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Short Communication

P2Y1 purinoceptor inhibition reduces extracellular signal-regulated protein kinase 1/2 phosphorylation in spinal cord and dorsal root ganglia: implications for cancer-induced bone pain

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It remains unclear as to whether P2Y1 purinergic receptor (P2Y1R) and the molecules that act downstream, such as extracellular signal-regulated protein kinase 1/2 (ERK1/2), are involved in the development of cancer-induced bone pain (CIBP) *in vivo*. Here, we investigated the role of the P2Y1R in the modulation of CIBP-associated nociception in spinal cord and dorsal root ganglia (DRG). A CIBP model was established by inoculating Walker 256 gland carcinoma cells into the tibia of female rats. Tactile allodynia and spontaneous pain were assessed using von Frey filaments and ambulatory scores. The results showed that both the paw withdrawal latency to tactile allodynia and the ambulatory score to spontaneous pain were significantly different between the CIBP group and the sham group on days 7–9 post-inoculation ($P < 0.01$). Furthermore, rats in the CIBP group also showed a progressive increase in ambulatory score, which is different from the sham group ($P < 0.01$). Furthermore, P2Y1R mRNA and phosphorylated ERK1/2 (p-ERK1/2) protein expression levels were increased in the spinal dorsal horn and DRG of the CIBP group relative to the sham group. However, intrathecal injection of the P2Y1R antagonist MRS2179 decreased P2Y1R mRNA and p-ERK1/2 protein expression in the spinal dorsal horn and DRG ($P < 0.01$). These results provide evidence that the inhibition of P2Y1R-mediated ERK1/2 phosphorylation in the spinal dorsal horn and DRG can attenuate nociception transmission.

Keywords cancer-induced bone pain; P2Y1 purinoceptor; ERK1/2; spinal cord; dorsal root ganglia

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Introduction

Breast cancer is the most frequently diagnosed cancer and also is the leading cause of cancer death among female individuals, accounting for 23% of the total cancer cases and 14% of cancer deaths [1]. Cancer-induced bone pain (CIBP) is the most common type of pain associated with breast and other forms of cancer. Bone metastases are a common cause of CIBP, with metastatic cancer, such as breast, prostate, or lung cancer, invading the axial skeleton in most cases [2,3]. Often, the survival rate among patients diagnosed with bone metastases is longer, particularly with breast or prostate cancer [2,4]. Cancer-induced bone pain has also been shown to correlate with reduced performance status, increased anxiety and depression, and reduced quality of life [5]. Therefore, it is important to relieve pain and preserve function to enable these patients to enjoy a high quality of life as long as possible. Cancer-induced bone pain manifests as spontaneous pain, hyperalgesia, and allodynia [6]. To clarify the mechanisms of CIBP, a rat model of bone cancer pain using breast cancer cells (i.e. Walker 256 cells) has been established [7–9]. Yao *et al.* [8] revealed that rats with CIBP were not sensitive to radiant heat, and CIBP appears distinct from neuropathic or inflammatory pain states.

A growing body of evidence indicates that extracellular nucleotides play important roles in the regulation of neuronal and glial functions in the nervous system via the P2Y1 purinergic receptor (P2Y1R). The P2Y1R can be activated by nucleotides and coupled to intracellular second-messenger systems via heterotrimeric G-proteins. The P2Y1 subtype was the first cloned P2Y purinergic receptor. Evidence has indicated that the P2Y1R is associated with the production of tactile and mechanical pain and is involved in the thermal modulation of pain [10,11]. G proteins can be coupled to P2Y1R by a variety of cell-signaling mechanisms, including the protein kinase C pathway, which in turn activates the mitogen-activated protein kinase (MAPK)-signaling pathway [12]. Of note, the phosphorylated extracellular signal-regulated protein kinase 1/2 (p-ERK1/2) participates in this process. The

activation of ERK and p38 (another member of the MAPK family) signaling promotes cancer cell proliferation *in vitro*. However, the underlying mechanism by which P2Y1R mediates ERK activation in CIBP remains unclear.

In this study, we used a rat CIBP model to investigate the effects of MRS2179, a specific inhibitor of P2Y1R, on changes in rat behavioral pain performance. The results provide new insights into molecular mechanisms associated with pain inhibition in the spinal horn cord and DRG via inhibition of P2Y1R-mediated ERK1/2 signaling.

Materials and Methods

Animals

Female Sprague–Dawley rats (150–180 g) were kept under a 12/12 h light/dark cycle regime with free access to food and water. All experiments were done at 6–7 weeks of age and were conducted with the approval of the Soochow University Animal Care and Use Committee (Suzhou, China).

Intrathecal catheterization

A polyethylene catheter with an outer diameter of 0.6 mm was used. Five days before intra-tibial injection of Walker 256 cells, under anesthesia with pentobarbital sodium (40 mg/kg, intraperitoneally), a polyurethane intrathecal catheter with an inner diameter of 0.3 mm (R-intrathecal catheterization, Pittsburgh, USA) was inserted 10 mm cephalad into the rat lumbar subarachnoid space at the L4–L5 intervertebrae, according to the modification of a method described previously [13]. Three days later, 2% lidocaine (10 μl) was injected intrathecally into rats without showing the impaired movement, and animals that showed lower-limb paralysis within 30 s indicated successful catheterization.

Bone cancer model

The Walker 256 cell injection protocol was performed as described previously [14]. In brief, under anesthesia with pentobarbital sodium (40 mg/kg, intraperitoneally), rats were fixed, and the left tibia was prepared for surgery. A skin incision was made parallel to the tibia to expose the tibial plateau. Under the guidance of a surgical microscope, a 24 G needle was then inserted into the medullary canal to create a pathway for the Walker 256 cells. A depression was then made using a microinjector. Animals in the sham group ($n = 18$) were generated by injection of 10 μl sterile saline into the intramedullary space of the tibia, whereas animals in the CIBP group ($n = 18$) were generated by injection of media containing 10^5 Walker 256 cells in 10 μl. For all animals, the injection site was sealed with a medical glue to confine the cells within the intramedullary canal, followed by irrigation with sterile water. Finally, incision closure was achieved using medical glue.

Drug delivery

Intrathecal injection of all drugs was accomplished via the above described intrathecal catheter ($n = 10$ per group). MRS2179 (a P2Y1 purinoceptor inhibitor, 100 pmol; Sigma, St Louis, USA) was freshly dissolved daily in 10 μl of normal saline (NS) vehicle. On days 7, 8, and 9 after carcinoma cell inoculation, rats received an intrathecal injection of MRS2179 (MRS2179 group) or NS (vehicle group) once daily ($n = 10$ per group). Nociceptive testing [paw withdrawal threshold (PWT) or ambulatory score] was measured 1 h before drug administration (baseline) 1, 2, 4, 6, 8, 10, 12, and 24 h after the first drug injection on day 7, as well as 3, 7, 9, 12, 15, and 18 days after carcinoma cell inoculation. On day 9, some animals were killed 4 h after being injected for analysis of the expression of P2Y1R and p-ERK1/2 [real-time reverse transcription-polymerase chain reaction (RT-PCR) and immunoblot analysis].

The dose of MRS2179 and time point (4 h) of administration employed here was based on preliminary experiments (data not shown) and previous studies employing other pain models [15,16].

Behavioral studies

Tactile allodynia Tactile allodynia was assessed using von Frey filaments (Stoelting, Wood Dale, USA). Animals were placed in individual plastic boxes (20 × 25 × 15 cm) on a metal mesh floor and allowed to acclimatize for 30 min. A series of eight calibrated von Frey filaments, ranging from 0.4 to 32.0 g in log increments, were used. Testing was initiated with the 4.5 g von Frey filament, the middle of the series of filaments. Each filament was applied to the plantar surface of each hind-paw for 5 s. A decreasing-weight series of filaments was used if the rat responded to the 4.5 g starting filament; an increasing-weight series of filaments was used if the rat did not respond to the starting filament. Brisk withdrawal or paw flinching was considered positive responses. The PWT was determined by sequentially increasing and decreasing the stimulus strength (i.e. the 'up-and-down' method), and the data were analyzed using the nonparametric method of Dixon, as described by Chaplan *et al*. [17,18].

Ambulatory-evoked pain Ambulatory-evoked pain was evaluated by ambulatory score in the tumor-injected hind limb. The scoring of each animal was characterized as follows: 0, normal activities; 1, mild claudication; 2, moderate claudication; 3, severe claudication; and 4, disuse. All of the behavioral data were obtained using at least 12 animals for each time point or group.

Real-time RT-PCR

RNA from lumbar spinal dorsal horn tissue and DRG (L4–5) stored in RNA*later*® Solution (Ambion, Austin, USA) was isolated following homogenization using a Tissuelyser (MM300; Qiagen, Hilden, Germany) with a single 6.5 mm stainless steel ball per sample ($n = 4$ for each group). A same amount of RNA was taken from each sample for reversing transcript, synthesizing cDNA and amplificating PCR according to the kit manufacturer's instructions. The sequences of the primer for *P2Y1R* (GenBank accession: NW000186) as follows: sense 5′-CC TGCCTGCGGTCTACATCTTA-3′ and anti-sense 5′-ACA CCGTCAGGACAATTATCACCA-3′; and the primers of 'β-actin' as an internal reference (GenBank accession: V00481): sense 5′-ACCTTCAACACCCCAGCCATGTA CG-3′ and anti-sense 5′-CTGATCCACATCTGCTGGAA GGTGG-3′. Dye staining (SYBR Green I mix; Roche, Madison, USA) was used for real-time PCR. Each PCR reaction was performed according to the following protocol: 3 min at 95°C for denaturation; 30 s at 60°C for annealing; 20 s at 72°C for extension; and 1 s at 80°C in the plate reader, a total of 40 cycles were run and used to produce a melting curve, and then the reaction was carried out at 72°C for 5 min. After several preliminary experiments, the best reaction and melting curve conditions were acquired, and then the fluorescence threshold cycle numbers (Ct values) were recorded. At least four to six duplicate wells were run, and the mean value for those duplicate wells was determined. Finally, we used the $2^{-\Delta\Delta Ct}$ method to calculate changes of gene expression.

Immunoblot analysis

Lumbar spinal dorsal horn tissue and DRG (L4–6) taken from the rats were homogenized as previously described [18]. The homogenates were incubated for 30 min in ice-cold water with constant agitation and then centrifuged at $13{,}000 \times g$ for 15 min at 4°C. The supernatants were used for immunoblot analysis. Protein concentrations were determined using the Bradford method, and the protein samples were stored at −80°C. Protein samples were resuspended in loading buffer containing Tris–HCl (250 mmol/l), sucrose (200 mmol/l), DTT (300 mmol/l), 0.01% Coomassie brilliant blue-G, and 8% SDS, pH 6.8, and denatured at 95°C for 5 min. Equivalent amounts of protein (40 μg) were separated using 10% SDS–polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. Membranes were blocked with 5% fat-free milk solution overnight at 4°C. Samples were probed with a 1 : 200 dilution of goat polyclonal antibodies against ERK1/2 and p-ERK1/2 (Santa-Cruz Biotechnology, Inc., Santa Cruz, USA) and a 1 : 5,000 dilution of rabbit anti-goat HRP-conjugated IgG (Huamei Chemical Corp., Shanghai, China). A rat monoclonal antibody against β-actin (Sigma, St Louis, USA) was used as a loading control. Protein bands were detected using an ECL kit (Pufei Chemical Corp., Shanghai, China). Scanning densitometry was used for semiquantitative analysis of the data.

Statistical analysis

All data were presented as mean ± SD. Data from the immunoblot and RT-PCR studies were analyzed using a one-way analysis of variance (ANOVA) followed by *post hoc* Dunnett testing. Data from the nociceptive tests were analyzed using a two-way ANOVA to analyze differences between groups at different time points, whereas repeated-measures ANOVA was used to evaluate the efficiency of treatment at different time points within groups; *pos hoc* Bonferroni's test was used to detect significant differences for both ANOVA analyses. A value of $P < 0.05$ was considered a significant difference.

Results and Discussion

Inhibition of the P2Y1 receptor attenuated nociceptive behavior in a CIBP model

As shown in **Fig. 1**, there were no significant differences in the overall mean baseline PWL to tactile allodynia or in the ambulatory score to spontaneous pain among the normal, sham, and CIBP groups ($P > 0.05$). Seven days after inoculation, there was a distinct reduction in the PWL in >56% of the CIBP group rats relative to the sham control group rats [**Fig. 1(A)**, $P < 0.01$]. Meanwhile, the ambulatory score progressively increased in the CIBP group [**Fig. 1(B)**, $P < 0.05$]. The median PWL to allodynia and the ambulatory score to spontaneous pain of the inoculated paw in CIBP rats differed significantly from the median PWL and ambulatory score in sham controls from day 6 through day 18 post-inoculation. In contrast, tactile allodynia and spontaneous pain in CIBP rats treated with the P2Y1 purinoceptor antagonist MRS2179 for 4 h were attenuated by 45.8% and 39.2%, respectively, relative to CIBP rats injected with vehicle ($P < 0.01$). This effect also recurred on day 9 of treatment following intrathecal injection of MRS2179 [**Fig. 1(A,B)**].

P2Y1R mediates ERK activation which is involved in CIBP

As shown in **Fig. 2(A)**, *P2Y1R* mRNA levels in the L4–5 lumbar spinal cord and DRG significantly increased in the CIBP group rats inoculated with Walker 256 cells for 9 days relative to the sham group ($P < 0.01$). In contrast, *P2Y1R* mRNA levels in the L4–5 lumbar spinal horn cord and DRG from the MRS2179-treated CIBP group (137.50% for spinal horn cord and 115.15% for DRG) were significantly lower than those of the vehicle-treated CIBP group (195.82% for spinal horn cord and 279.29%

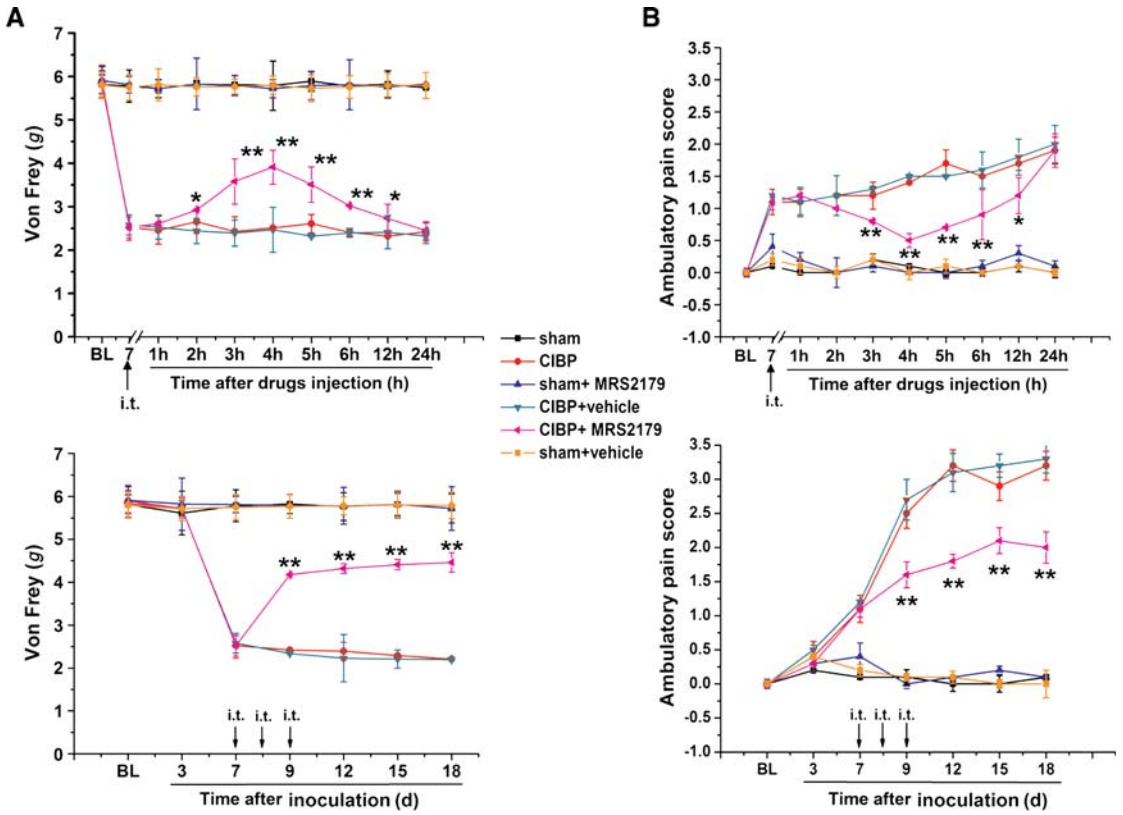

Figure 1 Inhibition of P2Y1 receptor attenuated nociception transmission in a rat model of CIBP Rats in CIBP and sham groups ($n = 10$ per group) were injected (intrathecal) with or without MRS2179 (100 pmol/10 μl) at the indicated times. (A) Tactile allodynia was assessed using von Frey filaments. The PWT was determined by sequentially increasing and decreasing the stimulus strength (gravity, *g*). (B) Spontaneous pain was evaluated by ambulatory score. The scoring of each animal was characterized as described in the Materials and Methods. The arrows represent intrathecal injection time points. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. CIBP + vehicle.

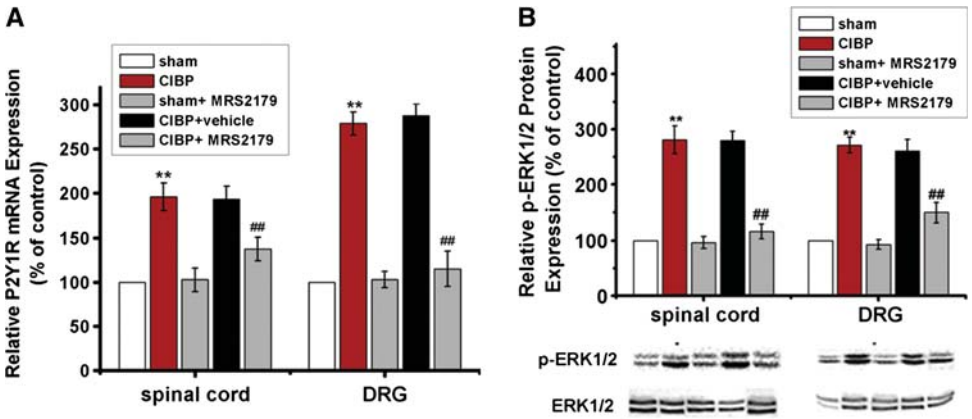

Figure 2 Inhibition of P2Y1R-mediated ERK1/2-signaling pathway Rats in the CIBP and sham groups ($n = 4$ for each group) were injected (intrathecal) with MRS2179 (100 pmol/10 μl) or vehicle on days 9 post-inoculation. (A) The temporal expression levels of P2Y1R mRNA in rat lumbar spinal horn cord or DRG (L4–5) were determined by real-time quantitative RT-PCR analysis. (B) Phosphorylated ERK1/2 protein levels were determined in rat spinal horn cord and DRG by immunoblot analysis. Total-ERK1/2 protein expression levels were used to ensure equal protein loading. Values are presented as mean ± SD. $^{**}P < 0.01$ vs. sham group; $^{\#\#}P < 0.01$ vs. vehicle-treated CIBP group.

for DRG) ($P < 0.01$). To further investigate whether ERK1/2 activation was involved in P2Y1R-mediated bone cancer pain, we investigated the effects of MRS2179 on the level of ERK1/2 phosphorylation in the CIBP rat model. The results of these experiments showed that inoculation of Walker 256 cells in the tibia of CIBP group rats for 9 days resulted in a 189.40% increase in the levels of ERK1/2 phosphorylation relative to the sham control group ($P < 0.01$) [**Fig. 2(B)**]. Of note, MRS2179 treatment of the CIBP group for 4 h resulted in a significant decrease

in ERK1/2 phosphorylation ($n = 4$, $P < 0.01$) relative to vehicle-treated CIBP group ($P < 0.05$) [**Fig. 2(B)**]. These data suggested that the inhibition of P2Y1R-mediated ERK1/2 signaling attenuates CIBP.

This study demonstrates a key role for the P2Y1 receptor in the induction of ERK1/2 activation and the maintenance of tactile allodynia and spontaneous pain in a rat model of CIBP. Our findings support the hypothesis that the P2Y1R-mediated ERK1/2 signaling participates in CIBP pathogenesis.

Pain is one of the most frequent and disruptive symptoms experienced by bone metastasis patients. A distinct feature of bone cancer pain is that the chronic pain state may be driven simultaneously by inflammation, tumor-released products, and tumor-induced injury to primary afferent neurons [19]. In this study, we also confirmed that the female rat model of bone pain from metastatic bone cancer can closely mimic the human disease [18,20]. Our previous studies [18] on pain behavior have shown that the time window comprising days 6–18 post-inoculation is a reasonable period in which one can investigate the mechanisms of bone cancer pain and the effects of analgesic drugs on that pain. The results of the current study demonstrated that female Sprague–Dawley rats experienced progressive tactile allodynia and spontaneous pain 7 days after being inoculation with Walker 256 cells in the tibia. These findings accord with the fact that patients with various kinds of bone cancer report progressive suffering from spontaneous and evoked pain at the end of their lives.

A growing body of evidence indicates that extracellular nucleotides play important roles in the regulation of the pain state in the nervous system via P2 purinoceptors [21]. P2 purinoceptors are divided into two families: ionotropic receptors (P2X) and metabotropic receptors (P2Y). P2Y receptors, which include eight types (i.e. P2Y1, 2, 4, 6, 11, 12, 13, and 14), are activated by nucleotides and coupled to intracellular second messenger systems through heterotrimeric G proteins. Studies have shown that microglia in the CNS express several subtypes of P2X and P2Y receptors, and that these receptors play a key role in pain signaling in the spinal cord under pathological conditions, such as peripheral nerve injury and inflammatory pain [11].

Different subtypes of P2Y receptors can couple G proteins, leading to the activation of signal transduction pathways and stimulation of specific physiological functions. Endogenous ATP, ADP, and UTP are the main subtype P2Y1 agonists. However, little is known regarding the potential regulatory role of P2Y1 in spinal cord pain modulation. Experimental evidence suggests that the P2Y1 receptor may regulate mechanical and thermal sensitivity and pain modulation in primary sensory neurons [21]. MRS2179 is the strongest antagonist of the P2Y1 receptor [12,22]. In this study, intrathecal injection of MRS2179 resulted in a significant increase in the von Frey threshold in CIBP group rats, suggesting that the P2Y1 receptor may play an important role in bone cancer pain. Under our experimental conditions, the P2Y1 receptor subtype was, in part, responsible for the change in pain-associated behavior. This finding was supported by our finding that MRS2179 treatment reversed both tactile allodynia and spontaneous pain in CIBP group rats.

As has been reported in [23], *in vitro* ERK activation in dorsal horn and DRG neurons, microglia, and astrocytes mediates pain in the temporal evolution of neuropathic pain. The results of this study showed that p-ERK1/2 protein expression was significantly reduced 7–18 days after intrathecal injection of MRS2179, suggesting that the P2Y1 receptor may mediate bone cancer pain through the ERK pathway in the spinal cord horn and DRG. Of note, our findings also suggest that ERK1/2 activation in the spinal cord horn and DRG participates in the development of hyperalgesia at different stages in a CIBP rat model. Inhibition of ERK1/2 activity may explain, at least in part, why intrathecal injection of the P2Y1R antagonist MRS2179 attenuated the expression of *P2Y1R* mRNA, the mechanism of which will require further studies to resolve.

In this report, treatment with a P2Y1R inhibitor prevented bone cancer-induced tactile allodynia and spontaneous pain at an early stage of tumor growth. However, at a later stage, the P2Y1R inhibitor did not completely block the well-established bone cancer pain. This result not only illustrates that P2Y1 is one of the main mediators of bone cancer pain, but also suggests that other receptors may play a role in the maintenance of the pain state. Candidates include P2X3, bradykinin, prostaglandin receptors, acid-sensing ion channel 3 and voltage-gated sodium channels, and TLR4 signals in glia [24,25]. Thus, our current results underscore the complexity of the CNS cascades and mediators that may underlie neuronal sensitization, the pathological manifestation of cancer pain.

In summary, the results of the current provide evidence that P2Y1 receptor signaling in the spinal horn cord and DRG may mediate bone cancer pain through the ERK pathway, thereby participating in the pathogenesis of CIBP. These findings have increased our understanding of the molecular mechanisms underlying pathological pain and have uncovered the P2Y1 receptor as a potential therapeutic target for CIBP.

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