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# Optimization and characterization of a rat model of prostate cancer-induced bone pain using behavioral, pharmacological, radiological, histological and immunohistochemical methods

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ABSTRACT

The major limitation of currently utilized rodent models of prostate cancer (PCa)-induced bone pain (PCIBP) involving intra-osseous injection of PCa cells, is their relatively short-term applicability due to progressive deterioration of animal health necessitating euthanasia. Here, we describe establishment of an optimized rat model of PCIBP where good animal health was maintained for at least 90-days following unilateral intra-tibial injection (ITI) of PCa cells. We have characterized this model using behavioral, pharmacological, radiological, histological and immunohistochemical methods. Our findings show that following unilateral ITI of $4 \times 10^4$ AT3B PCa cells (APCCs), there was temporal development of bilateral hindpaw hypersensitivity that was fully developed between days 14 and 21 post-ITI. Although there was apparent spontaneous reversal of bilateral hindpaw sensitivity that was maintained until at least day 90 post-ITI, administration of bolus doses of the opioid receptor antagonist, naloxone, rescued the pain phenotype in these animals. Hence, upregulation of endogenous opioid signaling mechanisms appears to underpin apparent spontaneous resolution of hindpaw hypersensitivity. Importantly, the histological and radiological assessments confirmed that tumor formation and development of osteosclerotic metastases was confined to the APCC-injected tibial bones. In our rat model of PCIBP, single bolus doses of morphine, gabapentin, meloxicam and amitriptyline produced dose-dependent relief of mechanical allodynia and thermal hyperalgesia in the bilateral hindpaws. The optimized rat model of PCIBP characterized herein has potential to provide new insights into the pathophysiological mechanisms associated with long-term (mal)adaptive pain due to advanced PCa-induced bony metastases and for screening novel compounds with potential for improved alleviation of this condition.


## 1. Introduction

Prostate cancer (PCa) is the second most common form of cancer and the sixth most common cause of cancer-related death among men worldwide (Ferlay et al., 2010). Globally, the prevalence rates of PCa varies more than twenty-fold with the highest incidence in Australia/New Zealand, western Europe and the United States of America (104, 93 and 86 per 100,000 in 2008, respectively) and the lowest incidence in south-central Asia (4 per 100,000) (Ferlay et al., 2010).

Although metastatic cells could theoretically intrude any organ, clinical experience is that certain cancers including PCa have ~70% propensity for targeting the skeleton (Roodman, 2004). Advanced PCa-induced bony metastases produce unremitting pain in >20% of patients who do not achieve adequate pain relief with currently available analgesics (von Moos et al., 2008). Poorly relieved pain such as PCa-induced bone pain (PCIBP) due to advanced metastatic lesions in the skeleton not only results in enormous patient disability and suffering, but also significantly impairs the quality of life of patients and their carers (von Moos et al., 2008). Hence, there is an obvious need for new drug treatments with improved efficacy and greater tolerability for alleviation of this condition.

Rodent models of PCIBP involving intra-osseous injection of PCa cells with subsequent development of hypersensitivity behaviors including mechanical allodynia and hyperalgesia in the hindpaws (Pacharinsak and Beitz, 2008), are crucial for improving our collective understanding of the pathobiology of PCIBP and for screening new molecules as potential novel analgesics for improved alleviation of this pain condition.

However, a significant limitation of the rodent models of PCIBP published to-date, is that they involve intra-osseous injection of very large numbers of PCa cells which results in profound bone destruction (De Ciantis et al., 2010; Feeley et al., 2006; Kolosov et al., 2011; Lamoureux et al., 2008; Liepe et al., 2005; Zhang et al., 2005). This in turn often leads to escape of PCa cells from bone and

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formation of metastases in adjacent soft tissues as well as the lungs and liver (Kolosov et al., 2011; Liepe et al., 2005; Luo et al., 2006). As a direct consequence, the general health of animals administered with intra-osseous injections of PCa cells often declines, characterized by a significant temporal decrease in body weight relative to the control (vehicle) and/or sham (heat-killed cells) injected groups (De Ciantis et al., 2010; Zhang et al., 2005). The consequences are that animals with progressively declining animal health will often require early euthanasia due to ethical concerns. Furthermore, declining animal health has the potential to confound the interpretation of behavioral pain endpoints. Clearly, these limitations of the currently available rodent PCa bone pain models mean that their use is restricted to relatively short-term experiments. Hence, use of these models makes it difficult to gain insight into the pathobiology of PCIBP associated with advanced bony metastases that almost certainly involves neuroplastic changes at multiple levels of the somatosensory system.

Hence, the aims of the study described herein were to establish and characterize an optimized rat model of PCIBP to enable investigation of the pathobiology of this condition at the more advanced stages. Briefly, we systematically examined the impact of a range of concentrations over a 125-fold range (4000 to 500,000) of AT3B PCa cells (APCCs) administered by unilateral intra-tibial injection in the rat on general animal health as well as on the temporal development of hindpaw hypersensitivity secondary to bone tumor development over a 90-day period. We also characterized the optimized rat model of PCIBP using tibial bone histology, micro-computed tomography (μCT) scans and pharmacological profiling using commonly available analgesics and adjuvant drugs.

## 2. Material and methods

### 2.1. Drugs and reagents

Morphine sulfate ampoules were purchased from Hospira Australia Pty. Ltd. (Melbourne, Australia). Gabapentin was provided by Dr. Ben Ross, from the School of Pharmacy, at the University of Queensland (QLD, Australia). Meloxicam sodium salt hydrate and amitriptyline hydrochloride were purchased from Sigma Aldrich (NSW, Australia). Naloxone hydrochloride was purchased from Abcam Biochemicals (MA, USA). Isoflurane and sodium benzylpenicillin (BenPen™) were purchased from Abbott Australasia, Pty. Ltd. (NSW, Australia). Topical antibiotic powder was purchased from Apex Laboratories, Pty. Ltd. (NSW, Australia). Pentobarbitone sodium (Lethabarb®) was purchased from Virbac (Australia) Pty. Ltd. (NSW, Australia). Medical grade $O_2$ and $CO_2$ were purchased from BOC Gases Australia Ltd. (QLD, Australia).

### 2.2. Cell culture

The AT3B prostate cancer cells were purchased from the American Type Culture Collection (ATCC; VA, USA). These cells were cultured, passaged and harvested as per ATCC guidelines. Briefly, the cells were propagated from the frozen stock and were cultured in 75 $cm^2$ Cellstar® flasks (Greiner bio-one) at 37 °C (5% $CO_2$:95% air) in RPMI 1640 medium with L-glutamine (Invitrogen, VIC, Australia) supplemented with 10% fetal bovine serum (Invitrogen). For detaching, the cells were first rinsed gently with Dulbecco's phosphate-buffered saline solution (Invitrogen), followed by trypsinization using 0.25% trypsin EDTA (1×) (Invitrogen). The detached cells were then collected by centrifuging with 11 ml of medium for 4 min at 200 ×g. After careful aspiration and discarding of the supernatant, the resulting pellet was re-suspended in 5 ml of phosphate buffered saline solution (PBS, Invitrogen) and cells were counted using a hemocytometer. After re-centrifugation of the final pellet for 4 min at 200 ×g, the cells were prepared in five concentrations *viz.* $4 \times 10^3$, $1 \times 10^4$, $4 \times 10^4$, $1 \times 10^5$, and $5 \times 10^5$ APCCs/10 μl PBS for intra-tibial injection. For preparing heat-killed cells (HKCs), APCCs were prepared in the same final concentrations and heated for 15 min at 85 °C. All cells were kept on ice until injection.

### 2.3. Surgical procedure

Male Wistar Han (HsdBrlHan:WIST) rats were purchased from Monash Animal Services (Melbourne, Australia) and used for experimentation herein. Rats were housed in groups of three in a temperature-controlled room (21 °C ± 2 °C) with a 12 h/12 h light–dark cycle and standard laboratory food and water were available ad libitum. Rats were acclimatized for at least 2–3 days prior to initiation of any experimentation to allow acclimatization. After surgery and intra-tibial injection, rats were housed in individual cages. All experiments were conducted during the light phase and all procedures mentioned herein were approved by the Animal Ethics (AE) Committee of the University of Queensland (QLD, Australia).

The unilateral intra-tibial injection procedure was performed as previously described (Zhang et al., 2005). Briefly, while groups of male Wistar Han rats (90–130 g) were deeply anesthetized with 3% isoflurane delivered in oxygen, a 1 cm unilateral rostro-caudal incision was made on the upper medial half of the lower hind limb. Once the tibial bone was exposed, a 23-gage needle was used to pierce the bone below the knee joint medial to the tibial tuberosity. Subsequently, a 10 μl injection comprising APCCs in one of the afore-mentioned concentrations or HKCs (sham) or PBS (vehicle) was made into the bone cavity using a Hamilton syringe (Hamilton 705 N (22 s/2″/2), NV, USA) and the bone was sealed using bone wax (Ethicon). To more fully characterize the model, a separate group of rats was included where a tibia was pierced unilaterally and immediately closed using bone wax (control) without intra-tibial injection. The hindpaw on the injected side is referred to as the 'ipsilateral' hindpaw and the non-injected side as the 'contralateral' hindpaw. The rats were kept warm and monitored closely during the post-surgical recovery period. Thereafter, the general health and body weights of rats were assessed every day throughout the experimental period.

### 2.4. Animal groups

The number of rats used per group for each of the experiments described herein is summarized as follows:

Experiment one (20 days): $5 \times 10^5$ APCCs (n = 6), vehicle (n = 4) and control (n = 4) rats.
Experiment two (21 days): $4 \times 10^3$ (n = 4), $1 \times 10^4$ (n = 4) and $4 \times 10^4$ (n = 4) APCCs, and vehicle (n = 4) injected rats.
Experiment three (28 days): $4 \times 10^4$ (n = 8) and $1 \times 10^5$ (n = 8) APCCs, HKCs (n = 6), vehicle (n = 6) and control (n = 6) rats.
Experiment four, cohort 1 (90 days): $4 \times 10^4$ APCCs (n = 10) and HKCs (n = 5) injected rats were used for assessing temporal development of pain behavior and general animal health.
Experiment four, cohort 2 (21 days): Separate groups of $4 \times 10^4$ APCCs (n = 4) and HKCs (n = 4) injected rats were used for tibial bone radiological and histological studies.
Experiment four, cohort 3 (21 days): $4 \times 10^4$ APCCs (n = 4) and HKCs (n = 3) injected rats for tibial bone immunohistochemical studies.
Experiment four, cohort 4 (28 days): $4 \times 10^4$ APCCs (n = 4) and HKCs (n = 4) injected rats for tibial bone marrow immunohistochemical studies.
Experiment five, cohort 1 (14–21 days): $4 \times 10^4$ APCCs injected rats (n = 12 for each dose tested) for dose–response curves for morphine, gabapentin, amitriptyline and meloxicam for relief of mechanical allodynia.