Original Paper

Blocking Mammalian Target of Rapamycin (mTOR) Alleviates Neuropathic Pain Induced by Chemotherapeutic Bortezomib

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Key Words

Mammalian target of rapamycin • Neuropathic pain • Bortezomib • Signal pathway

Abstract

Background/Aims: Bortezomib (BTZ) is targeted as a chemotherapeutic agent for the treatment of cancer. However, one of the significant limiting complications of BTZ is painful peripheral neuropathy during chemotherapy. Drugs preventing and/or treating the painful symptoms induced by BTZ are rare, since the underlying mechanisms leading to neuropathic pain remain largely unclear. The purposes of this study were to examine 1) the effects of blocking mammalian target of rapamycin (mTOR) on mechanical pain and cold hypersensitivity evoked by BTZ and 2) the underlying mechanisms responsible for the role of mTOR in regulating BTZ-induced neuropathic pain. **Methods:** Behavioral test was performed to determine mechanical and cold sensitivity in a rat model. Western blot analysis and ELISA were used to examine expression of mTOR and phosphatidylinositol 3-kinase (p-PI3K) signals, and the levels of substance P and calcitonin gene-related peptide (CGRP). **Results:** Systemic injection of BTZ significantly increased mechanical pain and cold sensitivity as compared with control animals ($P < 0.05$ vs. control rats). The expression of p-mTOR, mTOR-mediated phosphorylation of p70 ribosomal S6 protein kinase 1 (p-S6K1), 4E-binding protein 1 (4E-BP1), as well as p-PI3K was amplified in the dorsal horn of spinal cord of BTZ rats as compared with control rats. Blocking mTOR by intrathecal infusion of rapamycin attenuated mechanical pain and cold hypersensitivity. Blocking PI3K signal also attenuated activities of mTOR, which was accompanied with decreasing neuropathic pain. Inhibition of either mTOR or PI3K blunted enhancement of the spinal substance P and CGRP in BTZ rats. **Conclusions:** The data for the first time revealed specific signaling pathways leading to BTZ-induced peripheral neuropathic pain, including the activation of mTOR and PI3K. Inhibition of these signal pathways alleviates pain. Targeting one or more of these signaling molecules may present new opportunities for treatment and management of peripheral painful neuropathy observed during chemotherapeutic application of BTZ.

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Introduction

Bortezomib (BTZ), a reversible inhibitor of the proteasome complex, is used as a chemotherapeutic agent for the treatment of cancer such as multiple myeloma [1, 2]. However, the prior studies have demonstrated that dose-limiting painful peripheral neuropathy is one of the significant adverse events observed during BTZ therapy [3]. Generally, treatment options for these abnormal sensations have been restricted, partly due to a poor understanding of the underlying mechanisms leading to neuropathic pain induced by BTZ. Drugs preventing and/or treating the painful symptoms evoked by BTZ therapy are lacking. Therefore, it is significant to determine molecular mediators of BTZ-induced painful neuropathy to make therapeutic strategies and have management of chemotherapeutic patients with multiple myeloma.

Mammalian target of rapamycin (mTOR) is a serine threonine protein kinase. There are two distinct mTOR forms of protein complexes, mTOR complex 1 (mTORC1) and mTORC2. In general, mTORC1 is composed of raptor, mLST8 and mTOR, and is known to govern translation of most proteins by phosphorylation of specific downstream effectors including, p70 ribosomal S6 protein kinase (p70 S6Ks) and 4E-BPs [4].

Activation of mTOR, in particular, mTORC1 that is negatively regulated by rapamycin, leads to promotion of the phosphorylation of downstream effectors, such as p70 S6K1 and this further governs mRNA translation [4]. The mTORC1 is well known to play critical roles in the regulation of protein synthesis and growth and evidence supports the notion that mTOR plays a role in modulating long-term neuronal plasticity [5, 6]. Particularly, mTOR, S6K1 and 4E-BP1 are presented in the spinal cord dorsal horn and contribute to transmission and modulation of pain [6-8], i.e., intrathecal administration of rapamycin, an inhibitor of mTOR, produces antinociception in models of inflammation [9]. Local perfusion of rapamycin into the spinal cord attenuates formalin-induced neuronal hyperexcitability in the dorsal horn [10]. Note that rapamycin attenuates pain response and this is accompanied with downregulated mTOR, S6K1 and 4E-BP1 [12]. These findings indicate that mTOR and its downstream signals are activated in persistent pain conditions and contribute to the development of spinal pain sensitization.

The superficial dorsal horn is the first synaptic site from peripheral afferent nerves to the central nervous system [13, 14] and plays an important role in modulating pain [15, 16]. Especially, the superficial dorsal horn at the lumbar levels (i.e., L5 to L6) is the first synaptic site receiving pain input from the hind paw. Thus, in this study we determined the role played by mTOR in the superficial dorsal horn in regulating mechanical hyperalgesia and cold hypersensitivity in rats with systemic administration of BTZ. We hypothesized that BTZ regulates the protein expression of mTOR signal pathways in the superficial dorsal horn resulting in mechanical pain and cold hypersensitivity. Moreover, blocking spinal mTOR by intrathecal injection of rapamycin attenuates the amplified neuropathic pain response evoked by BTZ. We also hypothesized that BTZ amplifies the expression of phosphatidylinositol 3-kinase (PI3K), an upstream signal of mTOR [4]. We speculated that blocking spinal PI3K would attenuate activities of mTOR pathway thereby leading to reduction in neuropathic pain.

Moreover, the releases of neurotransmitters such as substance P and calcitonin gene-related peptide (CGRP) [17-19], within the dorsal horn play a key role in regulating pain responses [16]. Numerous neurotransmitters and related receptors in the dorsal horn have been targets to understanding of the underlying mechanisms responsible for pain [15, 16]. Accordingly, we further hypothesized that blocking mTOR and PI3K pathways attenuates the amplified levels of substance P and CGRP in the superficial dorsal horn of BTZ rats.

Materials and Methods

Animal model of neuropathic pain

All animal protocols were in accordance with the guidelines of the International Association for the Study of Pain and approved by the Institutional Animal Care and Use Committee of Jilin University. Adult

male Sprague-Dawley (200-250 g) were housed in individual cages with free access to food and water and were kept in a temperature-controlled room (25°C) on a 12/12 h light/dark cycle. BTZ (0.4 mg/kg body weight; Haoran BioTech Co., Shanghai, China) was administrated intraperitoneally (i.p.) once daily for 5 consecutive days. Control animals received an equivalent volume of saline.

Intrathecal catheter for administration of drugs

One end of polyethylene-10 tubing was inserted intrathecally through an incision in the cisternal membrane and advanced 7-9 cm caudal until the tip of the catheter was positioned at the lumbar spinal level (L5 to L6). The other end of the intrathecal tubing was sutured to the musculature and skin at the incision site and externalized to the back of the rat. Animals were allowed to recover and returned to the animal care facility where they were housed individually. Five days were allowed before the experiments were performed.

Behavioral test

To quantify the mechanical sensitivity of the hindpaw, rats were placed in individual plastic cages and allowed to acclimate for > 30 min. Mechanical paw withdrawal threshold (PWT) and hindpaw response to the stimulation of von Frey filaments was determined. A series of calibrated von Frey filaments (ranging from 0.5 to 18.0 g) were applied perpendicularly to the plantar surface of the hindpaw with a sufficient force to bend the filaments for 60s or until paw withdrew. In the presence of a response, the filament of next lower force was applied. In the absence of a response, the filament of next higher force was applied. To avoid injury during tests, the cutoff strength of the von Frey filament was 18.0 g, the tactile stimulus producing a 50% likelihood of withdrawal was determined using the "up-down" method [20]. Each trial was repeated 2 times at ~ 2 min intervals. The mean value was used as the force produced a withdrawal response.

Thermal Place Preference System (Coulburn Instruments) was used to perform the thermal place preference test in order to assess a cold avoidance behavior. Two connecting metal plates were surrounded by a plastic enclosure. The first plate was kept at room temperature (25°C) and the second plate was kept at cold temperature (12°C). The test was performed in darkness and each session lasted 3 minutes. During the session, the rats were left free to explore both plates. The time spent on the cold plate during the entire session was recorded using an infrared camera connected to a computer to determine cold avoidance behavior. To better control behavior, the rats were repeatedly placed on the apparatus with both plates held at room temperature (25°C) for 30 minutes 2 days before the beginning of the experiment. Note that rats spent an equal amount of time on each plate under these conditions, suggesting that animals showed no place preference. To avoid learning or any place preference unrelated to cold, the temperature of the plates was inverted between two consecutive sessions. Two trials were made and data were averaged. Note that all behavioral tests were performed in a blind fashion.

Experiment procedures

Following collection of baseline data, a Hamilton microsyringe (250µL) was connected to the intrathecal tubing to deliver 100 µl of dimethyl sulfoxide (DMSO) as vehicle control, antagonists to mTOR (rapamycin, 10µg; Selleck Chem) and inhibitor of PI3K (LY294002, 10µM; Sigma-Aldrich). The volume of rapamycin and LY294002 was 100 µl. An infusion pump was used to deliver vehicle and drugs and the pump was set to constantly over a period of 60 min. Note that a prior study [12] using the same approach to intrathecally infuse rapamycin (1, 5 and 10µg) and LY294002 (1, 5 and 10µM). It was shown that 10µg rapamycin and 10µM of LY294002 were effective to attenuate mechanical and thermal hyperalgesia. We selected those doses accordingly in our current study.

Sixty minutes after intrathecal infusion, mechanical and cold sensitivity of the hindpaws were again determined. Thus, six groups were included in this study as: saline rats + vehicle infusion (n=10); BTZ rats + vehicle infusion (n=15); saline rats + rapamycin (n=12); BTZ rats + rapamycin (n=16); saline rats+ LY294002 (n=8); and BTZ rats + LY294002 (n=12).

In a subset of experiments, in order to examine the effects of blocking mTOR and PI3K on substance P and CGRP vehicle (n=12), rapamycin (10µg, n=15) and LY294002 (10µM, n=15) were intrathecally given using an infusion pump, respectively. The pump was set to constantly deliver vehicle or the drugs over a period of 60 min. At the end of infusion, rats were euthanized by sodium pentobarbital (120 mg/kg, i.p.) and the superficial dorsal horn tissues (L4-L6) were obtained under an anatomical microscope for Western Blot and ELISA experiments.

Western blot analysis

Total protein of the superficial dorsal horn tissues was extracted by homogenizing dorsal horn sample in ice-cold immunoprecipitation assay buffer. The lysates were centrifuged and the supernatants were collected for measurements of protein concentrations. After being denatured by heating at 95°C for 5 min in buffer, the supernatant samples containing 20 µg of protein were loaded onto 4-20% Mini-PROTEA TGX gels and electrically transferred to a polyvinylidene fluoride membrane. The membrane was blocked in 5% nonfat milk in 0.1% Tween-TBS buffer and was incubated overnight with respective primary antibody, including: rabbit anti-p-mTOR /p-S6K1/p-4E-BP1 antibodies (1:200); rabbit anti-mTOR/S6K1/4E-BP1 antibodies (1:200); rabbit anti-p-PI3K p85 (1:250). Next, the membranes were washed and incubated with an alkaline phosphatase conjugated anti-rabbit secondary antibody (1:1000). All these primary antibodies were purchased from the Abcam Co. and/or Cayman Chem Co; and goat anti-rabbit secondary antibody was purchased from Santa Cruz Biotech. The immunoreactive proteins were detected by enhanced chemiluminescence. The bands recognized by the primary antibody were visualized by exposure of the membrane onto an x-ray film. The membrane was stripped and incubated with anti-rabbit antibody to show equal loading of the protein. Then, the film was scanned and the optical density of bands was analyzed using the Scion Image software (NIH, USA), and values for densities of immunoreactive bands/β-actin band densities from the same lane were examined. Each of the values was then normalized to a control sample.

ELISA measurements

To examine the levels of substance P and CGRP in the superficial dorsal horn of the spinal cord, ELISA methods were employed. Substance P was measured using substance P ELISA kit following the manufacturer's instructions (Abcam Co., Cambridge, MA). Briefly, the diluted tissue supernatant (100 µl) was placed in a 96-well goat anti-mouse IgG-coated plate and incubated for 2 hours. After incubation, the plate was washed using the provided washing buffer, and the color was developed by adding PNPP (200 µl) substrate after 45 min and determined by an ELISA plate reader with a wavelength of 575 nm. The amount of substance P was calculated by using a substance P standard curve. In the similar way, the CGRP content of the samples (100 µl supernatant) was determined using a commercial CGRP ELISA kit (Cayman Chemical Co.). Briefly, the diluted sample supernatant was placed in a 96-well plate incubated with pre-coated anti-rat IgG antibody overnight, washed and developed, and quantified [21].

Statistical analysis

All data were analyzed using two-way repeated-measures analysis of variance since the effects of pre- and post-infusion were compared among six treatment groups. Values were presented as means ± standard deviation of mean. For all analyses, differences were considered significant at $P < 0.05$. All statistical analyses were performed using SPSS for Windows version 13.0 (SPSS Inc., Chicago, USA).

Results

Effects of blocking mTOR and PI3K on mechanical and cold sensitivity

We obtained all the baseline data of mechanical and cold sensitivity, control vehicle, rapamycin and LY294002 were intrathecally infused. Fig. 1 (top panel) showed that BTZ decreased PWT in rats with vehicle infusion ($P < 0.05$ vs. saline control rats; $n = 15$) compared with saline control rats ($n = 10$). As rapamycin ($n = 16$) and LY294002 ($n = 12$) were infused, a decrease in PWT evoked by BTZ was blunted as compared with PWT in BTZ rat with vehicle infusion ($P < 0.05$, vehicle vs. rapamycin/ LY294002 in BTZ rats). Note that there was a smaller increase of PWT observed in saline control rats after infusion either rapamycin ($n = 12$) or LY294002 ($n = 8$), suggesting that blocking mTOR and PI3K had less effects on mechanical sensitivity in rats under healthy conditions.

Fig. 1 (bottom panels) further showed that BTZ decreased % time spent on the cold plate. As rapamycin and LY294002 were intrathecally infused, the shortened time spent on the cold plate evoked by BTZ was largely recovered. Similarly, a smaller increase of % time spent on the cold plate observed in saline control rats after infusion either rapamycin or

LY294002, suggesting that blocking mTOR and PI3K had less effects on cold sensitivity in rats under healthy conditions.

Expression of mTOR signal pathway

Fig. 2A showed the protein expression of p-mTOR, p-S6K1 and p-4E-BP1 as well as mTOR, S6K1 and 4E-BP1 in saline control rats and BTZ rats. BTZ significantly increased the protein levels of p-mTOR and mTOR-mediated p-S6K1 and p-4E-BP1 in the superficial dorsal horn as compared with control rats. Optical density for p-mTOR, p-S6K1 and p-4E-BP1 was 1.06 ± 0.16 ; 1.05 ± 0.16 ; 1.03 ± 0.15 in control rats, and 1.78 ± 0.26 ; 1.67 ± 0.25 ; 1.63 ± 0.21 in BTZ rats ($P < 0.05$ vs. control rats, $n=6-10$ in each group). Note that total protein of mTOR, S6K1 and 4E-BP1 levels was not significantly increased in BTZ rats. Furthermore, Fig. 2B demonstrated that the ratio of p-mTOR, p-S6K1 and p-4E-BP1 levels vs. total protein of mTOR, S6K1 and 4E-BP1 levels was significantly increased in BTZ rats.

In addition, Fig. 3A showed that expression of PI3K was upregulated in BTZ rats as compared with control rats ($n=6$ in each group). We further examined the effects of blocking PI3K on expression of p-mTOR and p-S6K1. Fig. 3B demonstrated that the protein expression of p-mTOR and p-S6K1 was significantly increased in BTZ rats ($n=6-10$) compared with saline control rats ($n=6-10$). When LY294002 was infused into the spinal cord of BTZ rat, the

Fig. 1. Effects of blocking mTOR and PI3K pathways on BTZ-evoked mechanical and cold hypersensitivity. Top panel: BTZ decreased PWT and intrathecal administration of rapamycin and LY294002 blunted the effects of BTZ. Bottom panel: BTZ decreased % time spent on cold plate. Rapamycin and LY294002 largely recovered the shortened time spent on cold plate induced by BTZ. * $P < 0.05$ vs. their respective pre-infusion; and ** $P < 0.05$ vs. post-infusion in all other groups. # $P < 0.05$, indicates significant difference between pre-infusion in respective groups. The number of animals is shown in the Fig. and they were the same in the bottom panel. Sal: saline; Veh: vehicle; BTZ: bortezomib; Rap: rapamycin; and LY: LY294002.

Fig. 2. Expression of mTOR pathways in control rats and BTZ rats. A, Top panel: typical bands and bottom panel: averaged data, showing that p-mTOR, p-S6K1 and p-4E-BP1 in the dorsal horn of the spinal cord were upregulated in BTZ rats. * $P < 0.05$ vs. control rats ($n=6-10$ in each group). There were insignificant differences in total protein expression of mTOR, S6K1 and 4E-BP1 in control rats and BTZ rats ($P > 0.05$, $n=6-10$ in each group). B, The ratio of p-mTOR, p-S6K1 and p-4E-BP1 levels vs. total protein of mTOR, S6K1 and 4E-BP1 levels was significantly increased in BTZ rats. * $P < 0.05$ vs. control rats ($n=6-10$ in each group).

amplified activities of p-mTOR and p-S6K1 evoked by BTZ were significantly attenuated (n=6-10 in each group). Nonetheless, total protein of mTOR and S6K1 levels was not significantly altered by LY294002 in BTZ rats. Fig. 3C demonstrated that the ratio of p-mTOR and p-S6K1 levels vs. total protein of mTOR and S6K1 levels was significantly increased in BTZ rats and this was inhibited after infusion of LY294002.

Levels of substance P and CGRP

In additional experiments, we examined the effects of BTZ on the levels of substance P and CGRP in the superficial dorsal horn of the spinal cord. Fig. 4 showed that substance P and CGRP were significantly increased in BTZ rats with vehicle infusion (n=12) as compared with control rats (n=10). Furthermore, blocking individual mTOR and PI3K signaling pathways by intrathecal infusion of rapamycin and LY294002 (n=15 in each group) significantly attenuated amplifications in substance P and CGRP evoked by BTZ. It is noted that LY294002 had a greater inhibitory effect on substance P and CGRP than rapamycin did.

Discussion

In this report, we observed that BTZ upregulated mTOR and its downstream signaling in the dorsal horn of the spinal cord.

Fig. 4. Effects of blocking mTOR and PI3K pathways on the levels of substance P and CGRP. BTZ significantly increased substance P and CGRP as compared with controls, and blocking mTOR and PI3K by rapamycin and LY294002 significantly attenuated the enhancement in substance P and CGRP evoked by BTZ. Note that LY294002 had a greater inhibitory effect on substance P and CGRP than rapamycin did. *P<0.05, indicated BTZ rats with vehicle infusion (n=12) vs. control rats (n=10) and BTZ rats with rapamycin (n=15) and LY294002 (n=15) infusion. # P<0.05, indicated BTZ rats with rapamycin vs. BTZ rats with LY294002.

Fig. 3. Effects of blocking PI3K on mTOR expression. A, Top panel: typical bands and bottom panel: averaged data, showing expression of upregulated p-PI3K in STZ rats as compared with control rats (n=6 in each group). *P<0.05, control vs. BTZ. B1: typical bands and averaged data: p-mTOR and p-S6K1 was amplified in the dorsal horn of BTZ rats with vehicle treatment as compared with saline control rats. B2: typical bands and averaged data: p-mTOR and p-S6K1 was amplified in the dorsal horn of BTZ rats with vehicle treatment as compared with saline control rats. *P<0.05 vs. control animals and BTZ animals infused with LY294002 (n=6-10 in each group). C, The ratio of p-mTOR and p-S6K1 levels vs. total protein of mTOR and S6K1 levels was significantly increased in BTZ rats. This was attenuated by infusion of LY294002. *P<0.05 vs. control rats and BTZ animals infused with LY294002 (n=6-10 in each group).

of mTOR signal by intrathecal infusion of rapamycin alleviated mechanical pain and cold hypersensitivity. Inhibition of PI3K further attenuated mTOR expression and neuropathic pain.

We consistently observed development of mechanical hyperalgesia and cold hypersensitivity in BTZ rats. Results of the current study also demonstrated that the expression of p-mTOR, mTOR-p-S6K1, p-4E-BP1 and p-PI3K pathway were upregulated in BTZ rats as compared with control rats. Blocking mTOR by intrathecal infusion of rapamycin attenuated mechanical hyperalgesia and largely restored shortened time spent on the cold plate evoked by BTZ. Blocking PI3K signal attenuated expression of mTOR in BTZ rats and also decreased mechanical and cold hypersensitivity. Moreover, enhanced spinal substance P and CGRP evoked by BTZ were blunted after inhibition of either mTOR or PI3K.

The PI3K/Akt pathway is an intracellular signaling pathway in regulation of the cell cycle. This important mechanism is directly related to cellular quiescence, proliferation, and longevity. PI3K can phosphorylate and activate Akt in the plasma membrane [17]. The Akt leads to several downstream effects which alter transcription of p70 ribosomal protein S6K1 or 4E-BP1 and activating cAMP response element-binding protein (CREB) and inhibition of p27 etc [4, 5, 22, 23]. Our present study demonstrated that blocking PI3K attenuated p-mTOR and p-S6K1 expression, and that intrathecal infusion of PI3K inhibitor significantly attenuated hyperalgesia and restored shortened time spent on the cold plate observed in BTZ rats. This suggests that PI3K is necessary to play a regulatory role in mediating the effect of mTOR on BTZ-evoked mechanical pain and cold hypersensitivity responses.

It is well known that stimulation of nociceptive receptors in the sensory nerves leads to the releases of substance P and CGRP from the peripheral terminal of dorsal root ganglions into the superficial dorsal horn [18, 24]. Numerous receptors are present on the presynaptic site of the nerve terminals and contribute to the releases of substance P and CGRP in regulating inflammatory pain [16]. It is assumed that the PI3K-mTOR pathway can indirectly affect these receptors such as transient receptor potential vanilloid 1 (TRPV1) and thereby decrease the levels of substance P and CGRP [16]. Another source of substance P and CGRP is likely originated from the dorsal horn interneurons [25, 26]. PI3K-mTOR signal pathway is likely to present in these interneurons containing two neuropeptides. Thus, blocking PI3K-mTOR can decrease the levels of these neuropeptides. Nevertheless, in our current study, the levels of substance P and CGRP, as two important neurotransmitters engaged in pain, were significantly increased in the superficial dorsal horn of BTZ rats. Moreover, the increased substance P and CGRP were significantly attenuated after respective injection of rapamycin and LY294002. Our data suggest that amplified expression of spinal mTOR and its downstream pathway p-S6K1 or 4E-BP1 are engaged in BTZ-induced mechanical hyperalgesia and cold hypersensitivity likely via the releases of substance P and CGRP.

Notably, we observed that LY294002 had a greater effect on substance P and CGRP than rapamycin did, indicating the role played by PI3K as a upstream pathway in regulating mechanical and cold hypersensitivity responses observed in BTZ rats via mTOR signals. This suggests that mTOR is only downstream pathway of PI3K. Nevertheless, to the best of our knowledge there is lacking evidence specifically showing the role played by mTOR and PI3K in regulating releases of spinal substance P and CGRP in a neuropathic pain model induced by BTZ. Results of the present report suggest that substance P and CGRP regulated by mTOR and PI3K at the spinal level contribute to mechanical pain and cold hypersensitivity in BTZ-induced neuropathy.

In conclusion, in BTZ rats, spinal p-mTOR and mTOR-mediated p-S6K1 and p-4E-BP1 are upregulated, resulting in mechanical and cold hypersensitivity. Intrathecal administration of rapamycin has analgesic effects on BTZ-induced neuropathic pain. Also, blocking PI3K blunts the amplified expression of p-mTOR and thereby alleviates BTZ-evoked neuropathic pain. Nonetheless, our data for the first time reveal specific signal pathways leading to mechanical pain and cold hypersensitivity evoked by BTZ, including the activation of PI3K and mTOR downstream pathways, and releases of substance P and CGRP. Our data provide a base for the mechanisms responsible for BTZ-induced peripheral painful neuropathy. Targeting one

or more of these signaling molecular mediators involved in activation of PI3K-mTOR at the spinal levels may present new opportunities for treatment and management of pain often observed in cancer patients during chemotherapeutic application of BTZ.

Disclosure Statement

No conflict of interests exists.

References

- Adams J, Kauffman M: Development of the proteasome inhibitor Velcade (Bortezomib). *Cancer Invest* 2004;22:304-311.
- Voorhees PM, Dees EC, O'Neil B, Orlowski RZ: The proteasome as a target for cancer therapy. *Cancer Res* 2003;9:6316-6325.
- Curran MP, McKeage K: Bortezomib: a review of its use in patients with multiple myeloma. *Drugs* 2009;69:859-888.
- Hay N, Sonenberg N: Upstream and downstream of mTOR. *Genes Dev* 2004;18:1925-1945.
- Banko JL, Poulin F, Hou L, DeMaria CT, Sonenberg N, Klann E: The transcription repressor 4E-BP2 is critical for eIF4F complex formation, synaptic plasticity and memory in the hippocampus. *J Neurosci* 2005;25:9581-9590.
- Costa-Mattioli M, Sossin WS, Klann E, Sonenberg N: The neuronal control of long-lasting synaptic plasticity and memory. *Neuron* 2009;61:10-26.
- Bao Y, Gao Y, Hou W, Yang L, Kong X, Zheng H, Li C, Zhang R: Engagement of signaling pathways of protease-activated receptor 2 and mu-opioid receptor in bone pain and morphine tolerance. *Int J Cancer* 2015;137:1475-1483.
- Geranton SM, Jimenez-Diaz L, Torsney C, Kulkarni SA, Leith JL, Lumb BM, Hunt SP: A rapamycin-sensitive signaling pathway is essential for the expression of persistent pain states. *J Neurosci* 2009;29:15017-15027.
- Price TJ, Rashid MH, Miller R, Pons M, Entrena JM, Cervero F: Decreased nociceptive sensitization in mice lacking the fragmentin-related protein: role of mGluR1/5 and mTOR. *J Neurosci* 2007;27:13958-13967.
- Xu Q, Fitzsimmons B, Steyer JA, Neill A, Newton AC, Hua XY, Yaksh TL: Spinal phosphoinositide 3-kinase-Akt-mammalian target of rapamycin signaling cascades in inflammation-induced hyperalgesia. *J Neurosci* 2011;31:2142-2152.
- Asante A, Waller J, Vokonas AH: Formalin-induced behavioural hypersensitivity and neuronal hyperexcitability mediated by rapid protein synthesis at the spinal level. *Mol Pain* 2009;5:27.
- Jiang H, Li S, Wu X, Zhong J, Lv A, Jiao J, Chen Z: Blocking mammalian target of rapamycin alleviates bone cancer pain and morphine tolerance via micro-opioid receptor. *Int J Cancer* 2016;138:2013-2020.
- Morgado C, Serra PP, Tavares I: Neuronal hyperactivity at the spinal cord and periaqueductal grey during experimental diabetic neuropathy: effects of gabapentin. *Eur J Pain* 2010;14:693-699.
- Silva M, Amorim D, Almeida A, Tavares I, Pinto-Ribeiro F, Morgado C: Pronociceptive changes in the activity of rostroventromedial medulla (RVM) pain modulatory cells in the streptozotocin-diabetic rat. *Brain Res Bull* 2013;96:39-44.
- Bouhassira D, Lantéri-Minet M, Attal N, Laurent B, Touboul C: Prevalence of chronic pain with neuropathic characteristics in the general population. *Pain* 2008;136:380-387.
- Hua X-Y, Yaksh TL: Dorsal Horn Substance P and NK1 Receptors: Study of a Model System in Spinal Nociceptive Processing; in (Malcangio M, ed) *Synaptic Plasticity in Pain*. Springer Science Business Media, 2009, vol. p. pp.109-138.
- Engel MA, Khalil M, Mueller-Tribbensee SM, Becker C, Neuhuber WL, Neurath MF, Reeh PW: The proximodistal aggravation of colitis depends on substance P released from TRPV1-expressing sensory neurons. *J Gastroenterol* 2012;47:256-265.

- 18 Lin Q, Li D, Xu X, Zou X, Fang L: Roles of TRPV1 and neuropeptidergic receptors in dorsal root reflex-mediated neurogenic inflammation induced by intradermal injection of capsaicin. *Mol Pain* 2007;3:30.
- 19 Puttfarcken PS, Han P, Joshi SK, Neelands TR, Gauvin DM, Baker SJ, Lewis LG, Bianchi BR, Mikusa JP, Koenig JR, Perner RJ, Kort ME, Honore P, Faltynek CR, Kym PR, Reilly RM: A-995662 [(R)-8-(4-methyl-5-(4-(trifluoromethyl)phenyl)oxazol-2-ylamino)-1, 2,3, 4-tetrahydro naphthalen-2-ol], a novel, selective TRPV1 receptor antagonist, reduces spinal release of glutamate and CGRP in a rat knee joint pain model. *Pain* 2010;150:319-326.
- 20 Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL: Quantitative assessment of tactile allodynia in the paw. *J Neurosci Methods* 1994;53:55-63.
- 21 Wang D, Zhao J, Wang J, Li J, Yu S, Guo X: Deficiency of female sex hormones augments PGE₂ and CGRP release within midbrain periaqueductal gray. *J Neurol Sci* 2014;346:107-111.
- 22 Al-Batran SE, Ducreux M, Ohtsu A: mTOR as a therapeutic target in patients with gastric cancer. *Ann Oncol* 2012;23:1931-1939.
- 23 Shor B, Gibbons JJ, Abraham RT, Yu K: Targeting mTOR globally in cancer: thinking beyond rapamycin. *Cell Cycle* 2009;8:3831-3837.
- 24 Bevan S, Quallo T, Andersson DA: TRPV1. *Handb Exp Pharmacol* 2014;222:207-227.
- 25 Chaouch A, Besson JM: [Peripheral and spinal mechanisms of nociception]. *Rev Neurol (Paris)* 1986;142:173-200.
- 26 Kozsurek M, Lukacsi E, Fekete C, Wittmann G, Rethelyi M, Puskar Z: Brain-derived neurotrophic factor-regulated transcript peptide (CART) is present in peptidergic C primary afferents and axons of excitatory interneurons with a possible role in nociception in the superficial laminae of the rat spinal cord. *Eur J Neurosci* 2007;26:1624-1631.