Systemic blockade of P2X3 and P2X2/3 receptors attenuates bone cancer pain behaviour in rats

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Pain remains an area of considerable unmet clinical need, and this is particularly true of pain associated with bone metastases, in part because existing analgesic drugs show only limited efficacy in many patients and in part because of the adverse side effects associated with these agents. An important issue is that the nature and roles of the algogens produced in bone that drive pain-signalling systems remain unknown. Here, we tested the hypothesis that adenosine triphosphate is one such key mediator through actions on P2X3 and P2X2/3 receptors, which are expressed selectively on primary afferent nociceptors, including those innervating the bone. Using a well-established rat model of bone cancer pain, AF-353, a recently described potent and selective P2X3 and P2X2/3 receptor antagonist, was administered orally to rats and found to produce highly significant prevention and reversal of bone cancer pain behaviour. This attenuation occurred without apparent modification of the disease, since bone destruction induced by rat MRMT-1 carcinoma cells was not significantly altered by AF-353. Using in vivo electrophysiology, evidence for a central site of action was provided by dose-dependent reductions in electrical, mechanical and thermal stimuli-evoked dorsal horn neuronal hyperexcitability following direct AF-353 administration onto the spinal cord of bone cancer animals. A peripheral site of action was also suggested by studies on the extracellular release of adenosine triphosphate from MRMT-1 carcinoma cells. Moreover, elevated phosphorylated-extracellular signal-regulated kinase expression in dorsal root ganglion neurons, induced by co-cultured MRMT-1 carcinoma cells, was significantly reduced in the presence of AF-353. These data suggest that blockade of P2X3 and P2X2/3 receptors on both the peripheral and central terminals of nociceptors contributes to analgesic efficacy in a model of bone cancer pain. Thus, systemic P2X3 and P2X2/3 receptor antagonists with central nervous system penetration may offer a promising therapeutic tool in treating bone cancer pain.
Introduction

A significant proportion of cancer patients with bone metastases, which cost more than $350,000 lives in the USA alone each year, suffer from severe pain (Mundy, 2002; Delaney et al., 2008; Breivik et al., 2009). Moreover, this chronic pain condition can have an unpredictable onset and increase in severity with progression of malignancy. Despite the availability of bisphosphonates, non-steroidal anti-inflammatory drugs and opioids, many patients with bone cancer pain report limited pain relief and adverse side effects, such as neuropsychiatric symptoms and gastric bleeding (World Health Organization, 1990; Zeh et al., 1995; Bruera and Kim, 2003). No new pharmacotherapy has emerged, and there is an urgent need for new bone cancer pain treatments.

The study of bone cancer pain has been advanced by animal models that show pathological changes that are distinct from those described in neuropathic and inflammatory pain models (Mantyh, 2006). In particular, unique central sensitization changes in the spinal cord, including astrogliosis and hyperexcitability in dorsal horn neurons, have been reported (Schwei et al., 1999; Medhurst et al., 2002; Urch et al., 2003). Importantly, the use of animal models has provided mechanistic insights for developing potential new analgesics, given that clinical mechanistic studies are difficult in this situation.

P2X3 and P2X2/3 receptors are preferentially expressed on the non-epidermic population of afferents and have been implicated in various neuropathic, inflammatory and visceral pain conditions (Burnstock, 2006). ATP is the endogenous ligand for P2X3 and P2X2/3 receptors and is present in abundance within cancer cells. ATP is released upon cell stress, lysis or stimulation, and thus may activate P2X3 and P2X2/3 receptors present on sensory afferent endings to cause pain (Burnstock, 1996). Despite previous reports of increased P2X3 expression on nerve fibres (Gilchrist et al., 2005; Nagamine et al., 2006) and reduced ATP-evoked current sensitivity of primary afferents to opioids in cancer pain models (Chizhmakov et al., 2009), no direct behavioural pharmacological evidence involving systemic treatments using purinergic antagonists in bone cancer pain has been published to date. A major hindrance has been the lack of suitable pharmacological tools. Recently, a selective P2X3 and P2X2/3 receptor antagonist, A-317491, was reported to be efficacious in various chronic neuropathic and inflammatory pain models (Jarvis et al., 2002; McGarraughtly et al., 2003). However, a significant drawback of A-317491 is its pharmacokinetic attributes, including poor CNS penetration, low oral bioavailability and high plasma protein binding (Wu et al., 2004; Sharp et al., 2006; Gever et al., 2010), thus limiting its value for in vivo model testing and forestalling any possible clinical use. More recently, AF-353 (previously known as RO-4), a member of a new diaminopyrimidines series of potent and selective P2X3 and P2X2/3 receptor antagonists, has been described with desirable medicinal characteristics, including high oral bioavailability and CNS penetration (Carter et al., 2009; Jahangir et al., 2009; Gever et al., 2010). Thus, AF-353 is a useful tool that can be administered systemically for evaluation of P2X3 and P2X2/3 receptor blockade as a novel therapy for chronic pain conditions.

In this study, we tested whether selective blockade of P2X3 and P2X2/3 receptors using AF-353 can attenuate nocifensive behaviours associated with bone cancer pain. AF-353 was administered systemically in a well-characterized model of bone cancer pain induced with MRMT-1 mammary gland carcinoma cells introduced to the tibia in rats. To understand the mechanisms potentially involved in the contribution of P2X3 and P2X2/3 receptors in cancer pain, in vitro co-culture of primary dorsal root ganglion (DRG) neurons with MRMT-1 carcinoma cells and in vivo dorsal horn electrophysiology were used. Our data show that oral administration of AF-353 attenuates bone cancer pain behaviour in both preventative and interventional paradigms without modifying cancer-induced bone destruction via actions on P2X3-expressing sensory neurons that innervate the tibia. In addition, AF-353 was found to reduce bone cancer-induced hyperexcitability in dorsal horn neurons and MRMT-1 carcinoma cell-induced enhanced immunoexpression of phosphorylated extracellular signal-regulated kinases (ERKs) in DRG neurons. Therefore, systemic blockade of P2X3 and P2X2/3 receptors represents a novel target for bone cancer pain therapy.

Material and methods

Animals and bone cancer surgical procedures

All procedures were performed in accordance with United Kingdom Home Office regulations (Animals Scientific Procedures Act, 1986). Adult Sprague-Dawley rats (180–250 g) were used and housed with free access to food and water at 22 °C with a 12 h alternating light/dark cycle. Cell preparation and surgical methods were similar to those previously reported (Medhurst et al., 2002). Syngeneic MRMT-1 rat mammary gland carcinoma cells were cultured in RPMI 1640 (Gibco) supplemented with 10% heat-inactivated foetal bovine serum, 1% l-glutamine and 2% penicillin/streptomycin (Gibco). On the day of surgery, MRMT-1 carcinoma cells adhered onto flasks were released by brief exposure to 0.1% w/v trypsin and collected by centrifugation in a 10-ml medium for 3 min at 225g. The resulting pellet was washed twice with 10 ml of Hanks’ balanced salt solution (HBSS) without calcium, magnesium or phenol red (Gibco) and re-centrifuged for 3 min at 225g. With the rat under halothane anaesthesia, an incision was made in a shaved and disinfected area on the anterior-medial surface around the proximal epiphysis of the tibia. MRMT-1 carcinoma cells suspended in 10 μl HBSS (containing approximately 2 x 10⁶ cells) were injected into the tibia and control animals received HBSS injection only. We have demonstrated that injection of heat-killed MRMT-1 carcinoma cells does not cause any significant differences in pain behaviour.
in comparison with vehicle control (HBSS-injected) animals (data not shown), which is in agreement with a previous study (Medhurst et al., 2002).

Pain behavioural assessments and X-ray radiology

Rats were assessed for pain behavioural changes before and during a period of 19 days following bone cancer surgery. To test for mechanical allodynia, von Frey filaments of various forces (0.36–12.6 g) were manually applied 10 times to the plantar surface of the ipsilateral hindpaw from either MRMT-1 carcinoma cell or HBSS-injected (control) rats and the frequency of positive withdrawals was recorded. The results on each day were presented as area under the curve calculated from a plot of the percentage of positive withdrawal responses at each filament against the filament force on a logarithmic scale. Hindlimb weight-bearing differences were measured using the incapacitation tester (Linton Instruments). Measurement was recorded as an average of three trials, with each trial measuring the weight over 3 s and expressed as contralateral–ipsilateral readings.

Radiographic images of ipsilateral tibia were taken following fixation on Day 20, using a digital camera inside an enclosed cabinet during exposure to an X-ray source (Faxitron MX-20) for 10 s at 25 kVp. Bone density was determined in both the proximal tibial end (near the MRMT-1 carcinoma cell or HBSS-injected site where bone deterioration was observed to be the most severe) and the neighbouring distal end of the femur by measuring the grey intensity on the X-ray radiographs in a boxed area of 2.5 mm × 2.5 mm in both regions. Statistical analysis of behavioural data was performed using two-way repeated measures ANOVA followed by the Tukey post hoc tests at individual day of assessment against the cancer + vehicle group. Bone density was compared across treatment groups for either the tibia or femur area using one-way ANOVA followed by the Tukey post hoc tests against the control + vehicle group.

AF-353 treatment in vivo

AF-353 (previously known as RO-4) is a recently described selective dual P2X3 and P2X2/3 antagonist with ideal pharmacokinetic characteristics and is orally bioavailable (Carter et al., 2009; Jahangir et al., 2009; Gever et al., 2010). AF-353 is highly selective with potent activity on P2X3 and P2X2/3 receptors (pIC50 at P2X3: 8.1, P2X2/3: 7.4, P2X1: 2, 4, 5, 7, 3<5) in contrast to other commercially available purinergic receptor antagonists such as pyridoxal-phosphate-6-azophenyl-2'-4'-disulphonic acid (pIC50 at P2X3: 6, P2X2/3: 6) and 2',3'-O-(2,4,6-trinitrophenyl) adenosine 5'-triphosphate monolithium trisodium salt (pIC50 at P2X3: 9, P2X2/3: 8.4, P2X1: 8.22) (Gever et al., 2006, 2010). Carcinoma cell-injected and control rats were subjected to one of the two separate schemes (prophylactic and reversal paradigms) for systemic AF-353 treatment (100 mg/kg, orally, twice daily) (n=5–13 rats per treatment group). The dose was chosen based on unpublished data that determined the dosing regimen would produce sufficient plasma exposure throughout the course of treatment. Pharmacokinetic analysis from animals dosed in this study later confirmed that at trough levels (12 h post-dose), excellent exposure of AF-353 was evidently observed at 2000 ± 360 ng/ml (~4.5 μM; n=4). The oral administration of drug or vehicle was performed by gavage without any anaesthetics. Drug or vehicle administration was carried out by an investigator blinded to carcinoma cell or control injection and not involved in behavioural assessment. Prophylactic treatment was given to carcinoma cell-injected and control rats during the period of Days 11–20 inclusively. Another set of carcinoma cell-injected rats that had demonstrated distinct mechanical allodynia on Day 16 (defined by an area under the curve > 400) received treatment with AF-353 during the period of Days 17–20 inclusively, to assess potential reversal of established pain behaviour. AF-353 was freshly prepared in vehicle containing 10% propylene glycol, 10% 0.01 M HCl and 80% distilled water, prior to each dosing. Vehicle was administered to both carcinoma cell-injected and control rats according to the prophylactic scheme only to minimize the number of animals used.

Fast Blue retrograde neuronal tracing and immunohistochemistry

To examine the sensory innervation of the tibia, Fast Blue (4% prepared in distilled water, 10 μl (EMS-Chemie) was injected into naïve rats using the same surgical method for injecting MRMT-1 carcinoma cells as mentioned above. Seven days following tracer injection, rats were deeply anaesthetized by an overdose of pentobarbital and transcardially perfused with heparinized saline followed by paraformaldehyde (4% in 0.1 M phosphate buffer) (VWR). DRG from L1 to L6 levels were dissected out of the animals and post-fixed in paraformaldehyde. After the DRG were cryoprotected in 20% w/v sucrose and embedded in optimal cutting temperature compound (BDH Laboratory Supplies), the DRG were sectioned at 15 μm with a cryostat and mounted on SuperFrost slides. Slides were then incubated with rabbit anti-P2X3 (1:2000, Neuromics) and sheep anti-calcitonin gene-related peptide (1:1000, Biomol) primary antibodies overnight. Following three washes with 10 mM phosphate-buffered saline the next day, donkey anti-rabbit conjugated to Alexa Fluor 488 (Molecular Probes) and donkey anti-sheep conjugated to Alexa Fluor 546 (Molecular Probes) were applied as secondary antibodies for 2 h. The slides were then washed with phosphate-buffered saline three times before being coverslipped with FluoroSave reagent (VWR). Images were captured using a fluorescence Carl Zeiss microscope (Imager Z1) and AxioVision 4.6 program (Carl Zeiss) at ×10 objective magnification. Fast Blue-, P2X3 and calcitonin gene-related peptide-positive DRG neurons were identified with a soma fluorescent intensity of >125% compared with a non-immunostained region of the same slide using AxioVision v4.6 program. Measurements were determined from a minimum of three DRG slide sections per animal (n=4 per group).

In vivo dorsal horn electrophysiology

In vivo electrophysiology recordings were performed as previously described (Urch and Dickenson, 2003). Briefly, animals were anaesthetized in a nitrous oxide/oxygen mixture with isoflurane before a tracheotomy was performed. Extracellular recordings of single ipsilateral deep wide dynamic range dorsal horn neurons (500–1000 μm depth) were made using Parylene-coated tungsten electrodes (A-M systems). Positive wide dynamic-range neurons were identified by their ability to respond to a range of von Frey filaments, noxious pressure and thermal stimuli. A train of 16 transcutaneous electrical stimuli (2 ms pulse width, 0.5 Hz) was applied to the receptive field at three times the threshold current for C-fibre. A post-stimulus histogram was constructed and Aδ-fibre (0–20 ms), Aβ-fibre (20–90 ms), C-fibre (90–300 ms) and post-discharge (300–800 ms) responses were separated and quantified according to latency. Data were captured and analysed using Spike 2 software and CED 1401 interface (Cambridge Electronic Design). Input was calculated as the number of action potentials in the C-fibre range evoked by the first pulse of the
train multiplied by the total number of pulses (16) and represents the activity prior to any subsequent potentiation evoked by the stimulus. Wind-up was calculated by the total number of action potentials in the C-fibre range after the train of 16 stimuli minus the input and provides a measure of cell hyperexcitability. Electrophysiological studies were conducted on Days 15–18 post-injection of MRMT-1 carcinoma cells. Following electrical stimuli, natural stimuli comprised mechanical punctate (application of various forces of von Frey filaments: 2, 6, 8, 15, 26 and 60 g for 10 s each), and thermal stimuli (application of a constant water jet at 35, 40, 45 and 48°C for 10 s each) were applied to the neuronal receptive field on the ipsilateral hindpaw. Sufficient intervals were allowed within and between stimuli to avoid sensitization of receptors. After three consecutive stable control trials (<20% variation for all parameters), neuronal responses were averaged to give pre-drug control values with which subsequent responses were compared. Spinal applications of AF-353 (10, 30, 100 μg in a volume of 50 μl, dissolved in 5% dimethyl sulfoxide/95% saline) were made directly onto the exposed surface of the spinal cord. The concentrations were chosen based on previous efficacy of a similar potent and selective compound, AF-792, in blocking bladder reflexes when administered intrathecally (Kaan et al., 2010). The effect of each dose was followed for an hour, with tests carried out at 10, 30 and 50 min after subsequent doses were made and their effects were again recorded for an hour each. One neuron only per animal was characterized and in total n = 4–11 were completed for each drug dose and stimulus. Responses following electrical stimuli were compared using one-way repeated measures ANOVA followed by the Bonferroni post hoc tests against the control group was used for analysing responses following natural stimuli.

**Extracellular ATP release from MRMT-1 carcinoma cells**

The determination of extracellular ATP release from MRMT-1 carcinoma cells was carried out using the CellTiter-Glo Luminescent Cell Viability Assay (Promega) similar to previously described protocols (Yip et al., 2009; Clark et al., 2010). Briefly, MRMT-1 carcinoma cells were released from flasks with trypsin, followed by centrifugation and resuspension in fresh media with or without AF-353 (25 μM). In total, 25,000 cells were plated onto 24-well plates, and media were quickly removed for determining extracellular ATP level. The baseline release level of ATP from MRMT-1 carcinoma cells was determined over a period of 2 h. The cells were also subjected to gentle mechanical trituration to evoke ATP release. ATP standards (100 μl of 5 × 10−10 M to 1 × 10−6 M) and 100 μl of unknown media samples (n ≥ 3) were run in duplicates using a microplate luminometer (Veritas microplate luminometer, Turner Biosystems). Two-way ANOVA followed by the Tukey post hoc tests were used to compare the levels of extracellular ATP release from MRMT-1 carcinoma cells over time and Student’s t-test was used to compare the levels of mechanically evoked ATP release.

**Co-culture of primary adult rat dorsal root ganglion neurons with MRMT-1 carcinoma cells**

Methods for culturing adult rat DRG neurons were carried out similarly to those previously described (Wong et al., 2006). Briefly, rats were sacrificed under deep anaesthesia with pentobarbital. All DRG at lumbar level were removed and transferred into F12 media (Gibco). After incubation with 0.125% collagenase, the DRG were mechanically dissociated to form single-cell suspension, which was then transferred onto a 2-ml cushion solution of 15% sterile bovine serum albumin and centrifuged at 100g for 7 min. The resulting pellet was resuspended in warm Bottenstein and Sato’s culture media, which contained F12 media 1% supplemented with 1% N2 supplement, 1% bovine serum albumin and 1% penicillin/streptomycin. Three thousand cells in Bottenstein and Sato’s culture media were plated onto glass cover slips pre-coated with 10 μg/ml poly-L-lysine in sterile 24-well plates and then allowed to incubate overnight at 37°C with 95% O2/5% CO2 prior to co-culture. MRMT-1 carcinoma cells were prepared as mentioned above and 40,000 cells/ml were plated onto culture dishes (35 mm diameter; NUNC) in Bottenstein and Sato’s culture media and then allowed to incubate at 37°C with 95% O2/5% CO2 overnight.

The following day, cover slips containing the DRG neurons were transferred into culture dishes containing either MRMT-1 carcinoma cells or Bottenstein and Sato’s culture media alone (as control). The co-cultures were incubated for 1 h to 4 days in vitro. For co-culture of up to 4 days in vitro, fresh Bottenstein and Sato’s culture media was replaced on Day 2. Experiments that included AF-353 (2.5 μM or 25 μM, prepared in Bottenstein and Sato’s culture media) under the same culture conditions were also performed for the 1 day, 2 days and 4 days in vitro co-culture time points. In summary, five experimental conditions were carried out: (i) Bottenstein and Sato’s culture media only (control + vehicle); (ii) AF-353 (25 μM) in Bottenstein and Sato’s culture media only (control + AF-353); (iii) Bottenstein and Sato’s culture media with MRMT-1 carcinoma cells (MRMT-1 + vehicle); (iv) AF-353 (2.5 μM) in Bottenstein and Sato’s culture media with MRMT-1 carcinoma cells (MRMT-1 + AF-353 (2.5 μM)); and (v) AF-353 (25 μM) in Bottenstein and Sato’s culture media with MRMT-1 carcinoma cells [MRMT-1 + AF-353 (25 μM)].

**Immunocytochemistry**

Immunocytochemistry procedures were similar to those previously described (Wong et al., 2006; Yip et al., 2009). At various time points in vitro, media was removed and DRG neurons were fixed in 4% paraformaldehyde for at least 20 min, followed by cold 100% methanol for 3–5 min. Cells were then washed with phosphate-buffered saline three times and incubated at 4°C overnight with primary antibodies. The primary antibodies used were polyclonal rabbit anti-Phospho-p44/42 mitogen-activated protein kinase (Thr202/Tyr204) (phosphorylated ERK) antibody (1:100, New England Biolabs), mouse anti-β-III-tubulin (1:4000, Promega) and guinea pig anti-P2X3 (1:100, Neurimics). The next day, after three washes with phosphate-buffered saline, the DRG neurons were incubated at room temperature for 1 h with secondary antibodies. The secondary antibodies used were donkey anti-rabbit Alexa Fluor 488 (1:1000, Molecular Probes), donkey anti-mouse Alexa Fluor 546 (for β-III-tubulin, 1:1000, Molecular Probes) and goat anti-guinea pig Alexa Fluor 546 (for P2X3, 1:1000, Molecular Probes). After another three washes with phosphate-buffered saline, cover slips containing the DRG neurons were mounted onto glass slides with 0.5 μl 4',6-diamidino-2-phenylindole (DAPI, 10 μg/ml) in FluorSave reagent. Cultured DRG neurons were visualized with a Carl Zeiss fluorescence microscope (Imager Z1) and images were taken with AxioVision 4.6 program (Carl Zeiss) at ×10 objective magnification.
The level of phosphorylated ERK immunoexpression was assessed by procedures similar to those previously described (Yip et al., 2009). The measurement of phosphorylated ERK immunofluorescent staining intensity of individual DRG neurons was carried out using the Axiosvision v4.6 program (Carl Zeiss). The data were expressed in two ways: (i) percentage of phosphorylated ERK-positive DRG neurons that had >150% of the average control phosphorylated ERK immunofluorescent staining intensity and (ii) the phosphorylated ERK intensity normalized to control DRG neurons of adjacent wells with Bottenstein and Sato’s culture media only. Each set of experiments of various culture durations was completed using primary DRG neurons cultured from one rat. The complete data set involved four to eight independent rats. In addition, immunocytochemistry staining for each set of experiments from the same animal was done simultaneously to minimize inconsistencies. A minimum of 60 DRG neurons from at least two wells were analysed from each animal under the individual co-culture conditions of each treatment and duration. Time-course levels of phosphorylated ERK immunofluorescence were compared using two-way ANOVA followed by Tukey’s post hoc tests between control and MRMT-1 carcinoma cell co-culture conditions. Data for experiments with AF-353 treatment were compared using one-way ANOVA followed by Tukey’s post hoc tests against control + vehicle or MRMT-1 + vehicle group within each individual time point.

Results

Systemic P2X3 and P2X2/3 receptor antagonism attenuates bone cancer pain behaviour without modifying bone destruction

Rats injected with MRMT-1 carcinoma cells into the tibia showed no signs of pain behaviour up to Day 14 post-injection, at which point significant development of mechanical allodynia and weight-bearing differences started to be seen, consistent with previous findings (Medhurst et al., 2002). Rats were given oral treatment with AF-353 (100 mg/kg, orally, twice daily) (n = 5–13) according to either prophylactic or reversal paradigms. Prophylactic treatment was started on Day 11 post-injection, since no significant differences in pain behaviour between carcinoma cell-injected or control rats were detected on Day 10. Carcinoma cell-injected rats that received vehicle treatment developed a progressive increase in mechanical allodynia and weight-bearing differences from Day 14, which became significant in comparison with control animals receiving either AF-353 or vehicle starting on Day 16 (Fig. 1). Prophylactic treatment with AF-353 in carcinoma cell-injected rats significantly attenuated, to levels similar to that observed in control rats, the development of mechanical allodynia from Day 14 to Day 19 (Fig. 1A) and weight-bearing differences on Days 16 and 19 (Fig. 1B). More importantly, to assess the efficacy of AF-353 in a scenario better resembling the clinical setting, AF-353 was administered blindly on Day 17 in one of the two groups of carcinoma cell-injected rats that demonstrated similar levels of bone cancer-induced mechanical allodynia on Day 16. Following the first administration of AF-353, mechanical allodynia was significantly reduced compared with carcinoma cell-injected rats that received vehicle alone and thus the bone cancer pain behaviour developed on Day 16 was reversed (Fig. 1C). No significance was observed in weight-bearing differences between the carcinoma cell-injected rats that received vehicle or reversal treatment of AF-353 (Fig. 1D). All behavioural assessments were undertaken by an observer blinded to the treatment given.

X-ray radiographic images of tibia taken at the end point of the study showed bone destruction caused by MRMT-1 carcinoma cells compared with control rats (Fig. 2A–D). Quantitative analysis of the bone density revealed significant bone destruction in carcinoma cell-injected rats that was specific to the tibia at the site of MRMT-1 carcinoma cell injection and not present in the femur (Fig. 2E). Prophylactic or reversal treatments of AF-353 did not affect the degree of tibia destruction in carcinoma cell-injected rats, indicating that the apparent analgesic effect of AF-353 in bone cancer pain was not due to underlying disease modification.

Sensory afferents innervating the rat tibia contain P2X3-positive neurons

P2X3 receptor subunits are known to be expressed selectively on primary afferent nociceptors, but whether they are expressed on bone-innervating afferents remains to be determined. Identification of sensory neurons innervating the tibia was via intratibial injection of the retrograde tracer, Fast Blue, in naïve rats. The cell bodies of these afferents were observed mainly in the L3 DRG (Fig. 3C) and to a much lesser extent in neighbouring lumbar DRG (L1, L2 and L4–L6) (Fig. 3A, B and D–F). We next used immunohistochemistry to gauge whether the retrograde-labelled neurons in L2 and L3 DRG expressed P2X3 receptors and the peptide CGRP (Fig. 4A–H). Indeed, Fast Blue-positive neurons were found to be P2X3-positive alone (Fig. 4I and L), calcitonin gene-related peptide-positive alone (Fig. 4J and L) and even both P2X3- and calcitonin gene-related peptide-positive (Fig. 4K and L).

P2X3 and P2X2/3 receptor antagonism reduces bone cancer-induced dorsal horn neuronal hyperexcitability in vivo

Many forms of persistent pain, including that related to bone metastases, appear to be associated with sensitization of peripheral nociceptors and enhancement of central processing of sensory information. A major site for central action has been identified to be within the dorsal horn of the spinal cord (Gordon-Williams and Dickenson, 2007). We therefore asked if the blockade of P2X3 and P2X2/3 receptors on the central terminals of nociceptors with AF-353 contributed to its analgesic efficacy. We have previously demonstrated neuronal hyperexcitability in the deep dorsal horn neurons of rats with experimental bone cancer (Urch et al., 2003, 2005). To investigate whether bone cancer-induced dorsal horn neuronal hyperexcitability can be modulated by P2X3 and P2X2/3 receptor antagonism, AF-353 was administered directly onto the spinal cord of carcinoma cell-injected rats.
Spinal administration of AF-353 dose-dependently reduced the responses of electrically evoked Aδ- and C-fibres and post-discharge with significant reductions observed at the highest dose of AF-353 tested (100 μg/50 μl/rat) (Fig. 5A). These effects were selective for noxious stimuli, since no significant effect was observed on Aβ-fibre responses. In addition, input—the strength of the response to the first stimulus used as a measure of pre-synaptic actions—was significantly affected, with increased responses at the lowest dose of AF-353 tested (10 μg/50 μl) and a decrease at the highest dose (100 μg/50 μl) (Fig. 5B). AF-353 did not have any effects on the post-synaptic event of wind-up, further suggestive of actions on spinal substrates presynaptic to the neuron under investigation. Dose-dependent reductions by AF-353 of naturally evoked neuronal responses were also evident following both mechanical punctate and thermal stimuli (Fig. 5C and D). The inhibitory effects of AF-353 became significant at 30 and 100 μg/50 μl compared with control values, when von Frey filaments with forces of 15g and above were applied as the mechanical stimuli (Fig. 5C). The highest dose of AF-353 tested (100 μg/50 μl) was significantly effective in reducing neuronal responses following application of the entire range of thermal stimuli, while 30μg/50μl was also significantly effective when stimuli of higher temperatures of 45°C and 48°C were applied (Fig. 5D).

ATP release from carcinoma cells is not affected by P2X3 and P2X2/3 receptor antagonism

Cancer cells have been previously reported to contain high levels of ATP, which is the endogenous ligand for P2X3 and P2X2/3 receptors, and may release ATP in response to stress, lysis or stimulation (Maehara et al., 1987; Pedersen et al., 1999). Here, we found ATP to be released from MRMT-1 carcinoma cells under basal in vitro culture conditions (n ≥ 3) (Fig. 6A). In the presence of AF-353, the level of extracellular ATP from MRMT-1 carcinoma cells was also not significantly different from vehicle treatment, suggesting ATP release is not affected by the blockade of P2X3 and P2X2/3 receptors. Mechanical disruption caused over a 4-fold increase in extracellular ATP release that was also not affected by the presence of AF-353 (n ≥ 3) (Fig. 6B).
Carcinoma cells-induced extracellular signal-regulated kinase activation in dorsal root ganglion neurons is reduced in the presence of P2X3 and P2X2/3 antagonist

Phosphorylation of ERKs has been previously reported to be a marker for DRG nociceptor activation both in vitro and in vivo (Obata and Noguchi, 2004; Cruz and Cruz, 2007; Ji et al., 2009). To determine whether MRMT-1 carcinoma cells can induce activation by phosphorylation of ERK in primary sensory neurons, a co-culture model was used. Immunostaining confirmed the presence of P2X3-positive DRG neurons when cultured alone or co-cultured with MRMT-1 carcinoma cells for various times in vitro (Fig. 7A–L). Activation of the ERK-signalling pathway was assessed by measuring the intensity of phosphorylated ERK in dissociated primary DRG neurons and analysed using two parameters: (i) proportions of DRG neurons that displayed ≥150% of the average control intensity and (ii) phosphorylated ERK staining intensity normalized to the average control value (n = 60 DRG neurons from 4 to 8 rats). Across all time points investigated, fewer than 10% of control DRG neurons cultured alone exhibited ≥150% of the average control phosphorylated ERK intensity (Fig. 7A–C, G–I and M). In comparison, there were significant increases in the percentage of DRG neurons co-cultured with MRMT-1 carcinoma cells when given either before (prophylactic) or after (reversal) development of bone cancer pain behaviour. The control bone density was obtained from the corresponding femur of each animal. Values are expressed as means ± SEM. **P < 0.01 and ***P < 0.001 versus the tibia density value of the control + vehicle group.

Figure 2  Cancer-induced bone destruction is not affected by P2X3 and P2X2/3 antagonist. (A–D) Radiographic images of the hindlimb showing MRMT-1 carcinoma cell-induced local bone destruction in the tibia. Insets are higher magnification of the dashed boxes. Scale bar = 5 mm. (A) Intra-tibial injection of HBSS media (control) in rats did not show any visible bone destruction. (B–D) Regardless of vehicle or AF-353 treatment, rats with intratibial injection of MRMT-1 carcinoma cells showed significant reduction in bone density compared with the control group. (E) Quantification of bone density shows systemic administration of the selective P2X3 and P2X2/3 receptor antagonist, AF-353, did not significantly affect the bone destruction specific to the tibia induced by MRMT-1 carcinoma cell injection when given either before (prophylactic) or after (reversal) development of bone cancer pain behaviour. The control bone density was obtained from the corresponding femur of each animal. Values are expressed as means ± SEM. **P < 0.01 and ***P < 0.001 versus the tibia density value of the control + vehicle group.
co-culture: 192.7±10.7%) and lasted for as long as 4 days in vitro (Fig. 7D–F, J–L and N).

To investigate whether the enhanced ERK signalling in DRG neurons induced by MRMT-1 carcinoma cells was mediated by P2X3 and P2X2/3 receptors, AF-353 was added into the co-cultures. In the presence of AF-353 (25 μM), the immunoexpression level of phosphorylated ERK in control DRG neurons when cultured alone was not significantly different compared with vehicle-treated control DRG neurons (Fig. 8A–B and I–J). However, AF-353 caused a concentration-dependent reduction of enhanced phosphorylated ERK immunoexpression in DRG neurons co-cultured with MRMT-1 carcinoma cells (Fig. 8C–J). At the highest concentration of AF-353 (25 μM) tested, increases in both the normalized phosphorylated ERK intensity and the proportion of cells displaying ≥150% of the average control phosphorylated ERK intensity were attenuated to levels comparable to control DRG neurons at all three in vitro time points of 1 day, 2 days and 4 days tested.

**Discussion**

Recent bone cancer pain models have yielded valuable insights into novel mechanisms unique to bone cancer pain conditions, and thus hold great promise for developing mechanism-based effective analgesics (Mantyh et al., 2002). Importantly, the bone cancer pain model used in this study and other related models, such as those established in mice, resemble clinical symptoms with a positive correlation between the progression of the disease and increase in pain intensity, in addition to bone destruction in the absence of invasion of adjacent soft tissues (Delaney et al., 2008).

In this study, we report the efficacy of a selective and orally bioavailable P2X3 and P2X2/3 receptor antagonist, AF-353, to lessen bone cancer pain behaviour. Furthermore, the neuronal hyperactivity in the spinal cord in vivo and the upregulated expression of phosphorylated ERK in DRG neurons in vitro induced by MRMT-1 carcinoma cells were both attenuated by the P2X3 and P2X2/3 receptor antagonist.

The P2X3 subunit, which can form homomeric P2X3 and heteromeric P2X2/3 receptors, is the most highly expressed P2X receptor subtype found on sensory afferents and shows relatively selective expression in the non-peptidergic subpopulation that binds isolectin B4 (Dunn et al., 2001). These receptors are located on the peripheral terminals of target organs (e.g. skin and bladder), cell bodies within the DRG and central presynaptic terminals in the lamina II of the spinal cord (Vulchanova et al., 1997, 1998; Bradbury et al., 1998; Cockayne et al., 2000). Study of the potential roles for the P2X3 and P2X2/3 receptors in pain behaviour has been greatly advanced with the use of knockout mice and other molecular techniques, including small interfering RNA and
oligonucleotides (Cockayne et al., 2000, 2005; Souslova et al., 2000; Barclay et al., 2002; Honore et al., 2002; Dorn et al., 2004). However, these approaches are not without problems, and so a major step forward was the description of an antagonist to enable further detailed pharmacological studies. Jarvis et al. (2002) demonstrated A-317491 as the first potent and selective non-nucleotide P2X3 and P2X2/3 receptor antagonist to be effective in reducing chronic inflammatory and neuropathic pain behaviour in animals. Subsequently, the same group reported that intrathecal, but not peripheral, administration of A-317491 reduced pain behaviour following peripheral nerve injury, suggesting that the targeted functional P2X3 receptors were located centrally (McGaraughty et al., 2003). As a result of very poor oral bioavailability and CNS penetration, in addition to very high protein binding (>99.9%), the use of A-317491 as an in vivo tool and its potential for further clinical development are limited (Wu et al., 2004; Sharp et al., 2006; Gever et al., 2010).

More recently, AF-353 (previously known as RO-4) was reported to be a selective P2X3 and P2X2/3 receptor antagonist with high oral bioavailability and CNS penetration, in addition to superior potency compared with A-317491 (Carter et al., 2009; Jahangir et al., 2009; Gever et al., 2010). These properties of AF-353 make it an excellent tool for the therapeutic evaluation of P2X3 and P2X2/3 receptors as a potential target in pain models, including disease states. Here, we found that oral administration of AF-353 significantly attenuated bone cancer pain behaviour, with significant effects seen in both mechanical allodynia and weight-bearing differences when dosed prophylactically. Additionally, AF-353 significantly prevented the development of cancer pain behaviour despite the ongoing and rapid progression of the disease. More importantly, cancer-induced mechanical allodynia could be reversed by AF-353 following the first oral dosing. As the disease progresses, the increasing severity of cancer pain can be due to underlying shifts in the expression and function of various nociceptive targets (Mantyh et al., 2002). The significant attenuation produced by AF-353 when dosed prophylactically was not seen as a significant change in weight-bearing differences induced by bone cancer when AF-353 was dosed in the reversal study. This could relate to the later onset of bone cancer-induced weight-bearing differences being more resistant than mechanical allodynia and analogous to movement-induced breakthrough pain (Mercadante, 1997). Breakthrough pain is a particular type of cancer pain condition with unknown underlying pathophysiology and abrupt onset and is poorly responsive to treatment (Davies et al., 2009). Involvement of multiple factors, including prostaglandins, endothelin, bradykinin, nerve growth factor, an

Figure 4 Immunohistochemical characterization of the retrogradely labelled Fast Blue DRG neurons innervating the rat tibia. (A–H) Fast Blue (blue) labelling was present in both P2X3- (green) and calcitonin gene-related peptide (CGRP)-positive (red) neurons at the L2 and L3 levels. Higher magnification of Fast Blue-labelled neurons present in L3 DRG neurons that were (I) P2X3-positive alone, (J) calcitonin gene-related peptide-positive alone and (K) both P2X3- and calcitonin gene-related peptide-positive were observed. (L) Quantification of the immunohistochemical characterization of Fast Blue-positive neurons in L3 DRG. Scale bar = 100 μm.
acidic environment and increased osteoclast activity in bone cancer pain, have been previously demonstrated in animal models (Honore et al., 2000; Wacnik et al., 2001; Sabino et al., 2002; Fox et al., 2004; Peters et al., 2004; Ghilardi et al., 2005; Halvorson et al., 2005; Sevcik et al., 2004; Peters et al., 2006). Targeting P2X3 and P2X2/3 receptors together with these other nocioceptive targets may offer additional benefits against the complexity of an established and progressing severity of bone cancer pain.

One possible approach for treating bone cancer pain is to target mechanisms that may slow or halt disease progression. Recent pharmacological agents such as bisphosphonates and osteoprotegerin have been demonstrated to reduce bone deterioration, resulting in reduced bone cancer pain behaviour (Honore et al., 2000; Walker et al., 2002; Sevcik et al., 2004; Roudier et al., 2006). In this study under either the prophylactic or reversal-dosing regimen, AF-353 did not significantly alter cancer-induced bone destruction of the tibia. Hence, the analgesic efficacy of blocking P2X3 and P2X2/3 receptors was not via modifying the disease progression. Various neurochemicals released from the tumour have been postulated to activate nocioceptors present on afferent fibres to convey cancer pain signalling.

Blockade of these nocioceptors has been suggested as a route for treating bone cancer pain without necessarily modifying the disease (Mantyh et al., 2002). P2X3 and P2X2/3 receptors and the endogenous ligand, ATP, may possibly fit within this role. Abundant levels of ATP within tumours can be released to activate P2X3 and P2X2/3 receptors on afferent nerve endings (Burnstock, 1996). ATP can also be released centrally from multiple sites including neurons and glia, with possible elevated ATP release from enhanced spinal astrogliosis that has been observed in this bone cancer pain model (Medhurst et al., 2002; Burnstock, 2006).

Our in vitro study showed that MRMT-1 carcinoma cells could release ATP in a manner that was unaffected by the blockade of P2X3 and P2X2/3 receptors. Using the retrograde neuronal tracer Fast Blue, selective lumbar DRG neurons innervating the intramedullary space of the tibia were identified. Co-localization studies demonstrated that these DRG neurons express both P2X3 subunit and calcitonin gene-related peptide, indicative of involvement of non-peptidergic and peptidergic C-fibre nocioceptors, respectively. Although a recent study reported that the mouse femur was primarily innervated by the calcitonin gene-related peptide peptidergic population with negligible expression of P2X3 receptors, this discrepancy may be attributed to differences in the species and...
bone type examined (Jimenez-Andrade et al., 2010). In addition, de novo expression of P2X3 receptors on peptidergic C-fibres may be induced by nerve growth factor (Ramer et al., 2001), which has been found to be released from cancer cells and facilitate cancer pain (Sevcik et al., 2005b). We propose a mechanism whereby AF-353 reduces bone cancer pain behaviour by blocking the activation of P2X3 and P2X2/3 receptors by the elevated release of ATP from MRMT-1 carcinoma cells within the tibia.

Unique changes within the spinal cord in bone cancer pain models, not commonly found in animals with chronic neuropathic and inflammatory pain conditions, have indicated the involvement of forms of central sensitization particular to cancer pain conditions (Gordon-Williams and Dickenson, 2007). In electrophysiological studies, central sensitization in bone cancer-induced pain behaviour has been associated with hyperexcitability of wide-dynamic-range dorsal horn neurons (Urch et al., 2003; Khasabov et al., 2007). Significantly increased dorsal horn neuronal responses were found in animals with bone cancer pain following electrical and natural stimuli over both the innocuous and noxious ranges, whereas reduced neuronal activity is observed in nerve-injured animals (Chapman et al., 1998; Urch et al., 2003). Evoked dorsal horn neuronal hyperexcitability was reduced when AF-353 was applied directly onto the spinal cord of carcinoma cell-injected animals. The dose-dependent reduction in electrically stimulated dorsal horn neuronal hyperexcitability was evident in the responses of Aδ-fibres, C-fibres and post-discharge, but not of Aβ-fibres. Spinal P2X3 and P2X2/3 receptors, which are located at the presynaptic sites of sensory afferents terminating in lamina III of the spinal cord (Bradbury et al., 1998), have been implicated in nociceptive transmission under neuropathic and inflammatory pain conditions, including those associated with visceral organs (McGaraughty et al., 2003; Kaan et al., 2010). Specific interference with cancer nociceptive signalling can apparently occur following blockade of spinal P2X3 and P2X2/3 receptors by AF-353. Furthermore, the reduction in the input to the dorsal horn but not wind-up by AF-353 suggests that the inhibitory mechanism is probably due to primary afferent signalling rather than a direct effect of post-synaptic dorsal horn neurons. Interestingly, the input was enhanced following administration of the lowest dose of AF-353. One possible explanation may be deprivation of interneuronal activity, especially since the wide dynamic range recording was made in the deep dorsal horn and the P2X3-expressing non-peptidergic afferents are known to make connections with interneurons from lamina II into the deeper lamina for further projection (Braz et al., 2005). Altogether, the input responses were dose-dependently reduced and are consistent with the selective presynaptic expression of P2X3 and P2X2/3 receptors within the spinal cord. Mechanical and thermal stimuli-evoked dorsal horn excitability was also dose-dependently reduced when AF-353 was applied directly onto the spinal cord. In particular, the attenuation of responses to mechanical stimuli was more clearly evident as the stimuli moved into the noxious range and was correlated with the attenuation of mechanical allodynia seen following systemic administration of AF-353. Our data are in agreement with previous studies that reported a reduction in µmeATP-induced dorsal horn long-term potentiation and electrically evoked dorsal neuronal hyperexcitability following inhibition of P2X3 and P2X2/3 receptors in the spinal cord (Sharp et al., 2006; Ikeda et al., 2007).

In addition to central sensitization, peripheral sensitization of sensory neurons has been reported to underlie cancer pain behaviour (Cain et al., 2001; Khasabova et al., 2007). To investigate the direct interaction between cancer cells and sensory neurons, a co-culture system of MRMT-1 carcinoma cells and dissociated primary rat DRG neurons was established. Recent co-culture studies have shown that murine NCTC 2472 fibrosarcoma cells could induce phenotypic and functional changes in nociceptors of DRG or nodose ganglia (Khasabova et al., 2007, 2008; Chizhmakov et al., 2009). The co-culture system used undoubtedly represents an over-simplification of the conditions occurring in vivo where, apart from ATP, other factors such as cytokines are likely to be released and interact with a variety of cell types in addition to carcinoma cells and DRG neurons. However, the co-culture does allow at least some possible mechanisms to be explored in a more controlled fashion and, we believe, may give some insights into the mechanisms underlying cancer pain.

Figure 6 ATP release from MRMT-1 carcinoma cell in vitro. (A) Extracellular ATP was detected in the culture media of MRMT-1 carcinoma cells in vitro under basal conditions. This basal release was not significantly altered in the presence of AF-353 (25 µM). (B) Mechanical stimulation induced a significant increase in the release of ATP from MRMT-1 carcinoma cells either in the presence or absence of AF-353. Values are expressed as means ± SEM. ***P<0.001 versus baseline release.
Figure 7 Temporal phosphorylated ERK (pERK) immunoreexpression in adult rat DRG neurons co-cultured with MRMT-1 carcinoma cells in vitro. (A–L) Immunoexpression of phosphorylated ERK (green) was significantly increased in β-III-tubulin-positive (A–F, red) and P2X3-positive (G–L, red) DRG neurons when co-cultured with MRMT-1 carcinoma cells at various time in vitro compared with control. Scale bar = 100 μm. (M and N) The percentage of phosphorylated ERK immunopositive DRG neurons and the normalized levels of phosphorylated ERK intensity were significantly increased from 8 h to 4 days in vitro in MRMT-1 carcinoma cell co-culture compared with control conditions. Values are expressed as means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 versus control.
behaviour. In this study, increased expression of phosphorylated ERK was observed in DRG neurons co-cultured with MRMT-1 carcinoma cells within hours and persisted for as long as 4 days in vitro. This is in agreement with other studies demonstrating activation of the ERK-signalling pathway via upregulation of phosphorylated ERK immunoexpression in multiple chronic inflammatory, neuropathic and visceral pain models (Obata and Noguchi, 2004; Cruz and Cruz, 2007; Ji et al., 2009). P2X3 and P2X2/3 receptor-mediated ERK activation in dorsal horn and DRG neurons have been reported in visceral and inflammatory models, respectively (Dai et al., 2004; Seino et al., 2006; Kaan et al., 2010). In the presence of the P2X3 and P2X2/3 receptor antagonist, AF-353, there was a significant reduction in phosphorylated ERK expression in DRG neurons co-cultured with MRMT-1 carcinoma cells. Our data suggest that the ERK-signalling pathway is also involved in cancer pain and that blockade of P2X3 and P2X2/3 receptors can reduce activation of phosphorylated ERK and thus prevent the activation of the intracellular nociception pathway. In addition to ATP release from cancer cells, factors such as nerve growth factor may be released to cause sensitization of P2X3 and P2X2/3 receptors and contribute to enhanced ERK signalling (Sevcik et al., 2005b; D’Arco et al., 2007).

**Conclusion**

In summary, we have shown the analgesic efficacy of an orally bioavailable P2X3 and P2X2/3 receptor antagonist, AF-353, in a clinically relevant chronic pain model. Our results add to the growing repertoire of evidence supporting an important role for P2X3 and P2X2/3 receptors in chronic pain states despite varied underlying pathological mechanisms. The non-disease modifying analgesic effect of P2X3 and P2X2/3 receptor blockade in bone cancer pain includes a reduction in cancer-induced dorsal horn

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**Figure 8** Selective P2X3 and P2X2/3 receptor antagonism with AF-353 attenuates elevated phosphorylated ERK (pERK) immunoexpression (green) in β-III-tubulin-positive DRG neurons (red) induced by MRMT-1 carcinoma cells in vitro. (A–D) The phosphorylated ERK immunoexpression level was significantly elevated in DRG neurons co-cultured with MRMT-1 carcinoma cells compared with control. The enhanced phosphorylated ERK immunoexpression induced by MRMT-1 carcinoma cells in DRG neurons was reduced in the presence of AF-353 (E–H). Scale bar = 100 µm. (I and J) The percentage of phosphorylated ERK immunopositive DRG neurons and the levels of phosphorylated ERK intensity elevated by MRMT-1 carcinoma cells were significantly reduced with AF-353 from 1 day to 4 days in vitro compared with control. Values are expressed as means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 versus the control + vehicle group; †P < 0.05, ††P < 0.01, †††P < 0.001 versus the MRMT-1 carcinoma cells + vehicle group.
hyperexcitability and enhanced phosphorylated ERK expression in DRG neurons. ATP released from cancer cells can drive peripheral sensitization of P2X3-expressing bone afferents, which can in turn result in central sensitization at the level of the spinal cord to cause persistent bone cancer pain. Blockade of P2X3 and P2X2/3 receptors at both peripheral and central sites of action can reduce the central sensitization responses, highlighting the need for a systematically available antagonist. Given that many signalling factors other than ATP are probably present in the bone cancer milieu and several mechanisms may contribute to pain behaviour, further studies are merited to investigate the effectiveness of this strategy, possibly in combination with treatments aimed at other targets. In conclusion, our results suggest that a systemically active and CNS-penetrating P2X3 and P2X2/3 antagonist may have therapeutic potential in attenuating bone cancer pain.

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Conflict of interest: Anthony PDW Ford is an employee of Afferent Pharmaceuticals, which owns an exclusive license for P2X3 antagonists developed at Roche Pharmaceuticals, including AF-353.

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