ORIGINAL ARTICLE

The Antipruritic Effect of 2,6-bis-(4-hydroxy-3 methoxybenylidene)-cyclohexanone (BHMC) in a Mouse Model of Induced Pruritus

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ABSTRACT

Introduction: Itch, an uncomfortable sensation leading to the urge to scratch, is often inadequately managed by conventional antihistamine drugs, which can be ineffective in certain pruritic conditions and cause undesirable side effects. Therefore, there is a need to identify new pharmacologically potent antipruritic compounds with fewer side effects. A synthetic curcuminoid analogue, 2,6-bis-(4-hydroxy-3-methoxybenzylidene)-cyclohexanone (BHMC), a derivative of curcumin - a bioactive compound found in turmeric - has demonstrated various pharmacological activities. Previous studies have shown that BHMC possesses antinociceptive and anti-inflammatory properties. This study aimed to investigate the antipruritic effects of BHMC in mice models of induced pruritus. **Materials and Methods:** The pruritus in mice was induced using compound 48/80, substance P, histamine, and serotonin to establish an itch-induced mouse model. BHMC was administered intraperitoneally (i.p.) at doses of 0.1, 0.3, and 1.0 mg/kg. **Results:** BHMC significantly reduced pruriceptive responses in all models tested and notably inhibited compound 48/80 and substance P-induced mast cell degranulation in skin tissues. **Conclusions:** These findings suggest that BHMC inhibits pruriceptive responses in mice, likely through the inhibition of mast cell degranulation and/or direct antagonism of peripheral histamine and serotonin receptors. This may warrant further exploration of the antipruritic effect of BHMC in clinical trials for the betterment of animal and human health.
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Keywords: Itch, Curcuminoid analogue, Mast cell degranulation, Histamine, Good health and wellbeing

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INTRODUCTION

Itch, also known as pruritus, is defined as "an unpleasant sensation that elicits the desire or reflex to scratch" with its pathophysiology is closely linked to pain and inflammation (1). For many years, pharmacological research has focused primarily on pain and inflammation, which are prominent in diseases such as cancer, arthritis, lupus, multiple sclerosis, etc. However, many details about the mechanisms underlying itch remain unknown, largely due to a relative scarcity of research in this area. This gap in knowledge has hindered the effective management of pruritus, with treatments often proving ineffective in certain cases. Itch is frequently mistaken for mild pain due to their similarities (2, 3). Both sensations share common chemical mediators and similar signal transduction pathways (2). Chemical mediators involved in inflammation, pain, and itch include serotonin, bradykinin, substance P, acetylcholine, histamine, etc. (4). Like pain, peripheral nerve fibers transmitting itch also form synapses with secondary neurons in the dorsal horn of the spinal cord (5). Pain and itch signals then ascend through the spinothalamic tract and project to the somatosensory region in the cerebral cortex (5, 6). Despite these similarities, recent studies have established that itch and pain are distinct sensations (6, 7). For example, while opioids are effective analgesics for pain, their use paradoxically enhances itch sensation in the central nervous system (1).

Antihistamines are the most commonly used

conventional antipruritic agents, as histamine is a key chemical mediator in itch (8, 9). However, antihistamines are not effective in some pruritus diseases, particularly chronic pruritus, and can lead to undesirable adverse effects such as drowsiness, nausea, and dry mouth (10). Additionally, systemic and topical corticosteroids are employed to treat pruritus diseases, mostly in chronic cases that respond weakly to antihistamines, such as psoriasis, atopic dermatitis, and contact dermatitis (11). However, prolonged usage of systemic corticosteroids—whether orally or by injection—could lead to severe adverse effects, including osteoporosis, adrenal suppression, growth suppression, immunosuppression, etc. Long-term treatment using topical corticosteroids could also cause striae, ulceration, and increased risks of changes in skin pigmentation and varying degrees of skin atrophy (12). Therefore, there is a pressing need to search for novel treatments with higher efficacy and fewer side effects.

2,6-bis-(4-hydroxy-3-methoxybenzylidene)-cyclohexanone (BHMC) is a synthetic curcuminoid derived from curcumin, which previous reported to exhibit potential antinociceptive (13, 14) and anti-inflammatory (15, 16) properties. BHMC in previous in vitro studies has shown to suppress various pro-inflammatory mediators, including monocyte chemoattractant protein-1 (MCP-1) (15), which is one of the chemoattractants for mast cells, monocytes, and T-cells (17). BHMC has also been shown to suppress several mediators released by mast cells after being activated, such as IL-6 and TNF-α (15). In the same study, BHMC also demonstrated suppression of inducible nitric oxide synthase (iNOS), an important enzyme in synthesizing the neurotransmitter nitric oxide (NO), which plays a crucial role in nociception, inflammation, and itch (15, 18). Previous in vivo studies have also shown that BHMC exhibited antinociceptive properties in chemical and thermal-induced nociception models (13, 14). As pruriception is closely related to nociception and inflammation in signal transmission and chemical mediators, this study aims to investigate the potential ameliorative effects of BHMC in mouse models of induced pruritus. We employed chemical-induced itch models in mice as the main study model. Subcutaneous (s.c.) injection of Compound 48/80, substance P, histamine and serotonin at relatively low concentrations are known to elicit pruritus responses in mice, as they either cause rapid mast cell degranulation or directly activate the receptors that transmits the sensation of itch (19-21). Conventional anti-itch medications, eg. oxatomide. azelastine, and cimetidine (antihistamines) and ketanserin (5-HT2 antagonist) were used as reference drug, where applicable.

MATERIALS AND METHODS

Animals

The experiments reported in this study were approved by the Animal Care Unit Committee, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (ACUC_UPM/FSPK/PADS/UUH/F01). Male ICR mice, 6-8 weeks old (25–35 g), were used throughout the study. Mice were housed in the animal facility of the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, under a 12-hour light/dark cycle and provided with standard pellet food and drinking water ad libitum. Each animal was used only once throughout the experiment. All animals were acclimatized to the animal facility for at least one week before experimentation.

Drugs

The following reagents and drugs were used in the present experiment: substance P, oxatomide and azelastine were purchase from Santa Cruz Biotechnology, Inc. (Texas, USA), formalin, Tween 20, absolute ethanol (100%), toluidine blue, compound 48/80, histamine, serotonin, loratadine, ketanserine, DPX mountant and xylene were purchase from Sigma-Aldrich Chemicals Co. (St. Louis, MO, USA). BHMC was chemically synthesized at the Laboratory of Natural Products Discovery, Institute of Bioscience, Universiti Putra Malaysia, using the same protocols as previously reported (15). The structure was determined by 1H NMR and mass spectrometry with a purity of more than 99.0% as determined by HPLC. Compound 48/80, histamine, serotonin, cimetidine and ketanserin were dissolve in physiological saline (0.9% NaCl). Substance P was dissolve in PBS solution. BHMC and oxatomide were dissolved in vehicle (absolute ethanol: Tween 20 in saline; 5:5%; v/v) to the desired concentration while azelastine is suspended in 0.5% (w/v) carboxymethyl cellulose sodium salt solution. All drugs and BHMC were prepared freshly before the experiments. BHMC in vehicle or vehicle alone (control) were administered intraperitoneally (i.p.) 30 min prior to pruriceptive testing. The vehicle used alone had no effects on the pruriceptive responses in mice.

Experimental Design

A total of 120 male adult ICR mice were used in this study. They were distributed into 4 experimental groups (30 mice each) and further disseminated randomly into 5 treatment groups (n =6), namely control group, BHMC treated groups (0.1, 0.3 or 1.0 mg/kg) and reference drug group. The 4 experimental groups are: 1) compound 48/80-, 2) substance P-, 3) histamine- and 4) serotonin-induced scratching test group.

Assessment of the antipruritic property of BHMC

Behavioural observation. Behavioural observation was conducted as previously described with minor modifications . On the day of the experiment, the mice were first acclimatized for one hour in observation cages (20 cm x 20 cm x 26.5 cm) prior to testing. Following acclimatization, mice were treated with an i.p. injection of BHMC at doses of 0.1, 0.3, or 1.0 mg/kg, similar dose which it exhibited antinociceptive (13, 14) and anti-inflammatory effects (16) in vivo. Control mice received an equivalent volume of vehicle (10 ml/kg). After 30

minutes of treatment, mice received a subcutaneous (s.c.) injection of compound 48/80 (100 µg), substance P (150 nmol), histamine (100 nmol), or serotonin (100 nmol) 50 µl/site into the rostral back region as per previously described (22) with minor modification. Control mice received the same volume of saline (0.9%). Each mouse was gently placed into the observation chamber immediately after injection and videotaped for 30 minutes. The time spent scratching the rostral back region using the hind paw was evaluated during video playback. Pruriceptive activity of the mediators was indicated by the time spent scratching the rostral back region.

Assessment of Mast Cell Degranulation

Three days before the experiment, the hair at the rostral back area of each mouse was shaved. On the day of the experiment, mice were treated with i.p. injection of BHMC at the doses of 0.1, 0.3, 1.0 mg/kg (10 ml/kg). Control mice received similar volume of vehicle (10 ml/kg). After 30 minutes of treatment, mice received subcutaneous injection of compound 48/80 (100 µg/50 µl) or substance P (150 nmol/50 µl) into the rostral back area. The mice were rapidly sacrificed via cervical disclocation after 10 mins. The subcutaneous tissue of the rostral back area of the mice were surgically isolated with an area of approximately 1.5 x 1.5 cm2 and fixed in 10% formalin for at least 24h before proceed to embedding and sectioning. Tissues were attached to microscope slides and stained with 0.1% toluidine blue. After staining, slides were mounted with DPX mountant and covered with cover slips. Slides were observed under light microscope (DM 2500; Leica, Germany) with camera (DFC420 C; Leica, Germany). The counting of intact and degranulated mast cells was performed in five fields of each slide and the result expressed as the percentage of degranulated mast cells (23).

Statistical analysis

All data is represented as Mean ± S.E.M.. Statistical analysis was done via one-way ANOVA followed by Dunnett's post hoc test. The difference between means of the treated and a control group was considered significant at $p < 0.05$.

RESULTS

Compound 48/80-induced scratching

Negative control group mice that received s.c. compound 48/80 had spent 100.69 ± 6.29 sec scratching their rostral back. Pretreatment of 0.1, 0.3 and 1.0 mg/kg of BHMC significantly reduced the time spent scratching to 64.94 ± 5.50 sec, 52.20 ± 2.13 sec, and 36.03 ± 2.13 sec, respectively. The reference drug, oxatomide (10 mg/kg i.p.), also showed significant suppression compared to the control group (Fig. 1).

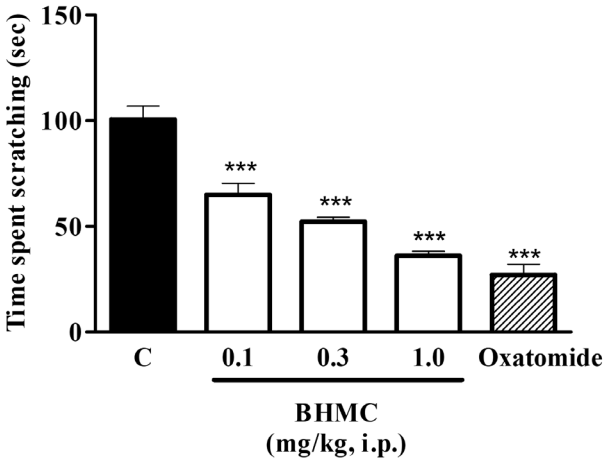

Fig. 1 : Effect of BHMC in Compound 48/80-induced scratching test in mice. Each column represents the mean ± S.E.M. (n=6/group). The mice were pre-treated with vehicle (C, control), oxatomide (10 mg/kg, i.p.) or BHMC (0.1, 0.3 and 1.0 mg/kg, i.p.) 30 minutes before injection of compound 48/80 (100 µg/50 µl, s.c.) into rostral back area of mice. Then the mice were observed for 30 minutes Statistical analysis was determined by one-way ANOVA followed by Dunnett's post hoc test. The asterisks denote the significance levels as compared to control, *** $p< 0.001$.

Substance P-induced scratching.

Mice spent 34.88 ± 3.10 second scratching rostral back area after the injection of substance P subcutaneously in the negative control group. However, only the 1.0 mg/kg dose of BHMC produced a statistically significant reduction in scratching time compared to control mice, which is 9.95 ± 2.79 sec. The reference drug, azelastinealso significantly reduced scratching time to 4.92 ± 1.61 sec (Fig. 2).

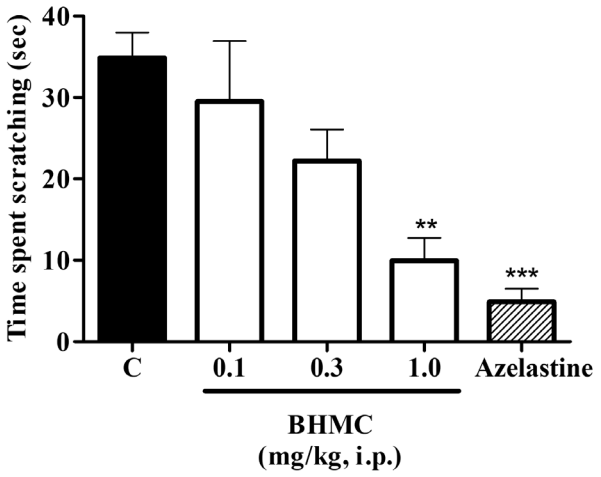

Fig. 2 : Effect of BHMC in substance P-induced scratching test in mice. Each column represents the mean ± S.E.M. (n=6/group). The mice were pre-treated with vehicle (C, control), azelastine (10 mg/kg, i.p.) or BHMC (0.1, 0.3 and 1.0 mg/kg, i.p.) 30 minutes before injection of Substance P (150 nmol/50 µl, s.c.) into rostral back area of mice. Then the mice were observed for 30 minutes. Statistical analysis was determined by one-way ANOVA followed by Dunnett's post hoc test. The asterisks denote the significance levels as compared to control, ** $p< 0.01$ and *** $p< 0.001$.

Histamine-induced scratching.

As shown in Fig. 3, subcutaneous injection of histamine produced 35.59 ± 2.64 sec of scratching time for negative control group mice. BHMC (0.1, 0.3, and 1.0 mg/kg i.p.) significantly reduced scratching time following histamine injection to 23.74 ± 2.1 sec5, 20.48 ± 2.07 sec, and 11.88 ± 1.67 sec, respectively. Cimetidine also significantly reduced scratching behavior to 17.60 ± 2.79 sec, as compared to the control group.

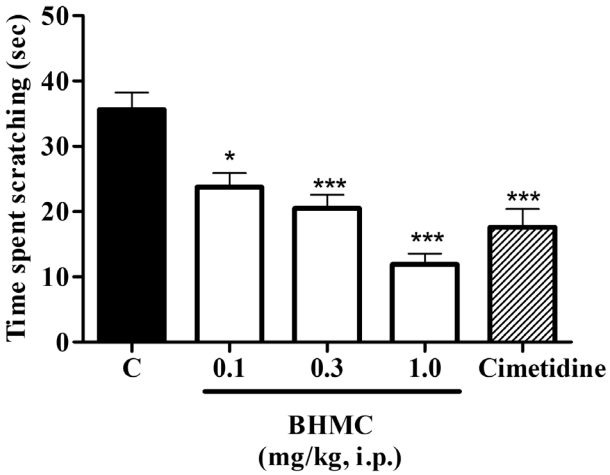

Fig. 3 : Effect of BHMC in histamine-induced scratching test in mice. Each column represents the mean ± S.E.M. (n=6/ group). The mice were pre-treated with vehicle (C, control), cimetidine (10 mg/kg, i.p.) or BHMC (0.1, 0.3 and 1.0 mg/kg, i.p.) 30 minutes before injection of histamine (100 nmol/50 µl, s.c.) into rostral back area of mice. Then the mice were observed for 30 minutes. Statistical analysis was determined by one-way ANOVA followed by Dunnett's post hoc test. The asterisks denote the significance levels as compared to control, * p< 0.05 and *** p< 0.001.

Serotonin-induced scratching

The results illustrated in Fig. 4 demonstrate that subcutaneous serotonin injection caused itch-like responses of 37.28 ± 5.18 sec. BHMC (0.1, 0.3 and 1.0 mg/kg, i.p.) produced significant reduction in serotonin induce scratching with dose dependent manner of to 14.94 ± 2.93 sec, 14.46 ± 4.24 sec and 13.79 ± 2.12, respectively. Positive control, ketanserin also produced significant reduction of time spent scratching to 4.72 ± 0.77 sec (Fig. 4).

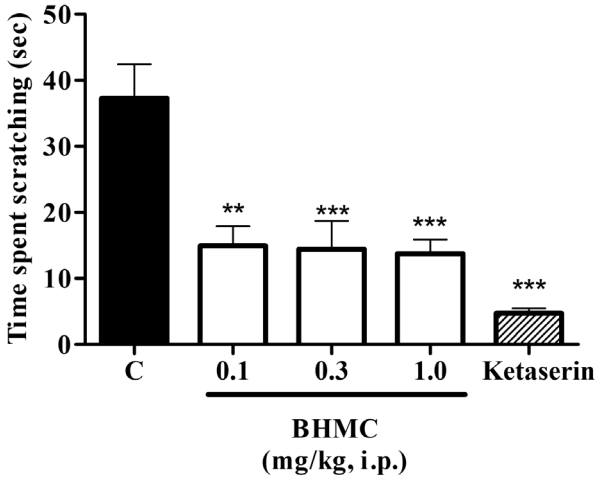

Fig. 4 : Effect of BHMC in serotonin-induced scratching test in mice. Each column represents the mean ± S.E.M. of (n=6/ group). The mice were pre-treated with vehicle (C, control), ketanserin (10 mg/kg, i.p.) or BHMC (0.1, 0.3 and 1.0 mg/kg, i.p.) 30 minutes before injection of serotonin (100 nmol/50 µl, s.c.) into rostral back area of mice. Then the mice were observed for 30 minutes. Statistical analysis was determined by one-way ANOVA followed by Dunnett's post hoc test. The asterisks denote the significance levels as compared to control, ** p< 0.01 and *** p< 0.001.

Compound 48/80-induced mast cell degranulation

As shown in Fig. 5, in negative control group where mice were injected intradermal with compound 48/80 and pretreated with vehicle, 91.76 ± 1.45% of mast cell degranulated. Pre-treatment of BHMC (0.1, 0.3 and 1.0 mg/kg, i.p.) significantly inhibited degranulation of mast cell induced by compound 48/80 ata dose dependent pattern to 66.97 ± 4.85%, 59.83 ± 6.26% and 51.06 ± 3.08% respectively. Oxatomide (10 mg/kg, i.p.) also showed significant of inhibition in mast cell degranulation to 62.73 ± 3.17%. The finding demonstrated that treatment with BHMC, with comparable efficacy as oxatomide, was able to retain more mast cells from being triggered by compound 48/80 for degranulation. This may be correlating with the reduction of itch-like responses as reported in the previous sub-sections.

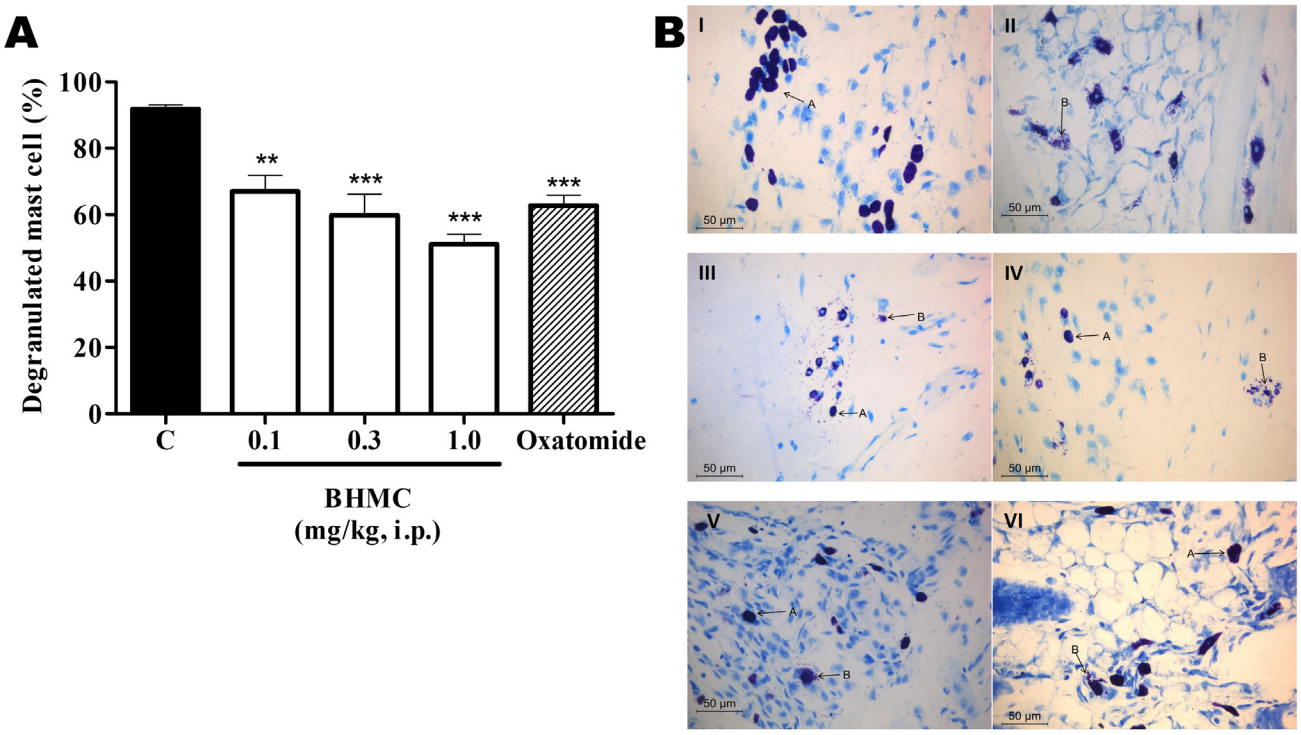

Fig. 5 : Effect of BHMC in compound 48/80-induced mast cell degranulation. (A) Each column represents the mean ± S.E.M. (n=6/group). The mice were pre-treated with vehicle (C, control), oxatomide (10 mg/kg, i.p.) or BHMC (0.1, 0.3 and 1.0 mg/kg, i.p.) 30 minutes before injection of compound 48/80 (100 µg/50 µl, s.c.) into rostral back area of mice. 10 minutes later subcutaneous tissues were isolated for toluidine blue staining. Statistical analysis was determined by one-way ANOVA followed by Dunnett's post hoc test. The asterisks denote the significance levels as compared to control, ** $p < 0.01$ and *** $p < 0.001$. (B) Representative figures from skin sections of compound 48/80-induced mast cell degranulation. Mice were given subcutaneous injection of saline (I) or compound 48/80 (100 µg/site, II-VI), and 10 min later the subcutaneous connective tissue was isolated for toluidine blue staining. Mice were pre-treated with BHMC (III-V) or oxatomide (VI) 30 minutes before injection of compound 48/80. (I) saline injected mice, (II) Vehicle treated mice, (III) 0.1mg/kg BHMC treated mice, (IV) 0.3mg/kg BHMC treated mice, (V) 1.0mg/kg BHMC treated mice and (VI) oxatomide (10mg/kg) treated mice. Letter A indicates intact mast cell and letter B indicates degranulated mast cell.

Substance P-induced mast cell degranulation

The results illustrated in Fig. 6 demonstrate that subcutaneous injection of substance P induced degranulation of mast cell at 79.47 ± 3.36%. Only BHMC at the dose of 1.0 mg/kg (i.p.) significantly reduced substance P-induced mast cell degranulation to 27.48 ± 2.77%. Azelastine (30mg/kg, i.p.) also significantly reduced mast cell degranulation induced by substance P to 42.27 ± 3.90%.

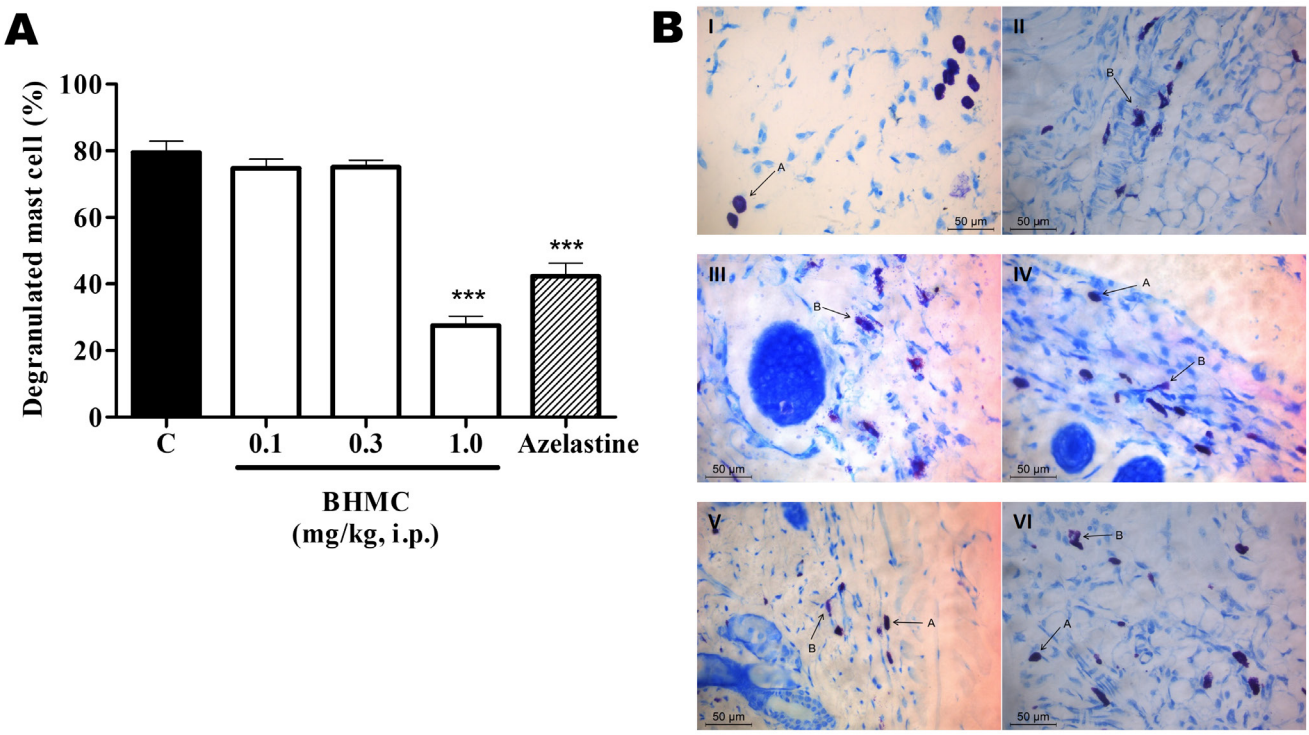

Fig. 6 : Effect of BHMC in substance P-induced mast cell degranulation. (A) Each column represents the mean ± S.E.M. (n=6/group). The mice were pre-treated with vehicle (C, control), azelastine (30 mg/kg, i.p.) or BHMC (0.1, 0.3 and 1.0 mg/kg, i.p.) 30 minutes before injection of substance P (150 nmol/50 µl, s.c.) into rostral back area of mice. 10 minutes later subcutaneous tissues were isolated for toluidine blue staining. Statistical analysis was determined by one-way ANOVA followed by Dunnett's post hoc test. The asterisks denote the significance levels as compared to control, *** $p < 0.001$. (B) Representative figures from skin sections of substance P-induced mast cell degranulation. Mice were given a subcutaneous injection of saline (I) or substance P (100 nmol/site, II-VI), and 10 min later the subcutaneous connective tissue was isolated for toluidine blue staining. Mice were pre-treated with BHMC (III-V) or azelastine (VI) 30 minutes before injection of compound 48/80. (I) saline injected mice, (II) Vehicle treated mice, (III) 0.1mg/kg BHMC treated mice, (IV) 0.3mg/kg BHMC treated mice, (V) 1.0mg/kg BHMC treated mice and (VI) oxatomide (10mg/kg) treated mice. Letter A indicates intact mast cell and letter B indicates degranulated mast cell.

DISCUSSION

The present study demonstrated that BHMC, a structural analogue of curcumin, exhibits significant antipruritic effects in mouse models of itch induced by compound 48/80, substance P, histamine, and serotonin. Additionally, BHMC inhibited mast cell degranulation in skin tissue triggered by compound 48/80 and substance P.

In this study, BHMC significantly inhibited scratching behavior induced by compound 48/80, a polymer known to promote mast cell degranulation and frequently used to induce itch in pruritic studies (24). Mast cells play a crucial role in both inflammation and pruritus, making them a focal point in studies of allergic reactions and anaphylaxis (9, 17). Mast cells are heavily granulated cells containing various mediators such as histamine, serotonin, substance P, and proteases (17, 25). The release of these mediators activates peripheral nerve endings, leading to inflammation and itch (17). BHMC may exert its antipruritic effects by inhibiting mast cell degranulation induced by compound 48/80 or by preventing the activation of peripheral nerve endings by the chemical mediators released from mast cells. This is supported by the results of skin histology stained with toluidine blue, showing significant inhibition of mast cell degranulation by BHMC. Since compound 48/80 is composed of polymers of varying sizes, the mechanisms by which it induces mast cell degranulation may vary, necessitating further investigation into how BHMC inhibits this process (25).

Subcutaneous injection of substance P into the rostral back area of mice induced hind paw scratching, confirming substance P's involvement in itch (26). Studies have shown that substance P binds to the neurokinin 1 receptor (NK1) on mast cells, and intradermal injection of substance P can cause mast cell degranulation (17, 27). In this study, pretreatment with BHMC significantly inhibited both scratching behavior and mast cell degranulation induced by substance P, particularly at a dosage of 1.0 mg/kg. This suggests that BHMC's antipruritic effects may be dose-dependent.

Substance P has a dual role in itch, as it can directly activate peripheral nerve fibers and induce mast cell degranulation (27). Previous studies have also shown

that substance P can induce scratching in mast cell-deficient mice, suggesting the existence of a mast cell-independent pathway in pruriception (26, 28). Although histamine is involved in pruritus, studies suggest that it is not essential for substance P-induced itch (29). Substance P injection has been shown to increase cutaneous leukotriene B4 concentrations, which also induce scratching behavior, indicating its role in itch (30, 31). Since azelastine significantly reduced cutaneous leukotriene B4 in substance P-injected mice (29), BHMC may exert its antipruritic effect by suppressing leukotriene B4 production. Given the crucial role of mast cells in substance P-induced pruriception, using mast cell-deficient mice could help clarify the specific pathways through which BHMC exerts its effects.

Both compound 48/80 and substance P are known to induce mast cell degranulation, leading to the release of histamine and serotonin. To further investigate the mechanism of BHMC's action, additional tests were performed to assess its effect on histamine-induced and serotonin-induced scratching. Histamine is a well-established pruritogen stored primarily in mast cells, with smaller amounts released by basophils, keratinocytes, and enterochromaffin cells (32). Histamine sensitizes peripheral nerves by binding to histamine receptors, particularly the H1 receptor (H1R), a G-protein-coupled receptor (GPCR) that belongs to the Gq subunit. Activation of H1R leads to the activation of phospholipase C (PLC), which hydrolyzes phosphatidylinositol bisphosphate (PIP2) into diacylglycerol (DAG) and inositol triphosphate (IP3) (33, 34). DAG can activate transient receptor potential vanilloid 1 (TRPV1) by directly binding to its capsaicin site (35). Co-expression of H1R and TRPV1 in dorsal root ganglia is essential for histamine-induced itch signal transduction (36). BHMC's antipruritic effects may involve antagonism or inverse agonism at H1R, H2R, and TRPV1 receptors, similar to the actions of cimetidine, an H2R antagonist that significantly reduces scratching behavior (37).

Serotonin (5-hydroxytryptamine or 5-HT) is another key pruritogen involved in itch (38, 39). The 5-HT2 receptors have been implicated in pruritus in mice (40). In this study, BHMC, like ketanserin, significantly suppressed scratching behavior. Ketanserin has a high affinity for the 5-HT2A receptor and moderate affinity for the 5-HT2C receptor (41). In addition to 5-HT2, other serotonin receptors, including 5-HT2B, 5-HT3, 5-HT4, 5-HT6, and 5-HT7, are involved in excitatory pathways (42). Similar to H1R, 5-HT2 receptors are GPCRs in the Gq subgroup, activating similar signaling molecules (42). Meanwhile, 5-HT4, 5-HT6, and 5-HT7 receptors are GPCRs belonging to the Gs subgroup, similar to H2R (42). Activation of these receptors leads to the production of cAMP, which can directly bind to cAMP-gated ion channels, causing depolarization of neurons (43, 44). The similarities between histamine and serotonin receptors suggest that BHMC's antipruritic pathway may involve antagonism or inhibition of second messenger activation following receptor binding.

A previous study showed that BHMC administered via the i.p. route at similar doses did not inhibit spontaneous motor activity in the rota-rod test (14). This finding rules out the possibility that the observed reduction in scratching behavior was due to muscle relaxant or sedative effects of BHMC.

CONCLUSION

BHMC, a synthetic derivative of curcumin, demonstrated significant antipruritic effects in mouse models of itch induced by compound 48/80, substance P, histamine, and serotonin. In addition to inhibiting mast cell degranulation induced by compound 48/80 and substance P, BHMC also inhibited the activation of peripheral pruriceptors by substance P, histamine, and serotonin. Future studies using mast cell-deficient mice could help elucidate the mast cell-independent pathways involved in BHMC's antipruritic effects. Given the potential involvement of multiple receptors and second messengers, further research is necessary to fully understand the mechanisms underlying BHMC's antipruritic actions.

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