Department of Oncology[1], The First Affiliated Hospital of Jinan University, Guangzhou; Department of Oncology[2], The People's Hospital of Jiangmen, Jiangmen, Guangdong, China

Efficacy and mechanism of action of etanercept in bone cancer pain

Yayong Liao[1, 2], Meng Xu[1, *]

Received October 31, 2016, accepted December 2, 2016

**Corresponding author: Meng Xu, Department of Oncology, The First Affiliated Hospital of Jinan University, No. 613, Huangpu Avenue West, Tianhe District, Guangzhou 510630, Guangdong, China*
xumeng5105@126.com

Pharmazie 72: 219–222 (2017) *doi: 10.1691/ph.2017.6901*

Bone cancer pain treatment remains a major clinical challenge, and the mechanism underlying bone cancer pain remains insufficiently understood. The purpose of the current study was to evaluate the efficacy of etanercept, a TNF-α inhibitor, against bone cancer pain in an established bone cancer pain model. The mechanical pain threshold value was tested, and the expression of inflammatory factors, NOS, MrgC and NR2B were assessed. The results showed that etanercept decreases the mechanical pain threshold value and inhibits expression of inflammatory factors, NOS, MrgC and NR2B. Etanercept may moderate bone cancer pain *via* the MrgC-NR2B signaling pathway.

1. Introduction

Chronic pain caused by bone cancer is a big problem in clinical treatment and seriously affects the patients' life quality (Xu et al. 2016b; Zhao et al. 2016). Research about bone cancer pain has achieve great progress, while effective therapies for treating bone cancer are lacking (Fuseya et al. 2016).

A mouse model of bone cancer pain was used to investigate the role of QX-314 in bone cancer pain relief, and the results showed that systemic administration of QX-314 inhibits some bone cancer pain through selective inhibition of TRPV1-expressing afferents in rats (Sun et al. 2016a). Moreover, Sun et al. (2016c) have investigated the effects of Mas oncogene-related gene (Mrg) C receptors (MrgC) on the expression and activation of N-methyl-D-aspartate receptor subunit 2B (NR2B), and neuronal nitric oxide synthase (nNOS) in a mouse model of bone cancer pain. They demonstrated that the MrgC-activated spinal Gi-NR2B-nNOS signaling pathway plays an important role in the development of bone cancer pain (Sun et al. 2016c). Additionally, BAM8-22 attenuated bone cancer pain in mice dose dependently, and anti-MrgC antibody reversed the effects of BAM8-22(Li and Zhang 2015). So far, the complete understanding of bone cancer pain is far from sufficient, while many pathophysiological components including tumor-stimulated osteolysis, nerve compression, stimulations of ion channels, and locally generated inflammatory cytokines have been suggested (Lu et al. 2015; Wang et al. 2015; Xu et al. 2016a). In particular, it has been shown that inflammation reactions are necessary for the development of bone cancer pain, as the expression of IL-1β, IL-6, IL-18, and TNF-α persistently participated in the progression of bone cancer pain (Kotak et al. 2015; Mao-Ying et al. 2012).

Etanercept is a clinical drug approved for the treatment of rheumatoid joints and ankylosing spondylitis, which is confirmed a competitive inhibitor of TNF-α, a polypeptide hormone involved in the development of the immune system, in host defense and immune surveillance (Ohtori et al. 2015; Senel et al. 2011). Moreover, direct injection of etanercept has also been proved to relieve osteoarthritic (OA) pain in animal and clinical studies (Sun et al. 2016b).

In this experiment, we investigated the role and underlying mechanism of etanercept in bone cancer pain. We firstly constructed a chronic pain model in mice, and then explored the effect of etanercept on pain relief and the related mechanism. The results have confirmed that etanercept can notably decrease the mechanical pain threshold and inflammatory factors. Moreover, we detected the expression of NOS and proved etanercept to inhibit iNOS and NNOS. Finally, etanercept moderates bone cancer pain through the MrgC-NR2B pathway.

All of our efforts will provide a theoretical basis and new insights into the treatment of bone cancer pain.

2. Investigations, results and discussion

2.1. Etanercept decreases mechanical pain threshold

Firstly, we measured the pain threshold value of each animal group. According to the results, the pain threshold increased in the bone cancer mice group, while its mechanical pain threshold value has fallen significantly ($p<0.5$) after etanercept treatment (Fig. 1).

2.2. Etanercept inhibits expression of proinflammatory factors

Next, kinds of cytokine secretion levels in animal tissues were tested. The results shown in Fig. 2 confirm that proinflammatory factors, including the TNF-α, IL-1β, and IL-6 levels were increased, while etanercept treatment notably inhibited the expression levels ($p<0.05$). The results tell that etanercept can inhibit the local inflammatory response.

2.3. Etanercept inhibits MrgC and NR2B expression

Previous studies have shown that the MrgC-NR2B pathway plays a key role in bone cancer pain, so we tested the related protein expression in both mRNA and protein levels. The results shown in Fig. 3 tell that etanercept significantly inhibited the expression of MrgC and NR2B($p<0.05$). So we believe that etanercept may play an important role in bone cancer pain relief.

2.4. Etanercept decreases NOS expression

Chronic pain and related inflammatory response can be regulated by NOS enzyme activity and expression. In this part of work, RT-PCR was used to detect NOS expression. The results are shown in Fig. 4. Expression of INOS and nNOS was increased in the pain model mice, while the expression levels of INOS and nNOS fell significantly compared with the model group after etanercept treatment ($p<0.05$).

3. Discussion

Bone cancer pain is usually progressive and treatment remains a major clinical challenge, while the cellular and molecular mechanisms underlying bone cancer pain remain elusive (Ke et al. 2013;

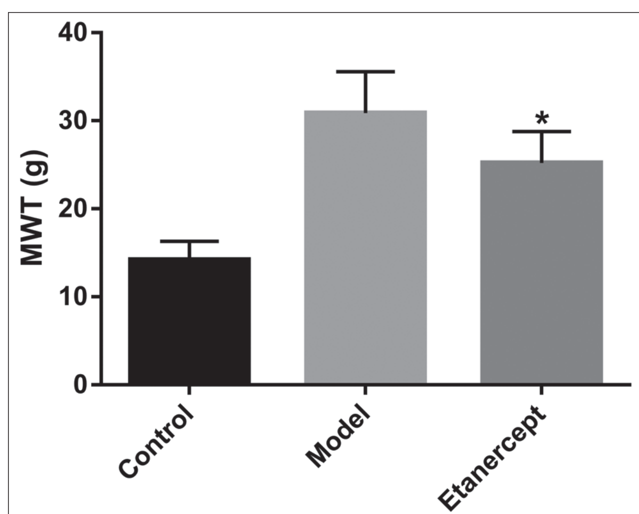

Fig. 1: Effects of etanercept on mechanical pain threshold. The mechanical pain threshold value was meausured before and after etanercept treatment. Error bars indicate mean±SD and * indicates significant difference compared with control group.

of this, we determined study the effect of etanercept on bone cancer pain. We firstly established a chronic pain model in mice, and then explored the effects of etanercept on the mechanical pain threshold. The results have confirmed that etanercept can notably decrease the mechanical pain threshold. Moreover, we detected the expression of NOS and inflammatory factors. TNF-α, IL-6, and IL-1, the results proved etanercept to inhibit iNOS, NNOS and the inflammatory factors. Finally, by monitoring the expression of MrgC and NR2B expression, we came to the conclusion that etanercept moderates bone cancer pain through MrgC-NR2B pathway. In conclusion, we found a high expression of inflammatory cytokines from the animal model of bone cancer for the first time and proved etanercept to moderate animal pain via the MrgC-NR2B pathway. These results suggest that etanercept could be a good choice for patients with bone cancer pain.

4. Experimental

4.1. Animal model

Adult male C3H/HeJ mice, 4–6 weeks old, 18–22 g, were purchased from the Vital River Experimental Animal Center, Beijing, China. These animals were exposed with free access to food and water, under a 12/12-h light/dark cycle, at 24 °C, and none of them die unexpectedly before the experimental endpoint.

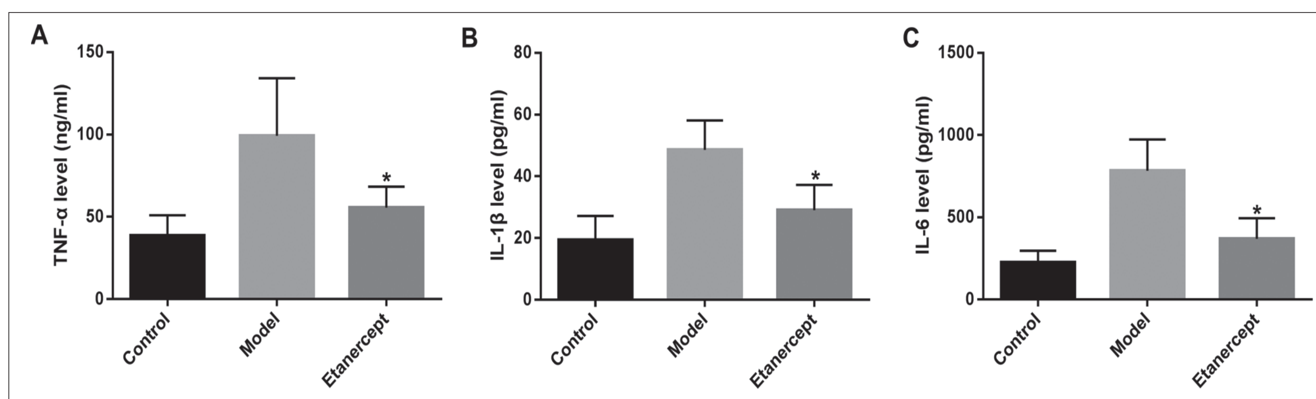

Fig. 2: Effects of etanercept on inflammatory response. The proinflammatory factors of TNF-α, IL-1β, and IL-6 levels were increased by Etanercept. Error bars indicate mean±SD and * indicates significant difference compared with control group. TNF-α: tumor necrosis factor. IL-1β: Interleukin-1β.

Yuan et al. 2013). Multiple factors are involved in the mechanism of bone cancer pain, and many therapies and treatments were proposed (Liu et al. 2013a; Song et al. 2016; Zhao et al. 2013). Particularly, bone cancer pain shares features of inflammatory and neuropathic pain, and much research is focused on inflammation related mechanisms (Hu et al. 2012; Zhang et al. 2015). In a study of the role of L-THP in rats with bone cancer pain, L-THP was regarded as clinically useful, and this may be related to an inhibition of TNF-α and IL-18 (Fei et al. 2015). Moreover, a study has confirmed spinal expression of CCR2, NR2B, and nNOS significant upregulation of nNOS in bone cancer pain rats (Yang et al. 2016). Additionally, spinal nitric oxide is involved in the mechanisms of pain generation and transmission during inflammatory and neuropathic pain, and it was found that an upregulation of nNOS and iNOS in the spinal cord is closely associated with bone cancer pain (Sainoh et al. 2016). As a tumor necrosis factor (TNF)-α inhibitor, etanercept is able to alleviate intractable discogenic low back pain (LBP) (Totoson et al. 2016). The compound also significantly increased NOS activity and decreased COX-2/arginase activities. Due to its effects on these inflammatory symptoms, etanercept was conceived a good choice for patients with rheumatoid arthritis (Bae et al. 2016). Furthermore, after injection in a rat model of acute ischemia, etanercept significantly suppressed optic nerve injury and the study suggests that etanercept might be a novel treatment agent for TNF-α-related diseases (Zhou et al. 2016).

However, the involvement of etanercept and the pertinent signaling is not determined yet in the model of bone cancer pain. In view

Fibrosarcoma NCTC 2472 cells (2087787; American Type Culture Collection, ATCC, Manassas, VA, USA) were cultured with the NCTC 135 medium (Sigma-Aldrich, St. Louis, MO, USA) with 10% horse serum (Gibco, Grand Island, NY, USA) in a 5% CO_2, 37 °C incubator.

A bone cancer pain model was established as follows. Firstly, we used 50 mg/kg pentobarbital sodium (1% in saline) to anesthetize the cultured mice with the intraperitoneal injection, and then, the skin overlying the right articulatio genu was given a superficial incision. Gonarthrotomy was performed to expose the femur condyles, which were then subjected to light depression caused by dental bur. Cortex were perforated with a 30-gauge needle. 20 μL α-MEM containing 2×10^5 NCTC 2472 cells were injected into the intramedullary space of the right femur for the mice in the model group. The injection hole was sealed with dental amalgam, and copious irrigation was implemented with saline before the wound was finally closed. Infection was prevented by operating under sterile conditions and with sterile equipment (Zhou et al. 2015).

4.2. Mechanical sensitivity measurement

Mechanical allodynia was assessed using von Frey filaments (Stoeltingcompany, USA) by researchers who were blinded to group assignment. The ipsilateral hind paw was pressed with one of a series of von Frey filaments with gradually increasing stiffness (2, 4, 6, 8, 10, 15 and 20 g) applied to the plantar surface for 5-6 s for each filament, and the interval between trials was ≥5 min. When the animal fastly lifted the hind paw, we recorded it positive paw withdrawal response. For each trial, the same hind limb was stimulated 10 times by a single von Frey filament prior to stimulation by the next larger filament. The minimal value that resulted in ≥6 responses to 10 stimulations was recorded (Chakravarthy et al. 2014).

4.3. Cytokine analysis

Firstly, the ankle tissues were excised after euthanasia and flash frozen in liquid nitrogen. Then, the tissue was pulverized and treated in a mixture of cell extraction solution, phosphatase and protease inhibitors. The tissue the supernatant was

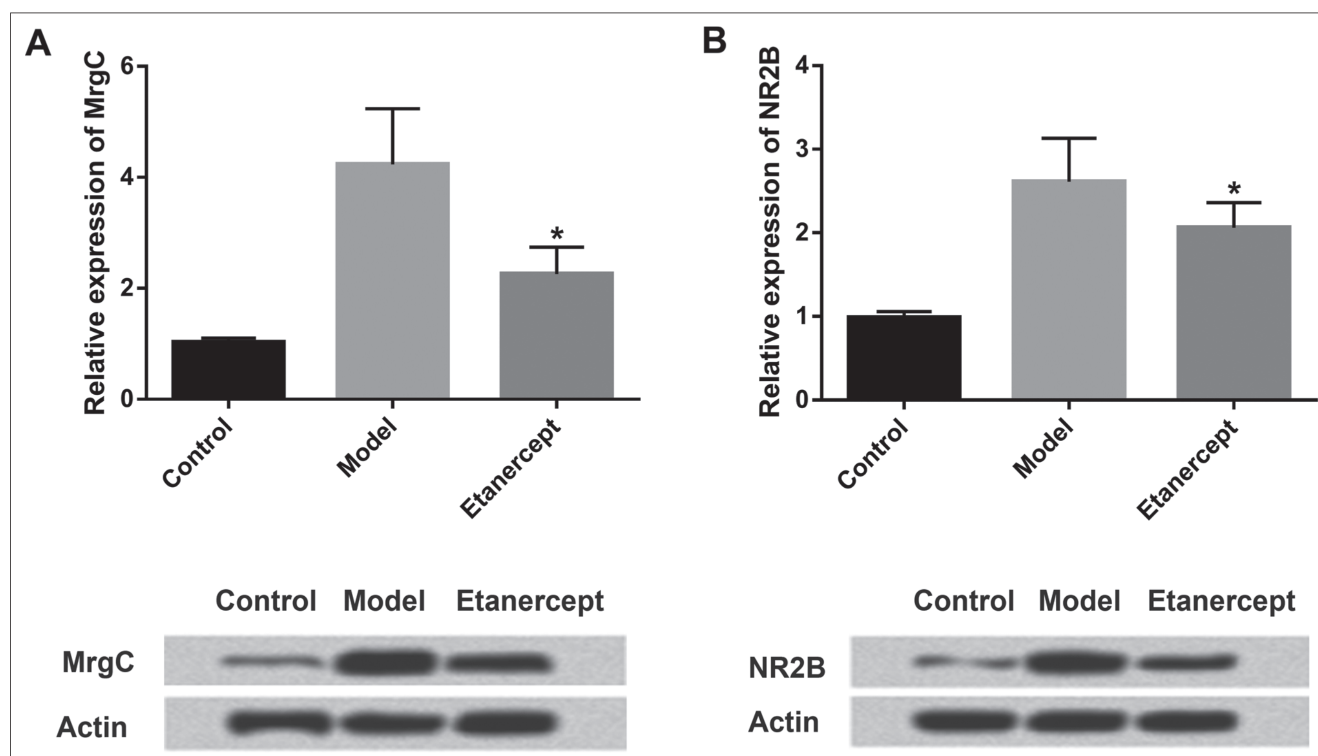

Fig. 3: Etanercept moderates bone cancer pain. The bond cancer pain related protein of MrgC and NR2B were monitored in both mRNA and protein levels. Error bars indicate mean±SD and * indicates significant difference compared with control group.

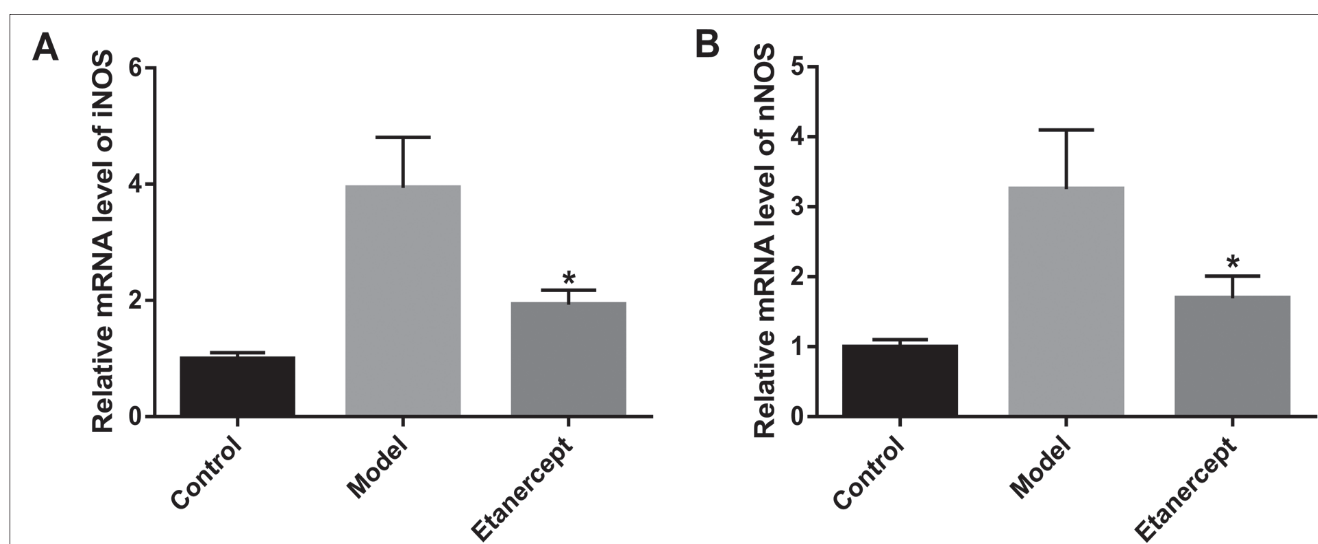

Fig. 4: Effects of etanercept on NOS. RT-PCR was used to test the nNOS and iNOS expression. Error bars indicate mean±SD and * indicates significant difference compared with control group.

homogenized or processed for cytokine expression, using Milliplex MAP 27-plex rat cytokine/chemokine magnetic bead panel (EMD Millipore, Billerica, MA, USA) on a LuminexFlexMAP 3D instrument (Luminex, Austin, TX, USA) (Sun et al. 2016b).

4.4. Western blotting

Tissue samples were homogenized with lysis buffer, and centrifuged at 13,000 rpm, 4 °C for 10 min. Protein concentration was determined with the BCA kit (Thermo Fisher Scientific Life Science Research, Shanghai, China). Protein sample was separated on SDS-PAGE, and then transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The membrane was blocked with 5% non-fat milk at room temperature for 1 h, and then respectively incubated with rabbit anti-mouse anti-MrgC polyclonal antibody (1:500 dilution; orb101320; Biorbyt), mouse anti-mouse anti-NR2B monoclonal antibody (1:1000 dilution; ab93610; Abcam, Cambridge, MA, USA), and rabbit anti-mouse anti-Gi polyclonal antibody (1:500 dilution; ab140333; Abcam). The membrane was washed and incubated with goat anti-rabbit IgG (1:5,000 dilution; ab6721; Abcam) or goat anti-mouse IgG (1:5,000 dilution; ab6789; Abcam). Protein band was visualized by ECL method (Santa Cruz, Santa Cruz, CA, USA), and protein quantification was performed with the IPLab software (Scanalytics, Fairfax, VA, USA). β-actin was used as reference control (Yang et al. 2016).

4.5. RT-PCR

Total RNA was isolated and purified using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. RT was performed using a Moloney murine leukemia virus reverse transcriptase kit (Promega Corp., Madison, WI, USA). The generated cDNA was then used as a template for PCR amplification with Taq DNA polymerase and ROX reference dye provided by the SYBR Premix Ex Taq kit (DRR041A; Takara, Dalian, China) using StepOnePlus RT-PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). β-actin, nNOS and iNOS were amplified using specific oligonucle-

otide primers to the Table. β-Actin was used as internal standard. PCR amplification was performed using the following thermocycling conditions: 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 66 °C (or 62 °C for iNOS and β-actin) for 30 s and 72 °C for 30 s, and a final elongation at 72 °C for 10 min. The relative expression was calculated using the ΔΔCq method and optimized with a standard curve to confirm specificity. Each sample (5 µl) was electrophoresed on 2% agarose gel (Biowest, Nuaillé, France) with ethidium bromide (Biomatik Corporation, Cambridge, ON, Canada) and the intensity of each band was analyzed using a gel imaging analytical system (UVP GDS 8000; UVP, Upland, CA, USA). The sample without reverse transcriptase was used as a negative control and showed no detectable band (Liu et al. 2013b).

4.6. *Statistical analysis*

The numerical values are expressed as the mean±SEM. Analysis of variance with the Bonferroni post hoc test was used to compare the values of the experimental groups. IBM SPSS Statistics software, version 21.0 (IBM Corp, Armonk, NY, http://www-01.ibm.com/software/analytics/spss/) was used for the statistical calculations. A statistical significance was defined when $p<0.05$.

Conflicts of interest: All authors declare that they have no conflict of interests. There was no funding to support the work.

References

Bae HW, Lee N, Gong JS, Rho S, Hong S, Chan YK (2016) Protective effect of etanercept, an inhibitor of tumor necrosis factor-α, in a rat model of retinal ischemia. BMC Ophthalmol 16: 1-6.

Chakravarthy K, Faltus R, Robinson G, Sevilla R, Shin J, Zielstorff M, Byford A, Leccese E, Caniga MJ, Hseih S, Zhang S, Chiu CS, Zhang-Hoover J, Moy LY, McLeod RL, Stoffregen D, Zhang W, Murtaza A, Cicmil M (2014) Etanercept ameliorates inflammation and pain in a novel mono-arthritic multi-flare model of streptococcal cell wall induced arthritis. BMC Musculoskelet Disord 15: 1-16.

Fei R, Jiao H, Cai H (2015) Analgesic effect of intrathecal administration of chemokine receptor CCR2 antagonist is related to change in spinal NR2B, nNOS, and SIGIRR expression in eat with bone cancer pain. Cell Biochem Biophys 72: 1-6.

Fuseya S, Yamamoto K, Minemura H, Yamaori S, Kawamata T, Kawamata M (2016) Systemic QX-314 reduces bone cancer pain through selective inhibition of transient receptor potential vanilloid subfamily 1-expressing primary afferents in mice. Anesthesiology 125: 204-218.

Hu JH, Yang JP, Lei L, Li CF, Wang LN, Ji FH, Cheng H (2012) Involvement of CX3CR1 in bone cancer pain through the activation of microglia p38 MAPK pathway in the spinal cord. Brain Res 1465: 1-9.

Ke C, Li C, Huang X, Fei C, Dai S, He W, Bu H, Gao F, Cai T, Hinton AO Jr, Tian Y (2013) Protocadherin20 promotes excitatory synaptogenesis in dorsal horn and contributes to bone cancer pain. Neuropharmacology 75: 181-190.

Kotak S, Mardekian J, Horowicz-Mehler N, Shah A, Burgess A, Kim J, Gemmen E, Boyd H, Koenig A (2015) Impact of etanercept therapy on disease activity and health-related quality of life in moderate rheumatoid arthritis patients population from a national British observational cohort. Value Health 18: 817-823.

Li Q, Zhang X (2015) Epigallocatechin-3-gallate attenuates bone cancer pain involving decreasing spinal Tumor Necrosis Factor-α expression in a mouse model. Int Immunopharmacol 29: 818-823.

Liu M, Yang H, Fang D, Yang JJ, Cai J, Wan Y, Chui DH, Han JS, Xing GG (2013a) Upregulation of P2X3 receptors by neuronal calcium sensor protein VILIP-1 in dorsal root ganglions contributes to the bone cancer pain in rats. Pain 154: 1551-1568.

Liu S, Liu YP, Song WB, Song XJ (2013b) EphrinB-EphB receptor signaling contributes to bone cancer pain via Toll-like receptor and proinflammatory cytokines in rat spinal cord. Pain 154: 2823-2835.

Lu CE, Liu Y, Sun B, Sun YE, Hou B, Zhang Y, Ma Z, Gu X (2015) Intrathecal injection of JWH-015 attenuates bone cancer pain via time-dependent modification of pro-inflammatory cytokines expression and astrocytes activity in spinal cord. Inflammation 38: 1880-1890.

Mao-Ying QL, Wang XW, Yang CJ, Xiu L, Mi WL, Wu GC, Wang YQ (2012) Robust spinal neuroinflammation mediates mechanical allodynia in Walker 256 induced bone cancer rats. Mol Brain 5: 16.

Ohtori S, Orita S, Yamauchi K, Eguchi Y, Ochiai N, Kishida S, Kuniyoshi K, Aoki Y, Nakamura J, Ishikawa T, Miyagi M, Kamoda H, Suzuki M, Kubota G, Sakuma Y, Oikawa Y, Inage K, Sainoh T, Sato J, Shiga Y, Abe K, Fujimoto K, Kanamoto H, Toyone T, Inoue G, Takahashi K (2015) Efficacy of direct injection of etanercept into knee joints for pain in moderate and severe knee osteoarthritis. Yonsei Med J 56: 1379-1383.

Sainoh T, Orita S, Miyagi M, Inoue G, Kamoda H, Ishikawa T, Yamauchi K, Suzuki M, Sakuma Y, Kubota G, Oikawa Y, Inage K, Sato J, Nakata Y, Nakamura J, Aoki Y, Toyone T, Takahashi K, Ohtori S (2016) Single intradiscal administration of the tumor necrosis factor-alpha inhibitor, etanercept, for patients with discogenic low back pain. Pain Med 17: 40-45.

Senel S, Kisacik B, Ugan Y, Kasifoglu T, Tunc E, Cobankara V (2011) The efficacy and safety of etanercept in patients with rheumatoid arthritis and spondyloarthropathy on hemodialysis. Clinical Rheumatol 30: 1369-1372.

Song ZP, Xiong BR, Guan XH, Cao F, Anne M, Zhou YQ, Zheng H, Tian YK (2016) Minocycline attenuates bone cancer pain in rats by inhibiting NF-κB in spinal astrocytes. Acta Pharmacol Sin 37: 753-762.

Sun Y, Zhang J, Lei Y, Lu C, Hou B, Ma Z, Gu X (2016a) Activation of spinal MrgC-Gi-NR2B-nNOS signaling pathway by Mas oncogene-related gene C receptor agonist bovine adrenal medulla 8-22 attenuates bone cancer pain in mice. Am J Translat Res 8: 1144-1154.

Sun YE, Jiang M, Hou B, Lu CE, Lei Y, Ma Z, Gu X (2016b) Mas-Related Gene (Mrg) C Activation Attenuates Bone Cancer Pain via Modulating Gi and NR2B. PLoS One11: e0154851.

Sun YE, Lu CE, Lei Y, Liu Y, Ma Z, Gu X (2016c) Mas-related G-protein-coupled receptor c agonist bovine adrenal medulla 8-22 attenuates bone cancer pain in mice. Int J Clin Exper Med 8: 20178-20187.

Totoson P, Maguin-Gaté K, Prigent-Tessier A, Monnier A, Verhoeven F, Marie C, Wendling D, Demougeot C (2016) Etanercept improves endothelial function via pleiotropic effects in rat adjuvant-induced arthritis. Rheumatology 55: 1308-1317.

Wang J, Zhang R, Dong C, Jiao L, Xu L, Liu J, Wang Z, Lao L (2015) Transient receptor potential channel and interleukin-17A involvement in LTTL gel inhibition of bone cancer pain in a rat model. Integr Cancer Ther 14: 381-393.

Xu S, Yao M, Xu L, Wang H, Li H, Huang B, Zhou X (2016a) [Effects of intrathecal injection PI3K antagonist on inflammatory cytokines in spinal cord of bone cancer pain model in rats]. Zhonghua Yi Xue Za Zhi 96: 297-300.

Xu Y, Liu J, He M, Liu R, Belegu V, Dai P, Liu W, Wang W, Xia QJ, Shang FF, Luo CZ, Zhou X, Liu S, McDonald J, Liu J, Zuo YX, Liu F, Wang TH (2016b) Mechanisms of PDGF siRNA-mediated inhibition of bone cancer pain in the spinal cord. Sci Rep 6: 27512.

Yang Y, Zhang J, Liu Y, Zheng Y, Bo J, Zhou X, Wang J, Ma Z (2016) Role of nitric oxide synthase in the development of bone cancer pain and effect of L-NMMA. Mol Med Rep 13: 1220-1226.

Yuan X, Wu J, Wang Q, Xu M (2013) The antinociceptive effect of systemic administration of a combination of low-dose tramadol and dexmedetomidine in a rat model of bone cancer pain. Eur J Anaesthesiol 31: 30-34.

Zhang MY, Liu YP, Zhang LY, Yue DM, Qi DY, Liu GJ, Liu S (2015) Levo-tetrahydropalmatine attenuates bone cancer pain by inhibiting microglial cells activation. Mediat Inflamm 2015: 752512.

Zhao J, Zhang H, Liu SB, Han P, Hu S, Li Q, Wang ZF, Mao-Ying QL, Chen HM, Jiang JW, Wu GC, Mi WL, Wang YQ (2013) Spinal interleukin-33 and its receptor ST2 contribute to bone cancer-induced pain in mice. Neuroscience 253: 172-182.

Zhao X, Liu HZ, Zhang YQ (2016) [Effect of P2X7 receptor knock-out on bone cancer pain in mice]. Sheng Li Xue Bao 68: 224-233.

Zhou YQ, Liu Z, Liu HQ, Liu DQ, Chen SP, Ye DW, Tian YK (2016) Targeting glia for bone cancer pain. Expert Opin Ther Targets 20: 1365-1374.

Zhou Z, Liang Y, Deng F, Cheng Y, Sun J, Guo L, Xu G (2015) Phosphorylated neuronal nitric oxide synthase in neuropathic pain in rats. Int J Clin Exper Pathol 8: 12748-12756.