Antinociceptive curcuminoid, KMS4034, effects on inflammatory and neuropathic pain likely via modulating TRPV1 in mice

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Editor’s key points

- Curcumin is a good analgesic and anti-inflammatory agent but has poor bioavailability.
- A novel curcuminoid KMS4034 was synthesized.
- Bioavailability was much better than curcumin.
- KMS4034 was an analgesic in various measures of behavioural pain.
- KMS4034 may be an effective analgesic.

Background. Curcumin, the active ingredient of turmeric (Curcuma longa), has a wide range of beneficial effects including anti-inflammation and analgesia. However, poor bioavailability of curcumin hinders its clinical application. To overcome this limitation, we modified the structure of curcumin and synthesized new derivatives with favourable pharmacokinetic profiles. Recently, curcumin has been shown to have an antagonizing effect on transient receptor potential vanilloid type 1 (TRPV1) ion channels. We investigated the antinociceptive activity of KMS4034 which had the most favourable pharmacokinetics among the tested curcumin derivatives.

Methods. To evaluate the mechanism of the antinociceptive effects of KMS4034, capsaicin (I_CAP) and heat (I_heat)-induced currents in TRPV1 expressing HEK293 cells were observed after the application of KMS4034. Nociceptive behavioural measurement using the hot-plate test, formalin test, and chronic constriction injury (CCI) model were evaluated in mice. Also, calcitonin gene-related peptide (CGRP) was stained immunohistochemically in the L4/5 dorsal horns in mice with neuropathic pain.

Results. I_CAP (P < 0.01) and I_heat (P < 0.05) of TRPV1 were significantly blocked by 10 μM KMS4034. Behaviourally, noticeable antinociceptive effects after 10 mg kg⁻¹ of KMS4034 treatment were observed in the first (P < 0.05) and second phases (P < 0.05) of the formalin and hot-plate tests. The mechanical threshold of CCI mice treated with 10 mg kg⁻¹ KMS4034 was significantly increased compared with control. Immunohistochemical CGRP expression was decreased in the lamina I–II of the lumbar dorsal horns in KMS4034-treated CCI mice compared with the control (P < 0.05).

Conclusions. KMS4034 may be an effective analgesic for various pain conditions.

Keywords: calcitonin gene-related peptide, curcuminoid synthesis, pain; inflammatory; neuropathic, TRPV1 receptor; nociceptive

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Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is an organic extract from the rhizome of the Indian spice turmeric (Curcuma longa). Curcumin has anti-inflammatory, 1, 2 antioxidant, 3 anticarcinogenic, 4 and antibiotic effects. 5 Curcumin is also antinociceptive for formalin-induced orofacial pain in rats 6 and diabetic neuropathic pain in mice. 7 Moreover, curcumin is very safe even at very high doses in humans. 8 Despite its therapeutic potential, curcumin has limitations as a drug because of its poor bioavailability with low serum levels.

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and rapid metabolism. To use curcumin as a drug, we synthesized curcuminoids, which are physicochemically more stable. Among the tested curcuminoids KMS4034 had the most favourable pharmacokinetics. Recently, curcumin has been found to have antagonizing effects on transient receptor potential vanilloid type 1 (TRPV1) ion channels. Since KMS4034 has a similar structure to curcumin it may also have effects on TRPV1.

To evaluate KMS4034 as a potential analgesic in mice, we used the formalin test, the hot-plate test and chronic constriction injury (CCI). We also investigated the effects of KMS4034 on TRPV1 ion channel function and the immunohistochemical expression of calcitonin gene-related peptide (CGRP) as an indirect molecular pain marker in the fourth and fifth lumbar spinal dorsal horns of the CCI mice.

Methods

The synthesis of KMS4034, structural stability, and the patch clamping experiment

The detailed methodology is described in the online supple-mental methods.

Animals

All experiments were performed on male ICR mice (Orient Bio, Inc., Seoul, Korea) weighing 30–35 g. Animals were randomly assigned to each group using a table of random numbers. The mice were housed 5 per cage and allowed free access to food and water. All experimental procedures were performed according to the ethical guidelines for the use of animals in research of International Association for the Study of Pain and Institutional Animal Care and Use Committee of Seoul National University. The mice were acclimatized for at least 3 days before any behavioural tests. Investigators were blinded to experimental groups when undertaking behavioural tests.

Formalin test

The formalin test was performed to determine the effect of KMS4034 on inflammatory pain responses. The mice were randomly assigned to receive 100 mg kg⁻¹ of gabapentin i.p. as a positive control, KMS4034 (0.1, 1, and 10 mg kg⁻¹) i.p. or vehicle control 30 min before formalin injection. All mice received an intraplantar injection of formalin (5% in distilled water) in the left hind paw. The duration of paw flinches, licking, and biting were recorded for 0–5 min after injection of formalin (first phase) and between 20 and 40 min (second phase). To evaluate KMS4034 as a potential analgesic in mice, we used the formalin test, the hot-plate test and chronic constriction injury (CCI). We also investigated the effects of KMS4034 on TRPV1 ion channel function and the immunohistochemical expression of calcitonin gene-related peptide (CGRP) as an indirect molecular pain marker in the fourth and fifth lumbar spinal dorsal horns of the CCI mice.

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Hot-plate test

The antinociceptive effect of KMS4034 on noxious thermal stimuli was determined by the hot-plate paw-shaking test. Mice received either KMS4034 (10 mg kg⁻¹) i.p. or vehicle control. A hot plate was electrically heated to 53.0 (0.1) °C. The latency for licking or shaking the hind paws or jumping off was measured. The hot-plate test was performed before and at different time points after injection (15, 30, 45, and 60 min). To avoid tissue damage, the maximum cut-off time was set to 30 s. These experiments were repeated three times at each time point.

CCl model

To assess mechanical allodynia, von Frey monofilaments (Stoelting Co., Wood Dale, IL, USA) were applied to the plantar surface of the left hind paw. The CCI model was established as previously described. Briefly, the animals were anaesthetized using facemask inhalation of 2 – 3% isoflurane in oxygen. Adequacy of anaesthesia was ascertained by the pedal withdrawal response to a noxious stimulus. The common sciatic nerve of the left hind limb was exposed and loosely ligated four times with 6–0 chromic gut (W812, Ethicon, Inc., Somerville, NJ, USA). The rescue analgesia protocol was tramadol and administration of it was not required in any animal.

At least 30 min after habituation, the von Frey monofilaments were applied perpendicular to the whole plantar surface in the CCI mice. Two weeks after the injury, baseline responses were assessed before KMS4034 injection (time point 0). The von Frey test was re-evaluated 30, 60, 90, and 120 min after injection.

Immunohistochemistry

Immunohistochemical staining for CGRP at the lamina I–II of the L4/5 dorsal horns was performed as previously described. Briefly, all mice were perfused intracardially with 4% paraformaldehyde under deep anaesthesia with isoflurane. The L4/5 spinal cord segments were dissected from neuropathic mice treated with 10 mg kg⁻¹ of KMS4034 or vehicle control. Immunohistochemical stainings for CGRP were performed with primary antibodies specific for rabbit-anti-CGRP (1:10,000, Calbiochem, Billerica, MA, USA), Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA), and SIGMA FAST DAB kit (Sigma Chemical Co., St Louis, MO, USA). CGRP expression patterns were analysed by an observer, blinded to the experimental group, using Image-Pro Plus 6.2 software (Media Cybernetics, Inc., Bethesda, MD, USA). Positively stained areas were measured at the lamina I–II and the average of sections for each mouse was recorded.

Data analyses

Statistical analysis was performed using SPSS (version 12.0, SPSS, Inc., Chicago, IL, USA). Inhibitory effect of KMS4034 on I°CAP and I°heat in TRPV1-expressing HEK293 cells was compared using the paired t-test. Antinociceptive effects of KMS4034 on formalin-induced inflammatory pain was analysed using Kruskal–Wallis test followed by Dunn’s multiple comparisons test. Statistical analysis of the hot-plate test and CCI model test were performed using Mann–Whitney U-test. The unpaired t-test was used for comparison of the results of immunohistochemistry. All data are expressed as median, interquartile, and full range or mean (SD) as appropriate. P < 0.05 was considered statistically significant.

Results

Plasma stability and pharmacokinetics of KMS4034

KMS4034 was constantly stable in the rat plasma for 120 min. At each time point, the concentrations were (15%) compared with initial concentration of KMS4034.
The mean plasma concentration of i.v. administered KMS4034 is shown in Supplemental Figure 2 and pharmacokinetic parameters are shown in Supplemental Table 1. KMS4034 was detected in the rat plasma for 4 h after the treatment. The mean values of area under the concentration–time curve (AUC) and total body clearance (CL) were 219 μg min⁻¹ ml⁻¹ and 48.1 ml min⁻¹ kg⁻¹, respectively. The amount of i.v. KMS4034 excreted in 24 h urine in the unchanged form (Ae₀–24 h) was only 0.15%, and the contribution of time-averaged renal clearance (CLr) to CL of KMS4034 was nearly insignificant. KMS4034 was eliminated mainly by in vivo metabolism.

KMS4034 inhibits I\_CAP and I\_heat in TRPV1-expressing HEK293 cells

We tested whether KMS4034 inhibits I\_CAP in TRPV1-expressing HEK293 cells. To prevent tachyphylaxis produced by repetitive capsaicin application that results from Ca\(^{2+}\) influx through TRPV1, we used the extracellular Ca\(^{2+}\)-free condition. By this approach, we could obtain reproducible I\_CAP by sequential applications of 200 nM capsaicin for 3 s at 100 s intervals with little desensitization (Supplemental Fig. 3A). Pretreatment with KMS4034 (10 μM, 200 s) blocked I\_CAP to 21.1 (7.1)% (n=6, P<0.01 vs vehicle) significantly, and I\_CAP recovered to the control level [90.8 (7.1)%, P<0.01 vs KMS4034] after 10 min washout period (Supplemental Fig. 3B). Next, we tested the effect of KMS4034 on heat (≏45°C, 5 s)-induced currents (Supplemental Fig. 3C and D). KMS4034 also significantly blocked I\_heat [29.5 (6.5)%, n=4, P<0.05 vs vehicle].

Antinociceptive effects of KMS4034

In the formalin test the control group produced a typical nociceptive response in both first and second phases. The licking time of the first phase was decreased when the mice were treated with 1 (P<0.01) or 10 (P<0.05) mg kg⁻¹ of KMS4034 (Fig. 1A). In the second phase, the total licking and flinching times were significantly decreased by gabapentin (P<0.05) and 10 mg kg⁻¹ of KMS4034 (P<0.05) (Fig. 1B). KMS4034-injected mice showed increased paw withdrawal latency.
time against noxious thermal stimuli, while the control group did not show any behavioural changes over time. The maximal paw withdrawal latency effect of KMS4034 (P < 0.01) was obtained at 30 min after KMS4034 injection (Fig. 1c). The mechanical threshold of CCI treated mice was decreased compared with the baseline mechanical threshold of unoperated mice. The mechanical threshold was significantly increased at 60 min after KMS4034 treatment (P < 0.01) and decreased thereafter (Fig. 1o).

KMS4034 decreases CGRP expression in the dorsal horn of CCI neuropathic mice

We performed immunohistochemical analyses to observe CGRP expression in the ipsilateral L4/5 spinal cord of vehicle-(Fig. 2a) or KMS4034-treated group (Fig. 2b). To quantify the expression of CGRP in the lamina I–II of the L4/5 spinal cord, we measured the percentage of positively stained areas (Fig. 2c). CGRP expression in CCI neuropathic mice was higher in the control group than those in the KMS4034-treated group (P < 0.05 vs control).

Discussion

In this study, KMS4034 showed antinociception against noxious thermal, inflammatory, and neuropathic pain in mice. In addition, KMS4034 decreased CGRP protein expression in the lamina I–II of the L4/5 spinal cord.

The AUC of intravenously administered 10 mg kg\(^{-1}\) of KMS4034 was ~30 times greater than that of the same dose of curcumin.\(^{18}\) The elimination half-life (T\(_{1/2}\)) of curcumin was 28.1 (5.6) min, whereas T\(_{1/2}\) of KMS4034 was 69.8 (21.7) min. Thus, KMS4034 is more bioavailable and remains longer in the circulation than curcumin.

TRPV1, a ligand-gated Ca\(^{2+}\)-permeable ion channel, is involved in nociceptive signalling and is a therapeutic target in pain management.\(^{19, 20}\) Pain from TRPV1 activation can occur as a result of non-selective stimuli such as heat, protons, and capsaicin.\(^{21-23}\) TRPV1 sensitization is crucial in the development of inflammatory pain.\(^{24}\) Upregulation of TRPV1 is also related to phenotypic changes seen in neuropathic pain.\(^{25}\) TRPV1 is, therefore, a promising target for developing analgesics.\(^{26}\) Indeed TRPV1 antagonists potentiated the antinociceptive effects of morphine and attenuated the development of tolerance to the antinociceptive effect of morphine.\(^{27, 28}\) Recently, we reported that curcumin which is similar to capsaicin in chemical structure, acted via TRPV1.\(^{10}\) This observation led us to hypothesize that the curcuminoid KMS4034 may also exert antinociceptive effects via modulation of TRPV1. Indeed, KMS4034 inhibited I\(_{\text{CAP}}\) and I\(_{\text{heat}}\) of TRPV1 in this study. These differing results between KMS4034 and curcumin\(^{10}\) may be explained by the differing structures. Structurally, the main functional groups of curcumin have a phenolic aromatic ring and two \(\alpha,\beta\)-unsaturated ketone groups existing in two tautomeric forms, keto or enol. The enol form is more energetically stable not only in the solid phase but in solution. Further studies to determine the functional groups of KMS4034 modulating TRPV1 are needed.

The formalin test was performed to investigate the hypothesis that KMS4034 has antinociceptive effects on inflammatory pain. In the formalin test, there are two distinct phases of

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**Fig 2** Immunohistochemical analysis of CGRP expression in the lamina I–II of the L4/5 spinal cord in the neuropathic pain model. After establishment of the neuropathic CCI model, the mice were treated with vehicle (a) or 10 mg kg\(^{-1}\) KMS4034 (a). (c) The statistical summary of the immunohistochemically positive stained area (*P < 0.05 vs vehicle, Mann–Whitney test). Data are shown as median, interquartile, and full range.
nociceptive behaviours, based on different noxious mechanisms. The first phase, which begins immediately after formalin injection and is sustained for ~5 min, is caused by C-fibre activation by peripheral stimuli. The second phase, which begins 15–20 min after injection, and is sustained for 20–40 min, reflects the inflammatory pain response.

Our results showed that KMS4034 significantly decreased pain-related behaviours in both phases of the formalin test. Interestingly, the antinociceptive effect of 10 mg kg⁻¹ of KMS4034 was comparable with that of 100 mg kg⁻¹ gabapentin.

A previous study suggested correlation between the second phase of the formalin test and the CCI neuropathic pain model. Consistent with that report, we found that KMS4034 was also antinociceptive in the CCI model in our study. The hot-plate test is useful for evaluating acute pain. We observed an increase in withdrawal latency compared with the baseline after KMS4034-treatment to noxious heat stimuli, which was probably mediated by inhibiting the heat-gated ion channel TRPV1. Peripheral nerve injury can generate neuropathic pain characterized by spontaneous pain, mechanical allodynia, and thermal hyperalgesia. Mechanical allodynia is easier to observe than thermal hyperalgesia and is more common in neuropathic pain. The peripheral nerve injury model used here mimicks causalgia in humans. After KMS4034 treatment, the mean mechanical threshold was increased in the CCI model, suggesting that KMS4034 may be effective against neuropathic pain.

CGRP, a 37-amino acid peptide, is distributed in both the central and peripheral nervous system. CGRP increases the excitability of neuronal cells and facilitates synaptic plasticity with postsynaptic action in the spinal dorsal horn. The level of CGRP is related to nociceptive transmission and excitation of postsynaptic neurones in the spinal dorsal horn. Intra-thecal injection of CGRP produces hyperalgesia in rats. In the neuropathic pain state, CGRP is increased in the spinal cord, which may be related to the development and maintenance of nerve injury-induced pain. TRPV1 ion channels are located in the peripheral nociceptive sensory fibres containing neurotransmitters such as CGRP and substance P. Several papers have recently reported a close linkage between TRPV1 activity and neuropeptide release. To assess the effects of KMS4034 on neuromodulation, we evaluated the expression levels of CGRP in the spinal dorsal horn after KMS4034 treatment. The expression of CGRP was decreased in both CCI neuropathic pain models. This implies that KMS4034 inhibits the expression of CGRP, a peptidergic neurotransmitter, in the spinal cord and ultimately decreases pain transmission. KMS4034 may produce antinociceptive effects by altering the transmission of nociceptive signals.

In conclusion, a novel curcuminoid KMS4034 has been shown to have a better pharmacokinetic profile compared with curcumin, and is able to attenuate noxious, thermal and inflammatory pain. Moreover, antinociceptive effects of KMS4034 were seen in the mechanical allodynia observed in a CCI model. KMS4034, therefore, may be an effective analgesic for various pain conditions.

Supplementary material
Supplementary material is available at British Journal of Anaesthesia online.

Authors’ contributions
J.Y.L. wrote a first draft of the paper; T.J.S. elaborated the first draft and corrected the paper according to the reviewers’ comments; H.J.K. designed the study; K.S.S., J.M.C., and T.G.Y. analysed and interpreted the behavioural data; Y.S.L. and H.H. synthesized KMS4034; H.J.C. tested its structural stability and performed pharmacokinetic analysis; Y.O. and S.J.J. performed the electrophysiological experiments; K.J.S. was in charge of synthesis of KMS4034 and gave valuable comments about pharmacological aspects of KMS4034.

Declaration of interest
None declared.

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