Analgesic effects of p38 kinase inhibitor treatment on bone fracture healing

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ABSTRACT

Traditional and COX-2 selective non-steroidal anti-inflammatory drug (NSAID) treatment inhibits fracture healing in animal models. This indicates that either the inflammatory phase following a bone fracture is necessary for efficient or sufficient bone regeneration to heal the fracture or COX-2 may have a specific function during bone regeneration unrelated to inflammation. These observations also indicate that NSAID use during fracture healing may be contra-indicated. Thus, identification of different analgesics for fracture pain or other orthopaedic surgical procedures would be of significant clinical benefit. Inhibitors of p38 kinase also have significant analgesic properties. However, p38 kinase is a critical regulator of inflammation. To assess the potential use of p38 kinase inhibition as a therapeutic strategy to manage fracture pain, the analgesic properties of SCIO-469, a p38 kinase inhibitor, were assessed in a rat fracture model and compared to other common analgesics. In addition, the effects of SCIO-469 treatment on ultimate fracture healing outcomes were measured by radiography and torsional mechanical testing. The data indicate that SCIO-469 was an effective analgesic. No adverse events related to fracture healing were observed in rats treated with SCIO-469. Immunohistochemistry showed that p38 kinase is activated primarily in the first days following a fracture. These observations suggest that p38 kinase inhibition may be an effective therapeutic strategy to manage orthopaedic-related pain. These observations also indicate that COX-2 has a specific function during bone regeneration other than promoting inflammation.

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1. Introduction

Pain management for bone fractures or orthopaedic surgical procedures relies upon the use of narcotics and non-steroidal anti-inflammatory drugs (NSAIDs) [30]. Narcotics act within the central nervous system to inhibit pain [11,29]. In contrast, NSAIDs work within the central nervous system and at the peripheral injury site to inhibit pain [6,48]. NSAIDs inhibit cyclooxygenase activity and reduce formation of prostaglandins [64]. High local concentrations of prostaglandins can sensitize nociceptors such that normally non-painful stimuli are able to illicit a pain response (allodynia) [66]. Reduced prostaglandin levels at the peripheral injury site lessen inflammation and inhibit allodynia. Persistent peripheral pain can trigger secondary hyperalgesia [7]. In turn, secondary hyperalgesia can lead to diffuse pain responses in unaffected body areas. NSAID treatment to reduce peripheral pain can diminish development of secondary hyperalgesia [52]. NSAIDs also have a direct effect in the central nervous system, since peripheral inflammation can induce cyclooxygenase expression and raise prostaglandin E2 levels in the central nervous system [21,52].

NSAID use can lead to severe side effects in a small proportion of users [49,55]. Among these complications are gastrointestinal bleeding and kidney malfunction [50,65]. In addition, NSAID use has been linked to impaired fracture healing in animal models and in retrospective clinical studies [3,5,17,51,57,58,60]. Thus, NSAID use to control bone fracture pain or after certain orthopaedic surgical procedures may not be advisable [12]. Similarly, narcotic use also can lead to side effects including drug dependency and impaired mental status which may delay rehabilitation [29].

The mechanism by which NSAIDs impair fracture healing is not known. However, use of cyclooxygenase-2 (COX-2)-selective NSAIDs and COX-2 knockout mice has demonstrated that inhibition of COX-2 or loss of COX-2 activity rather than COX-1 is primarily responsible for impaired fracture healing [57]. Since COX-2 is a critical regulator of the inflammatory response, these observations suggest that any treatment that might lessen inflammation also could lead to impaired fracture healing.

Drugs that inhibit p38 kinase are effective analgesics [27]. One such compound, SCIO-469, selectively inhibits p38 kinase, and has been demonstrated to be an effective analgesic in animal mod-
els and in a clinical trial for acute dental pain [43]. However, p38 kinase activity is associated with early inflammatory events [54]. Thus, use of a p38 kinase inhibitor to control bone fracture pain or pain following orthopaedic surgical procedures may impair healing in a manner similar to NSAIDs and COX-2-selective NSAIDs. In contrast, identification of a non-narcotic analgesic that effectively relieves pain following a fracture could have significant clinical value.

To determine whether p38 kinase inhibitors may be useful analgesics for fracture pain management, a procedure was developed to measure pain following a femur fracture in a rat model, and a study comparing analgesia with SCIO-469, acetaminophen, morphine, or celecoxib was completed. Further, the effects of SCIOS-469 treatment on fracture healing outcomes were measured by radiography and torsional mechanical testing. Immunohistochemistry was used to determine the period during fracture healing when p38 kinase was activated.

2. Materials and methods

2.1. Animals, drug dosage, and administration

All procedures were approved by the UMDNJ-New Jersey Medical School Institutional Animal Care and Use Committee prior to initiation of experiments and conform to ethical guidelines established by the International Association for the Study of Pain. Female Sprague-Dawley rats were fed a standard diet and kept caged in pairs in a constant temperature and humidity environment. All rats were between 250 and 300 g and approximately 12 weeks old at the beginning of the experiment. For the pain assessment studies, drugs were delivered by oral gavage or subcutaneous injection beginning 4 h after fracture, and were continued until groups showed 2 or 3 days of equivalent weight displacement between hind limbs (see below). The rats were gavaged with carrier (1% methylcellulose twice-a-day, control), 300 mg/kg acetaminophen (twice-a-day), 4 mg/kg celecoxib (once-a-day), 10 mg/kg celecoxib (once-a-day) or 30 mg/kg SCIO-469 (twice-a-day). Morphine was used at a 3 mg/kg dose and was delivered by subcutaneous injection in the scapular region of the rats once-a-day. p38α Kinase-specific inhibitor SCIO-469 was synthesized by Medicinal Chemistry at SCIOS Inc. (Fremont, CA, USA). SCIO-469 has an in vitro IC50 of 9 nM for the inhibition of p38α, and at least 2000-fold selectivity for p38α over p38β, and at least 2000-fold selectivity for p38α over an in vitro panel of 20 other kinases, including other MAP kinases [23]. The reported t1/2 in rats is ~30 min and ED50 is 30 mg/kg for SCIO-469 in the rat carrageenan-induced paw swelling model [34]. No significant differences were found in animal weight changes between treatment groups throughout these experiments. For the fracture healing outcome studies, the rats were gavaged twice-a-day with carrier for 14 days or with 30 mg/kg SCIO-469 for 7 or 14 days.

2.2. Closed femur fracture production

A standard closed femur fracture for rats was employed as described previously [3]. Briefly, rats were anesthetized by intraperitoneal injection of ketamine (50 mg/kg) and xylazine (10 mg/kg). Under aseptic conditions, a medial parapatellar incision (approximately 1 cm) was made in the right hind limb, and the patella was dislocated laterally. The medullary canal was entered through the intercondylar notch and reamed with a 21 gauge needle. A 0.71 mm diameter 304L stainless steel pin was then inserted into the canal, and was secured in the greater trochanter by tamping. The distal portion of the pin was cut flush within the intercondylar notch. The patella was reduced, and the soft tissue and skin were closed in two layers using resorbable sutures. The diaphysis of the pinned femur was fractured immediately using a 3-point bending device (Fig. 1) [3]. Six rats underwent the surgical procedure, including insertion of the intramedullary pin, but were not fractured in order to assess the amount of pain caused by the surgical procedure only.

2.3. Measuring pain

Differential weight displacement between the left (unfractured) and right (fractured) hind limbs was used to assess pain. Weight displacement on each hind limb was measured using an incapacitation meter (Stoelting Co., Wood Dale, IL) as an indicator of pain [39]. For this assay, 6 rats were tested in each treatment group except for the celecoxib 10 mg/kg group which consisted of 7 rats. For three consecutive days prior to fracture, rats were acclimated to the incapacitation meter. Beginning 24 h post-fracture, pain was measured prior to (defined as time zero) and 2 h after drug administration each day until groups showed 2 or 3 days of equivalent weight displacement or until 21 days after fracture for the carrier and morphine treatment groups. After a 1- or 2-day washout period, pain again was measured for 2-5 days to assess whether the rats had achieved normal weight-bearing. On days 4 and 7 post-fracture, pain was measured at time zero and at 1, 2, 4, and 8 h after drug administration to assess the kinetics of pain inhibition. Areas under the curve (AUC) were compared between each treatment group using ANOVA and post-hoc Bonferroni tests (SigmaStat version 3, SPSS Inc., Chicago, IL). Normal weight-bearing was considered to be approximately 45% weight displacement on the fractured hind limb as measured with the incapacitation meter.

2.4. Radiography

Radiographs were made using a Packard Faxitron (McMinnville, OR) and Kodak MinR-2000 mammography film (Eastman Kodak Co., Rochester, NY) while rats were under anesthesia or after sacrifice. Immediately following fracture, radiographs were made to verify the position and quality of each fracture. Additional radiographs were made at 5 and 8 weeks after sacrifice to determine the degree of healing. Fracture healing was evaluated from the radiographs using a 4-point scoring system [3]. For radiographic scoring, femurs were harvested, and the soft tissue was carefully removed to avoid damaging the callus. The isolated femurs were then radiographed (ventral-dorsal view) and the radiographs were scored in a blinded fashion by three independent investigators familiar with fracture healing in rats. Each radiograph was scored from 0 to 4 based on apparent bone bridging across the callus at the left and right periphery (1 point each) and by apparent bone bridging between the cortices of the femur on the left and right sides (1 point each). Radiographs with a score of 4 indicate that the fracture had been bridged completely with new bone, a score of 0 indicates that no new bone bridged the fracture, while scores of 1–3 indicate partial bridging of the fracture with new bone. The data were compared between treatment groups using ANOVA on ranks and post-hoc Dunn’s tests.

2.5. Immunohistochemistry using p38 MAPK antibodies

Time staged fracture callus specimens were collected from female Sprague-Dawley rats (~270 g) and processed for standard paraffin histology. Six rats were examined for each time point: 1, 2, 4, and 7 days post-fracture. Serial longitudinal sections (5 μm thick) along the dorsal-ventral plain were stained with Mason’s trichrome (Sigma Diagnostics, St. Louis, MO) to examine fracture histology or used to detect phospho-p38 kinase by immunohisto-
chemistry (IHC). Using standard procedures, specimens were de-waxed, re-hydrated, and endogenous peroxidase activity was neutralized with hydrogen peroxide. The specimens were blocked with 3% bovine serum albumin in PBS (BSA–PBS) for 60 min and then anti-phospho-p38 kinase (12F8) rabbit monoclonal antibodies (diluted 1:10,000 in BSA-PBS; Cell Signaling Technology, Danvers, MA) were applied and incubated overnight by rocking (Antibody Amplifier, ProHisto, Columbia, SC). After washing in PBS, the anti-phospho-p38 kinase antibody was detected with horseradish peroxidase-conjugated secondary antibody using a standard diaminobenzidine reaction. The IHC samples were counterstained with methyl green to identify cell nuclei. Sections were viewed using a standard light microscope, and digital images were captured (10–16 images per sample) using a Nikon DXM1200f camera. The number of phospho-p38 kinase positive cells in the fracture site of each specimen was counted using Image-Pro Plus Software Version 5.02.9 (Media Cybernetics, Silver Spring, MD). Only specimens from three rats were counted on day 2 because of the large number of phospho-p38 kinase positive cells.

2.6. Mechanical testing

The torsional mechanical properties of the ipsilateral and contralateral femurs were determined as described previously [3,57]. Animals in each treatment group were sacrificed 5 and 8 weeks after fracture by CO2 asphyxiation. Briefly, the femurs were retrieved, and the stainless steel pin and soft tissue were removed without disturbing the fracture callus or bone. The femurs were wrapped in saline-soaked gauze to prevent dehydration before mechanically testing. Femur length and external callus dimensions were measured using digital calipers. The femur ends were potted in 0.75-inch hexnuts using a low-melt temperature metal (Wood’s metal, Alfa Aesar, Ward Mill, MA). The gauge length of the potted femurs was recorded. Torsional testing was conducted using a servohydraulic testing machine (MTS Systems Corp., Eden Prairie, MN) with a 20-Nm reaction torque cell (Interface, Scottsdale, AZ). Testing was performed to failure at an actuator head displacement rate of 2 degrees per second and a data-recording rate of 20 Hz. The fractured and the contralateral femurs were tested in proper ana-
tomatical orientation. After mechanical testing, internal callus dimensions were measured and recorded. Peak torque and angle at failure were calculated from the load-deformation curves. The polar moment of inertia was calculated based on a hollow ellipse model and the recorded callus dimensions. Equations used to derive torsional rigidity, maximum shear stress, shear modulus, and the polar moment of inertia have been described [57]. Data were compared between treatment groups using ANOVA and post-hoc Holm–Sidak tests.

3. Results

3.1. Long-Term pain relief and return to weight-bearing

Pain is expected to diminish as inflammation recedes following bone fracture and as healing proceeds. Thus, it is likely that the analgesic efficacy of different drugs could vary depending upon the healing time. To account for this, pain relief was measured as weight-bearing on the fractured limb for at least 14 days after fracture (Fig. 2). Pain relief was compared between the different analgesics as the area-under-the-curve (AUC) defined by the time after drug administration versus the percent weight-bearing on the fractured limb each day at time zero and 2 h after drug treatment (Fig. 2A). As a control, weight-bearing was measured in rats that underwent the surgical procedure and insertion of the intramedullary pin but without fracture (Fig. 2A). A small drop in weight-bearing by the ipsilateral limb was detected in these mock-treated (pin-only) rats that peaked 2 days after surgery and returned to normal levels on day 4. Thus, the sharp decline in weight-bearing on the fractured limbs by other animals reflects effects of the fracture and not the surgical procedure or the presence of the intramedullary pin. All analgesics provided significant pain relief over 2 weeks when measured at 2 h after dosing as compared to the carrier (Fig. 3B). SCIO-469 treatment provided more pain relief than either the 4 or 10 mg/kg celecoxib dose over the first 14 days and a similar amount of pain relief as compared to acetaminophen or morphine treatment groups. Only SCIO-469 and morphine treatment led to a significant increase in pain relief at time zero over 14 days (Fig. 3A). Rats treated with SCIO-469 also returned to normal weight-bearing (measured at time zero) on day 12 post-fracture; while carrier-treated rats needed an additional 8 days to return to normal weight-bearing (Fig. 2A and D). Furthermore, when SCIO-469 treatment was discontinued on day 14 after fracture, normal weight-bearing was still evident on day 17 post-fracture. These observations demonstrate that SCIO-469 treatment provides good analgesia and suggests that SCIO-469 does not impair fracture healing.

In contrast, rats treated with celecoxib (10 mg/kg/day) returned to normal weight-bearing on day 16 after fracture (Fig. 2F). However, when celecoxib therapy was halted on day 17 post-fracture and pain was assessed on day 20 post-fracture, weight-bearing declined on the ipsilateral hind limb in the celecoxib-treated rats. This suggests that the return to normal weight-bearing on day 17 post-fracture in the celecoxib-treated rats was due to an analgesic effect. After celecoxib was discontinued, these rats did not return to normal weight-bearing until day 24 after fracture as compared to day 17 in the carrier-treated rats.

As expected, morphine treatment and acetaminophen treatment provided good pain relief (Fig. 2B and C: Fig. 3). Acetaminophen only provided significant pain relief after dosing (Fig. 3B) but was able to provide significant pain relief immediately after fracture during the inflammatory phase of fracture healing (Fig. 2C).

Fig. 2. Effects of different analgesics on weight-bearing following femur fracture. Weight-bearing was measured in mock-treated rats that underwent the surgical procedure and intramedullary pin placement but had no fracture or drug treatment (pin-only, A, n = 5). Rats that underwent the surgical procedure, intramedullary pin placement, and fracture were treated with the indicated doses of carrier (A, n = 6), morphine (B, n = 5), acetaminophen (C, n = 6), SCIO-469 (D, n = 6), low-dose celecoxib (E, n = 6), and high-dose celecoxib (F, n = 7) beginning 4 h after fracture and continuing for 14–21 days as indicated. Weight displacement was measured beginning on day 1 after fracture just before morning drug administration (closed symbols, zero time) and then again 2 h after drug administration (open symbols). Drug administration was stopped after the rats showed 2 or 3 days of normal weight-bearing (approximately 45% weight displacement on the fracture limb) before drug treatment. After an additional 2 or 3 days, weight displacement was again measured to determine whether normal weight-bearing had been achieved potentially through healing (C and D) or due to analgesia (E and F). Shown are mean values ± standard errors and a reference line at 45% weight-bearing.
In contrast, morphine appeared to have a longer-lasting analgesic effect that led to a significant increase in weight-bearing at zero-time between days 1 and 14 after fracture (Fig. 3A). This long-lasting effect that led to a significant increase in weight-bearing at zero-time between days 1 and 14 after fracture (Fig. 3A). This long-lasting effect that led to a significant increase in weight-bearing at zero-time (A) and 2 h after drug administration (B). Shown are the mean values (+ standard deviation) of the AUCs for each drug treatment group. Group size was 6 rats for the carrier, morphine (Morph.), acetaminophen (Acet.), low-dose celecoxib (Cx-4 mg), and SCIO-469 (SC-469) and 7 for the high-dose celecoxib (Cx-10 mg) group. SCIO-469 and morphine treatment had greater mean AUC values at time zero (A) than all other treatment groups. All analgesics had a greater mean AUC than the carrier treatment group at 2 h after drug administration, while the morphine, acetaminophen, and SCIO-469 treatment groups were also significantly greater than either celecoxib treatment group value (B). For (A) and (B), significant differences with the carrier, acetaminophen, low-dose celecoxib, and high-dose celecoxib treatment groups are indicated with an a, b, c, or d, respectively. All P values were less than 0.001 except (A), SCIO-469 vs. low-dose celecoxib (P = 0.035) and (B), low-dose celecoxib vs. high-dose celecoxib (P = 0.004).

3.2. Acute pain relief on days 4 and 7 post-fracture

Pain relief was measured in each animal immediately before drug dosing and then periodically throughout days 4 and 7 post-fracture to gauge the duration of pain relief afforded by each drug. We observed that all the analgesics provided pain relief as measured by increased weight-bearing on the fractured hind limb (Fig. 4). Rather than comparing pain relief at one specific time point after drug administration, we measured the amount of pain relief afforded by each analgesic over 8 h following drug administration as the AUC defined by time following drug administration versus percent weight-bearing. On day 4, all the analgesics performed comparably except morphine, which did not significantly improve weight-bearing on the fractured limb (Fig. 4B). On day 7 post-fracture, all the analgesics provided significant pain relief (Fig. 4D). However, SCIO-469 treatment and morphine treatment provided significantly better pain relief over 8 h than did either celecoxib dose (Fig. 4D). Acetaminophen provided a similar amount of analgesia when compared to SCIO-469 but less than morphine treatment. It should be noted that a high acetaminophen dose was used in order to match the anticipated analgesic effects of SCIO-469.

3.3. Radiographic assessment of fracture healing outcomes

Femur fracture healing success was assessed by radiography at 5 and 8 weeks after fracture in rats treated with 1% methylcellulose (carrier) or with SCIO-469 (30 mg/kg, twice-a-day) for 7 or 14 days after fracture (Figs. 5 and 6).

In carrier-treated rats, fracture healing appeared to proceed normally, and was consistent with the results of previous studies [3,57]. By 5 weeks, new bone formation had approached the fracture gap in the carrier-treated rats, and the presence of a calcified callus indicated that endochondral ossification had occurred (Fig. 5). Fracture bridging and callus remodeling were evident after 8 weeks of healing (Fig. 5). The mean radiograph scores increased from 0.8 at 5 weeks after fracture to 2.67 after 8 weeks (Fig. 6). These radiographic observations are typical of normal fracture healing [3,58].

Fracture healing appeared to proceed normally in rats treated with SCIO-469. Five weeks after fracture, radiographs from the SCIO-469-treated rats had an x-ray dense callus surrounding the fracture site similar to the radiographs from the carrier-treated rats (Fig. 5). Interestingly, the radiographs in the rats treated with SCIO-469 for 7 days indicated that bridging of the fracture site occurred sooner than in carrier-treated rats (mean scores of 0.8 in carrier-treated versus 2.1 in SCIO-469-treated rats, Fig. 6). While the mean radiograph score for the rats treated with SCIO-469 for 14 days (mean score 1.6) was higher than for carrier-treated (means score 0.8), the difference was not significant. Eight weeks after fracture, radiographs from the SCIO-469-treated rats had higher scores than the carrier-treated rats, but the differences were not significant.

3.4. Immunohistochemistry

The early events following fracture were assessed in histological specimens stained with Masson’s trichrome (Fig. 7). One day after fracture, the fracture hematoma was present with soft tissue swelling based upon the dispersed muscle fibers (Fig. 7A). By 2 days after fracture, the hematoma and soft tissue swelling were still present, but there was an apparent large influx of cells to the fracture site (Fig. 7C). Mesenchymal cells were abundantly apparent at the fracture site 4 days after fracture (Fig. 7E). Later stages of healing on day 7 after fracture were similar to previous observations (not shown) [15,57].

The natural course of p38 kinase activation during fracture healing was assessed by the detection of phosphorylated p38 kinase (p38-p38; Fig. 7 and Table 1). Phospho-p38 kinase positive cells were observed on 1 (Fig. 7B) and 2 days (Fig. 7D) after fracture, but few were evident on day 4 after fracture (Fig. 7F). The number of phospho-p38 kinase positive cells at the fracture site (callus) was counted on 1, 2, 4, and 7 days after fracture in untreated rats (Table 1). A significant number of phospho-p38 kinase positive cells were counted on day 1 after fracture that increased 10-fold on day 2 after fracture and then rapidly declined to near undetectable levels on day 7 after fracture.
3.5. Mechanical properties of healing femurs

When the mechanical properties of the femurs from the carrier-treated and both SCIO-469 treatment groups were compared, those of the SCIO-469-treated rats had similar or better mechanical properties than the carrier-treated group. No difference was found in peak torque at either 5 or 8 weeks after fracture between the carrier-treated and SCIO-469 treatment groups (Fig. 8A). However, maximum rigidity was higher after 5 weeks of healing in the rats treated with SCIO-469 for 7 days, and was consistent with the higher radiograph scores in this treatment group (Fig. 8B and Fig. 6). No differences were detected in callus material properties (maximum shear stress and shear modulus) between the carrier-treated and SCIO-469-treated rats after 5 weeks of healing (Fig. 8C and D). In contrast, callus maximum shear stress and shear modulus after 8 weeks of healing were significantly higher in the rats treated for 14 days with SCIO-469 as compared to the carrier-treated rats or the rats treated with SCIO-469 for 7 days (Fig. 8C and D). For comparative purposes, the mechanical testing data from the ipsilateral and contralateral femurs are shown in Table 2.

4. Discussion

The study’s main outcome is that p38α kinase inhibition with SCIO-469 provides good pain relief in a rodent model of fracture pain without inhibiting healing. SCIO-469 treatment did not interfere with callus formation, fracture bridging or the mechanical properties of the fractured femur. These results correlate with previous reports in which p38 kinase inhibition provided pain relief and reduced inflammation [4,14,37,61–63]. However, the results contrast with other studies showing that the inhibition of COX-2 significantly impairs fracture healing [3,16,57,58]. Since p38 kinase or COX-2 inhibition reduces inflammation, we can deduce that the inhibitory effects of celecoxib or NSAID treatment on fracture healing do not directly relate to a reduction in inflammation but to a specific property of COX-2. Additional experiments to measure inflammation following fracture in rats treated with these inhibitors will be required to validate this conclusion.

Of the analgesics tested, only acetaminophen provided significant pain relief immediately after fracture (Fig. 2). The basis for this difference is unknown, but may relate to the differences in peripheral versus central nervous system effects. Acetaminophen can act within the central nervous system to relieve pain [2,18]. SCIO-469 is not thought to cross the blood-brain barrier, and so its analgesic effects must occur peripherally [46]. Celecoxib can act peripherally and via the central nervous system to provide analgesia. The celecoxib doses used in this study are at or below minimum levels required for analgesia in rat models of hyperalgesia, but these doses inhibit fracture healing [9,56,58]. Morphine acts within the central nervous system to relieve pain. In this study, morphine treatment
increased pre-dosing analgesia on days 1 and 2 after fracture which dropped on day 3 (Fig. 2B). Morphine treatment in subsequent days appeared to increase post-dosing analgesia in a manner similar to SCIO-469 treatment. In rats without surgery or fracture, 3 or 10 mg/kg subcutaneous doses of morphine caused a significant 30% decrease in weight displaced on the hind limbs as measured with the incapacitance meter (data not shown). The morphine-treated rats appeared to displace more weight on their abdomen and rump rather than directly on their hind limbs. Thus, whether the higher pre-dosing analgesia reflects a morphine effect or experimental variability remains to be determined.

The rat closed femur fracture model has been used extensively to investigate the biology of bone regeneration and the effects of NSAIDs and COX-2 inhibitors on bone fracture healing [3,4,16,57]. However, bone fracture models have not been used extensively as pain models despite the obvious clinical association [19,28,31]. We became concerned that the analgesic effects of COX-2 inhibitors led to impaired fracture healing rather than healing being impaired by decreased prostaglandin synthesis. We reasoned that analgesia-treated rats would be more likely to weight-bear and use the fractured limb and that this would lead to re-injury and delayed healing. Data presented here indicate that SCIO-469 induced analgesia but did not impair healing in the rat closed femur fracture model. Previously, we found that acetaminophen treatment does not inhibit fracture healing, whereas celecoxib treatment does [3,57,58]. Celecoxib treatment provided the least pain relief but was the only test drug to inhibit fracture healing. Thus, we conclude that the inhibition of fracture healing caused by celecoxib treatment does not relate to any analgesic effects but to the role of COX-2 and prostaglandin synthesis in bone regeneration.

Morphine effects on fracture healing outcomes were not measured since opioid treatment did not impair fracture healing in another animal model [33]. However, opioid receptor antagonist may reduce osteoblast activity [44,45]. Additional studies to directly test morphine or other opioids effects on bone regeneration are needed to resolve this apparent discrepancy.

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The pain response following a fracture is poorly characterized. Bone is a highly innervated tissue [24,25]. The initial pain after a fracture is usually severe, and may be caused by the physical damage of the nociceptors that innervate the periosteum, bone, and marrow [25]. Fracture fixation or callus formation that stabilizes the fracture site diminishes the pain indicating that a portion of the pain is produced by mechanotransducers [8,22,53]. During the inflammatory phase of healing, cytokines, eicosanoids, and other inflammation-related factors can activate nociceptors to produce a painful response [20,36]. In addition, the inflammation-related mediators can sensititize and induce the production of additional mechanosensitive nociceptors in the bone so that nor-
nally non-noxious mechanical stimulation of the bone or callus is now perceived painfully. COX-2 inhibitors can significantly reduce fracture pain by reducing the synthesis of these inflammation-related mediators [35,38,41,52,59]. Similarly, pharmacological inhibition of p38 kinase can reduce the synthesis of inflammation-related mediators and reduce pain [1,26,32,63]. The persistent excitation of the nociceptors innervating the bone may induce changes in the dorsal horn of the spinal cord and higher brain, which could facilitate the transmission and perception of pain by the central nervous system [13]. When nerve damage occurs following fracture, peripheral and central sensitization may be maintained and accompanied by unnecessary sprouting of nociceptors [13]. These changes may contribute to the instances of chronic pain observed after fracture in patients with complex regional pain syndrome [10].

We found that inhibition of COX-2 or p38 kinase produced analgesia during the inflammatory phase of fracture healing in this model. Unlike acetaminophen, the analgesic response to SCIO-469 treatment did not begin until 3 days after fracture during the inflammatory phase of healing (Fig. 2) and after the apparent peak of p38 kinase activation on day 2 (Fig. 7 and Table 1). The reasons for the delayed analgesic response are unknown, but may relate to the painful stimuli produced by other signaling pathways. In carrier-treated rats, increased weight-bearing on the fractured limb began to increase after 10 days of healing (Fig. 2). This may relate to the resolution of inflammation following fracture and development of a callus with sufficient mechanical integrity to enable limited weight-bearing in the carrier-treated rats. The SCIO-469-treated rats returned to normal weight-bearing sooner than carrier, morphine, acetaminophen, or celecoxib-treated rats and unlike the celecoxib-treated rats, the SCIO-469-treated rats maintained normal weight-bearing even after drug cessation (Fig. 2). Curiously, celecoxib treatment (10 mg/kg), and perhaps morphine treatment, appeared to provide some analgesia the day after fracture and then declined as inflammation increased (Fig. 2). This may reflect that celecoxib provides some centrally acting analgesic effects, similar to acetaminophen, but that the doses used were insufficient to elicit a large peripheral analgesic response during the peak period of inflammation. In other experiments, we have shown that a 4 mg/kg celecoxib dose can reduce but not eliminate callus prostaglandin synthesis suggesting that the celecoxib doses used in the present study may not have been sufficient to reduce inflammation-related

### Table 1

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<th>Days post-fracture</th>
<th>Sample size</th>
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</tbody>
</table>

![Fig. 7. Immunohistochemical detection of phospho-p38 kinase in time-staged fracture callus specimens. Fractured femurs were collected at 1 (A, B), 2 (C, D), 4 (E, F), and 7 days (not shown) after fracture. Specimens from 6 rats were examined at each time point. Histological specimens were stained with Masson’s trichrome to determine callus morphology: H, hematoma; M, muscle; Ms, mesenchymal cells; O, cortical bone. The asterisk indicates the fracture site and all specimens are oriented with the external callus pointing upwards. Activated p38 kinase (phospho-p38 kinase) was detected in the samples by immunohistochemistry (B, D, and F). The boxes in (A), (C), and (E) represent the areas shown in (B), (D), and (F), respectively, from serial sections of the same specimens. Representative cells positive for phospho-p38 kinase are indicated (>). The marker at the bottom of (A), (C), and (E) is 250 μm and for (B), (D), and (F) is 25 μm.](https://example.com/image-url)
mediator levels below the pain threshold [58]. Our data support the hypothesis of Jimenez-Andrade et al. that initial fracture pain is promoted by the activation of mechanotransducers expressed by nociceptors, while secondary pain that occurs within hours to days of fracture is due to the release of prostaglandins and other inflammation-related factors in response to tissue injury that activate and sensitize nociceptors [28]. Consistent with this interpretation, activated p38a kinase (phospho-p38 kinase) peaked between 1 and 4 days after fracture (Fig. 7 and Table 1).

The ability of SCIO-469 treatment to reduce time to normal weight-bearing suggests that SCIO-469 treatment may promote healing. Radiographic examination and mechanical testing of fractured femurs after 5 or 8 weeks of healing also suggest that SCIO-469 treatment may promote healing. However, the effect was not consistent across all examined parameters. The mechanical testing results lacked sufficient statistical power to determine whether any positive effect of SCIO-469 treatment on fracture healing exists beyond those detailed above. Additional experiments will be required to identify p38a kinase inhibitor treatment regimens that may indeed promote bone regeneration and whether any such positive effect relates to enabling load-bearing sufficient to promote healing. However, a positive effect of p38a kinase inhibition on fracture healing outcomes would be consistent with the observations in which p38a kinase inhibition restored cartilage and bone

---

### Table 2

<table>
<thead>
<tr>
<th>Contralateral and Ipsilateral Femur Biomechanical Values.</th>
<th>Vehicle-treated</th>
<th>SCIO-469 (7 days)</th>
<th>SCIO-469 (14 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fractured</td>
<td>Left</td>
<td>Fractured</td>
</tr>
<tr>
<td><strong>5 Weeks post-fracture (mean ± SD)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample size</td>
<td>10</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>Peak torque (Nmm)</td>
<td>301 ± 70</td>
<td>345 ± 74</td>
<td>398 ± 118</td>
</tr>
<tr>
<td>Max. rigidity (Nmm²/rad) (×10³)</td>
<td>22.8 ± 11.9</td>
<td>29.5 ± 12.7</td>
<td>35.3 ± 8.5</td>
</tr>
<tr>
<td>Max. shear stress (MPa)</td>
<td>16.0 ± 3.0</td>
<td>158.1 ± 91.3</td>
<td>22.7 ± 13.2</td>
</tr>
<tr>
<td>Shear modulus (GPa)</td>
<td>0.34 ± 0.16</td>
<td>6.60 ± 3.21</td>
<td>0.568 ± 0.22</td>
</tr>
<tr>
<td><strong>8 Weeks post-fracture (mean ± SD)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample size</td>
<td>9</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>Peak torque (Nmm)</td>
<td>452 ± 103</td>
<td>435 ± 136</td>
<td>450 ± 137</td>
</tr>
<tr>
<td>Max. rigidity (Nmm²/rad) (×10³)</td>
<td>46.5 ± 19.8</td>
<td>36.1 ± 9.8</td>
<td>47.8 ± 29.8</td>
</tr>
<tr>
<td>Max. shear stress (MPa)</td>
<td>66.5 ± 12.3</td>
<td>174.0 ± 50.8</td>
<td>65.0 ± 19.2</td>
</tr>
<tr>
<td>Shear modulus (GPa)</td>
<td>2.34 ± 0.74</td>
<td>7.55 ± 2.51</td>
<td>2.57 ± 1.52</td>
</tr>
</tbody>
</table>

---

### Fig. 8.

Mechanical properties of healing fractures. Rats were euthanized at 5 (gray bars) and 8 weeks (black bars) after fracture. The healing femurs were resected and healing was assessed by torsional mechanical testing to failure. Shown are mean values ± standard errors for peak torque (A), maximum rigidity (B), maximum shear stress (C), and shear modulus (D). Values statistically higher than carrier-treated (Carrier) values are denoted with asterisks (\( P < 0.05 \)). Group sizes were 10, 11, and 8 for the carrier-treated, SCIO-469 7 day treatment group (SCIO-469-7d), and SCIO-469 14 day treatment group (SCIO-469-14d), respectively. At 8 weeks after fracture, the group sizes were 9, 10, and 10 for the carrier-treated, SCIO-469-7d, and SCIO-469-14d groups, respectively.
structure in a murine arthritis model [40]. Despite the statistical limitations, no data indicated that SCIO-469 negatively affected fracture healing outcomes.

The rats did not attain normal 50:50 weight-bearing between the intact and fractured hind limbs over the study period. The reason for this discrepancy is unknown, but may reflect the continued presence of the stainless steel pin in the fractured femur. Though the pin is trimmed flush with the femoral condyles, over time, the pin often migrates slightly into the articular space. This may lead to persistent painful or behavioral changes in weight-bearing by the rats.

In summary, these experiments demonstrate that SCIO-469, a p38α kinase inhibitor, is a potent and effective analgesic for pain caused by bone fractures in an animal model. These observations suggest that treatment with a p38α kinase inhibitor, which can nominally lessen the inflammatory response, does not impair fracture healing. Additional experiments will be required to determine the safety and efficacy of p38α kinase inhibition as an analgesic for fractures or other orthopaedic injuries.

Conflicts of interest

J.A.C. and M.M. have no conflict of interest. S.M. and L.H. are former employees of SCIOS, Inc. J.P.O.C. is a consultant for Celgene, Inc. and an owner and officer of Accelloc Inc.

Acknowledgements

Author contributions: J.A.C. performed the rat fracture studies, data collection, and aided with data analysis and manuscript preparation. Markus Meyenhofer performed the histology and immunohistochemical localization of phospho-p38 kinase. J.P.O.C. designed the experiments, aided with data collection and was primarily responsible for data analysis and manuscript preparation. S.M. and L.H. provided SCIO-469 and aided in experimental design.

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References