in mice with established collagen-induced arthritis, suggesting that the anti-inflammatory activity of GW2580 may be mechanism- and model-dependent (Robinson and Paniagua, 2007). Together, the data suggest further investigations of GW2580 in situations of enhanced bone turnover such, as osteoporosis, tumor-induced bone destruction, and orthopaedic implant failure, as well as other pathologies such as atherosclerosis and human immunodeficiency virus infection where CSF-1 signaling has been implicated.

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