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Unveiling the antinociceptive mechanisms of Methyl-2-(4-chloro-phenyl)-5-benzoxazoleacetate: insights from nociceptive assays in mice

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Abstract. – OBJECTIVE: Methyl-2-(4-chlorophenyl)-5-benzoxazoleacetate (MCBA), a synthetic benzoxazole derivative with established antipsoriatic efficacy, was investigated for potential antinociceptive effects. This study employs various nociceptive assays in mice to elucidate MCBA's antinociceptive mechanisms.

MATERIALS AND METHODS: MCBA's antinociceptive potential was tested against various nociception models induced by formalin, glutamate, capsaicin, a transient receptor potential vanilloid 1 (TRPV1) receptor agonist, and phorbol 12-myristate 13-acetate, a protein kinase C (PKC) activator. It was then assessed using the hot plate test and examined within the acetic acid-induced writhing test. During the acetic acid-induced writhing test, MCBA was pre-challenged against selective receptor antagonists such as naloxone, caffeine, atropine, yohimbine, ondansetron, and haloperidol. It was also pre-challenged with ATP-sensitive potassium channel inhibitor (glibenclamide) to further elucidate its antinociceptive mechanism.

RESULTS: The results showed that oral administration of MCBA led to a dose-dependent and significant inhibition ($p < 0.05$) of nociceptive effects across all evaluated models at doses of 60, 120, and 240 mg/kg. Moreover, the efficacy of MCBA's antinociceptive potential was significantly counteracted ($p < 0.0001$) by specific antagonists: (i) directed at adenosinergic, alpha-2 adrenergic, and cholinergic receptors using caffeine, yohimbine, and atropine, respectively; and (ii) targeting ATP-sensitive potassium channels, employing glibenclamide. Antagonists aimed at opioidergic and serotoninergic receptors (naloxone and ondansetron, respectively) had poor utility in inhibiting antinociceptive activity. Conversely, the dopaminergic receptor antagonist haloperidol potentiated locomotor abnormalities associated with MCBA treatment.

CONCLUSIONS: MCBA-induced antinociception involves modulation of glutamatergic-, TRVP1 receptors- and PKC-signaling pathways. It impacts adenosinergic, alpha-2 adrenergic, and cholinergic receptors and opens ATP-sensitive potassium channels.

Key Words:
Benzoxazole, Anti-nociception, Adenosinergic receptors, Cholinergic receptors, Glutamatergic signaling pathway, Health Care, Wellbeing, Medicine.

Introduction

Pain is an unpleasant experience encompassing intricate physiological and psychological aspects triggered by various factors such as injury, inflammation, and psychological distress[1]. It serves as a protective mechanism, alerting individuals to potential harm or underlying health issues[2]. The significance of pain on well-being and quality of life cannot be understated, it can lead to physical limitations, emotional distress, sleep distur-

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bances, and decreased social functioning, thus impacting daily activities, work productivity, and overall enjoyment of life[3-5].

The current landscape of pain management with available analgesics falls short of providing comprehensive relief due to the intricate nature of pain modulation. Pain involves a complex interplay of mediators and receptors at both peripheral and central levels, which regulate the sensitivity of nociceptive neurons[6,7]. These mediators, encompassing neurotransmitters and neuromodulators, activate diverse receptor classes, thus initiating a cascade of signaling pathways that contribute to the perception of pain[8,9]. However, our understanding of how these cascades orchestrate nociceptor sensitization and pain remains in its infancy. Consequently, researchers worldwide are diligently working to unravel the components involved in this intricate process and to develop novel agents targeting these components.

In addition to the challenges posed by pain modulation, currently available analgesic drugs, including opioids and nonsteroidal anti-inflammatory drugs (NSAIDs), have limitations in their efficacy[10-14]. Opioids, exemplified by morphine, have long been the gold standard for pain management but are plagued by adverse effects such as dependence, tolerance, and the potential for addiction following prolonged usage[15,16]. Moreover, existing analgesics often alleviate pain as a symptom without addressing its root cause, further compounding the issue. Therefore, there is an urgent imperative to explore alternative analgesic strategies that can overcome these limitations and offer more effective pain relief.

The shortcomings of current pain management approaches extend beyond opioids. Non-opioid medications, including NSAIDs, also have their drawbacks, such as gastrointestinal complications and cardiovascular risks[17-19]. Additionally, existing analgesics frequently provide suboptimal pain relief, thus leaving individuals with persistent discomfort and a compromised quality of life[20]. This unmet need drives the quest for the discovery and development of novel analgesic drugs with innovative pharmacological actions. Furthermore, the quest for new analgesics not only aims to address the limitations of current medications but also presents an opportunity to revolutionize pain treatment through personalized approaches[20,21]. By identifying specific pain pathways, receptors, or molecular targets, researchers can design drugs that precisely modulate these targets, thus offering tailored and more effective pain management strategies. This personalized treatment approach acknowledges the individual variations in pain perception and response, paving the way for more personalized and patient-centric care.

Benzoxazole compounds have gained attention in the field of medicinal chemistry due to their potential therapeutic activity, including analgesic properties[22,23]. Although research has shed light on their ability to impede the synthesis of inflammatory mediators *via* dual inhibition of cyclooxygenase and lipoxygenase pathways[23], the extent of our knowledge regarding their influence on different pain-related receptors and channels remains largely unexplored. These compounds exhibit a common structural motif consisting of a benzene ring fused with an oxazole ring, which serves as a foundation for evaluating their pharmacological properties and potential applications[24]. However, it is important to note that while benzoxazole compounds share this common structural motif, their therapeutic activities can vary significantly. The specific chemical structure, substitution patterns, and functional groups attached to the benzoxazole core can greatly influence their pharmacological properties, including their analgesic effects[25].

One benzoxazole compound that is currently being investigated for its potential to relieve pain is methyl-2-(4-chloro-phenyl)-5-benzoxazoleacetate (MCBA). This recently synthetic derivative of benzoxazole has exhibited notable anti-psoriatic properties in a previous study[26]. However, despite its observed efficacy in treating psoriasis, the specific effects of this compound on pain reduction have yet to be thoroughly explored. This unexplored aspect piqued our interest because it affords us the opportunity to probe a compound that has previously demonstrated efficacy within an alternative medical domain. It can unveil the analgesic attributes inherent to MCBA. Therefore, the aim of this study was to elucidate the potential analgesic properties of MCBA and investigate the underlying mechanisms involved. Through a comprehensive investigation, this research can enhance our understanding of MCBA and, to some extent, provide insights into the broader field of benzoxazole-based analgesics. Insights gained from the study of MCBA can be extrapolated to optimize other benzoxazole-based compounds. This can open doors to a broader range of therapeutic applications and the development of more potent medications.

Materials and Methods

This study is part of a larger project that began in 2018 and is still ongoing. It builds upon earlier research[26] where we successfully synthesized and evaluated two compounds, 2-(4-chlorophenyl)-5-benzoxazoleacetic acid and its derivative, methyl-2-(4-chlorophenyl)-5-benzoxazoleacetate (MCBA), for their effectiveness against psoriasis. The present study was conducted from June 2022 to July 2023, with a focus on investigating the potential pain-relieving effects of MCBA and uncovering the mechanisms behind its antinociceptive effects.

Synthesis of Methyl 2-(2-(4-Chlorophenyl)benzo[d]oxazol-5-yl)acetate (MCBA)

The synthesis of MCBA was carried out as described previously[26] as follows: Methyl 2-(3-amino-4-hydroxyphenyl) acetate (2.0 g, 0.011 mol) was dissolved in 15 mL of absolute ethanol in a 50 mL round-bottom flask; 4-chlorobenzaldehyde (1.54 g, 0.011 mol) was then added, and the mixture was stirred under continuous reflux for 4 h. The viscous material obtained after evaporation under reduced pressure was dissolved in 15-20 mL of hot glacial acetic acid. To this, $Pb(CH_3COO)_4$ (5.7 g, 0.013 mol) was added and stirred until it reached room temperature (25°C). The resulting crystalline solid was filtered, washed with distilled water, recrystallized with ethanol, and dried to yield 2 g of MCBA.

Animal Husbandry and Care

Male BALB/c mice, aged 8 to 12 weeks and weighing 20 to 25 g, were housed under standard research facility conditions. These conditions entailed a temperature range of 20-26°C, humidity maintained between 30-70%, and a 12-hour light/dark cycle. The animals were provided with clear polycarbonate cages (a cage size of 290 x 220 x 140 mm), nesting materials, and water and food *ad libitum*. The diet contained all the necessary nutrients and vitamins. The animals' food was sourced from the Jordanian Feed Company. All cages were cleaned regularly to maintain hygiene and prevent any infections or illnesses. The mice were acclimatized for a minimum of 5-7 days. During this time, the mice were monitored for any signs of illness or distress and furnished with appropriate care and housing. All aspects of animal manipulation and handling adhered strictly to the guidelines outlined in the "Guide for the Care and Use of Laboratory Animals"[27]. Euthanasia post-antinociceptive tests involved a combination of isoflurane overdose and cervical dislocation, thus ensuring a procedure that aligns with humane and ethical standards. The protocol concerning the use of animals was approved by the Scientific Research Ethics Committee of Isra University under reference number SREC/21/12/018.

Determining Maximum Tolerated Dose

The oral dose of MCBA to be administered to the mice was determined based on the expected toxicity levels with the procedure of this investigation drawn from prior research[28,29]. In this regard, determining the maximum tolerated dose (MTD) involved testing four specific oral doses (120, 240, 360, and 480 mg/kg) and comparing the vehicle and drug groups with untreated mice. The MTD was determined to be the highest dose, not causing unbearable side effects and a significant reduction in locomotor activity. These effects included various signs of toxicity, such as loss of coordination and balance, tremors, and seizures. This experiment was conducted by an expert toxicologist who was blind to the animal groups and drug doses. Following drug administration, individual mice were observed for 30 minutes, and seizure intensity was assessed using the previously described method[28] employing the following scoring system: 0 to indicate the absence of seizures, 1 for akinesia, 2 for myoclonic seizures, 3 for rearing seizures, and 4 for tonic-clonic seizures. Subsequently, mice were placed in an activity cage equipped with an actophotometer model (UGO Basile cage with a digital counter, photocell, and a light source) for 10 minutes, during which the automatic recording of total light beam interruptions (activity score) was used to assess locomotor activity.

Antinociceptive Effect of MCBA Against Formalin-, Capsaicin-, Glutamate-, Phorbol 12-Myristate 13-Acetate- (PMA-)-Induced Nociception

By implementing a well-established protocol[30,31], distinct experimental groups were structured with seven mice allocated to each specific group. The study subjects were administered distinct treatments comprising MCBA (60, 120, and 240 mg/kg, p.o.). CAPZ [transient receptor potential vanilloid 1 (TRPV1) antagonist at 0.17 mmol/kg po] was the positive control for the capsaicin

test. Diclofenac sodium (20 mg/kg, p.o.) was the positive control for the formalin, glutamate, and PMA tests. Morphine (5 mg/kg, p.o.) was the positive control for the hot plate test. A precisely timed pretreatment strategy was employed with the test compound administered 30 minutes prior to intraplantar injection (20 ul) of formalin (2% v/v), capsaicin (1.6 µg), glutamate (10 µmol), or PMA (0.05 µg) into the right hind paw. Subsequently, the assessment of nociceptive behavior commenced with a keen focus on quantifying the duration of paw licking, a well-established surrogate for pain response, throughout specific time intervals following the nociceptive agent injections. For formalin-induced nociception, the observation period spanned the early phase from 0 to 5 min post-injection, thus capturing the immediate nociceptive response as well as the late phase from 15 to 30 min, recording the sustained nociceptive response.

For capsaicin-induced nociception, the observation period spanned from 0 to 5 min post-injection, thus capturing the immediate nociceptive response. Glutamate-induced nociception was monitored from 0 to 15 min allowing for recording of sustained nociceptive behavior. In the case of PMA-induced nociception, the observation period extended from 15 to 45 min post-injection, thus targeting the delayed nociceptive response. The selection of these precise time intervals was based on established literature and previous experimental evidence[30,32].

Central Anti-Nociceptive Evaluation of MCBA Using the Hot Plate Test

The assessment of MCBA's central antinociceptive potential was executed through the utilization of the hot plate test adhering to established methodologies[33,34]. Initially, untreated animals were situated on a hot plate (Ugo Basile, Italy) maintained at a temperature of 50 ± 0.4°C, thus facilitating the meticulous selection of animals demonstrating a latency of response (4-8 s) to the thermally-induced nociceptive stimuli. Next, the selected mice (n = 7) were subjected to a pretreatment regimen involving the oral administration of either the vehicle (2% DMSO), MOR (5 mg/kg; positive control), or MCBA (60, 120 and 240 mg/kg) for 30 min preceding the commencement of the test. The nociceptive response was assessed by recording the time it takes for the animal to exhibit paw licking, jumping, or shaking upon hot plate placement and indicates sensitivity to thermal nociceptive stimulus.

Involvement of Various Pain Modulation Pathways in the Antinociceptive Activity of MCBA During the Acetic Acid-Induced Writhing Test

The present investigation examined the potential participation of diverse pain modulation pathways in the acetic acid-induced writhing test using a previously described protocol[30]. This study encompassed a range of receptor types, including cholinergic, alpha-2 adrenergic, GABAergic, adenosenergic, dopaminergic, and opioid receptors along with ATP-sensitive potassium channel inhibitors in the antinociceptive activity of MCBA. To assess the contribution of opioid receptors, mice (n = 7) received pre-treatment of naloxone [(Nal); 5 mg/kg, i.p.], an opioid receptor antagonist, 30 min before the oral administration of either the vehicle or MCBA (120 mg/kg, p.o.). For investigating nonopioid receptor modulation, separate groups of mice (n = 7/group) received pre-treatments of caffeine [(CAF); 3 mg/kg, i.p.], haloperidol [(HAL); 0.2 mg/kg, i.p.], atropine [(ATR); 10 mg/kg, i.p.], yohimbine [(YOH); 0.2 mg/kg, i.p.], flumazenil [(FLZ); 2 mg/kg, i.p.], or ondansetron [(OND); 0.5 mg/kg, i.p.] 15 min before orally administering either the vehicle (10 mL/kg, p.o.) or MCBA (120 mg/kg, p.o.). Additionally, the potential contribution of ATP-sensitive potassium channels in MCBA's antinociceptive properties was explored through careful pretreatment. A group of seven mice received glibenclamide [(GLIB); 10 mg/kg, i.p.] 15 min before orally administering either the vehicle (10 mL/kg) or MCBA (120 mg/kg). Subsequently, a well-established acetic acid-induced abdominal writhing test was conducted to assess antinociceptive efficacy. Cumulative writhing responses were systematically recorded over a 25-minute observation period with assessments starting 5 min after acetic acid injection.

Statistical Analysis

The results of the animal tests were expressed as mean ± standard error of the mean (S.E.M). To identify significant differences among the experimental groups, a one-way analysis of variance (ANOVA) was performed, followed by Dunnett's test, which compared each group to a control group. These analyses were conducted using GraphPad Prism (GraphPad Software, San Diego, CA, USA), version 8. A p-value below 0.05 ($p < 0.05$) was considered statistically significant.

Table I. Impact of different oral doses of MCBA on mice function and locomotor activity.

Dose (mg/kg)	Adverse effects	Seizure score	Locomotor activity (number of light beam interruptions)
Untreated mice (Negative control)	-	0	833.6 ± 29.44
0 (Vehicle control)	No unbearable side effects observed	0	754.9 ± 42.19
120	No unbearable side effects observed	0	712.0 ± 42.28
240	Some mice (2 out of 7) showed mild salivation	0	739 ± 51
360	All mice exhibited at least one of the following effects: salivation and/or urination, stereotypic head movement (head twitch), Straub tail and akinesia.	0	621 ± 32**
480	All mice exhibited at least one of the following effects: salivation and/or urination, stereotypic head movement (head twitch), Straub tail, and/or myoclonic seizure.	0.8 ± 0.4**	54 ± 21****

Data obtained from the analysis of locomotor activity were displayed as the mean ± standard error of the mean (S.E.M). The symbol "**" was employed to signify a significant difference ($p < 0.01$) from the negative control group, while "****" was used to indicate even more significance ($p < 0.0001$) compared to the negative control group.

Results

The results of the acute toxicity study revealed that MCBA induced different reactions in mice depending on the dosage administered, as shown in Table I. At a dose of 120 mg/kg, the compound was well-tolerated and exhibited no significant deviation from the untreated and vehicle control groups. At 240 mg/kg, the compound was similarly well-tolerated with only a few instances (2 out of 7 mice) of mild salivation. There was no significant reduction in locomotor activity. Conversely, when the dosage was escalated to 360 and 480 mg/kg, the mice exhibited signs of imbalance, stereotypic head movement, increased saliva production (Figure 1), urination, as well as Straub tail, akinesia, and/or myoclonic seizure. No mortality was observed in mice treated with doses of 120, 240, and 360 mg/kg. However, mice administered a dosage of 480 mg/kg recorded three deaths out of seven subjects. The study indicates that doses up to and including 240 mg/kg were deemed safe and did not result in fatalities or significant adverse effects. Therefore, proceeding with 240 mg/kg as the therapeutic dose for anti-nociceptive investigations appears to be a prudent choice based on the study's results.

The antinociceptive potential of MCBA was thoroughly assessed using the formalin-induced paw-licking test, with the results depicted in Figure 2. Notably, MCBA exhibited remarkable and statistically significant ($p < 0.05$) reductions in the response latency to nociceptive stimuli during both the early and late phases of the formalin-induced paw-licking test *vs.* vehicle-treated group. Specifically, in the early phase, doses of 60, 120,

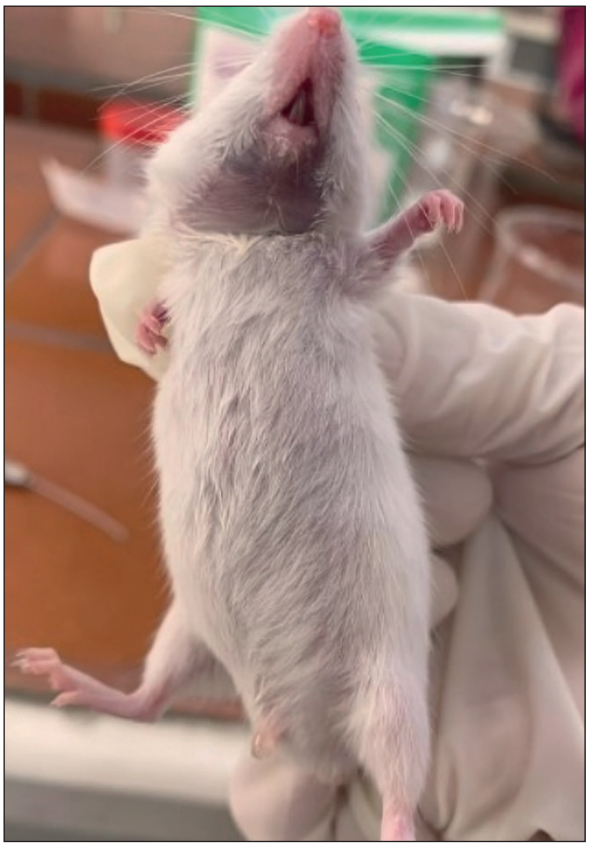

Figure 1. A mouse exposed to a dose of 360 mg/kg of MCBA exhibited excessive salivation. Notice the wet area below the mouth saturated with saliva.

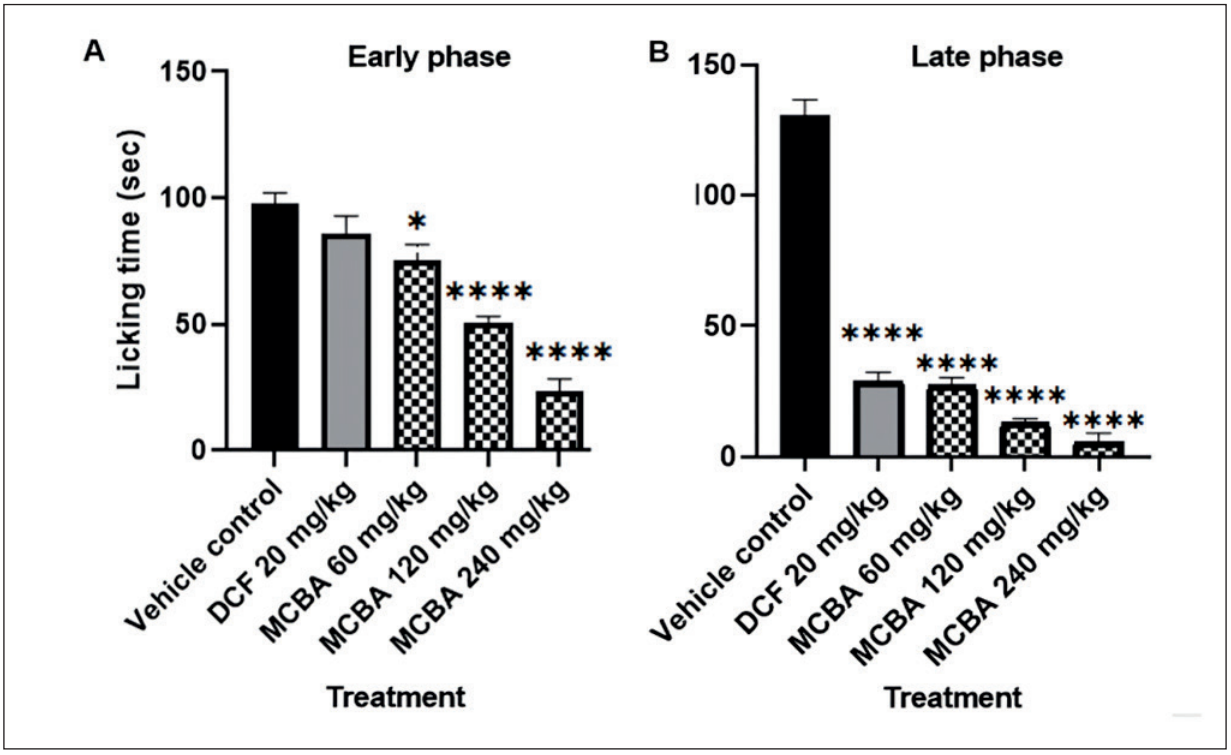

Figure 2. Effect of MCBA on the formalin-induced paw licking test in mice. The study investigated the influence of MCBA on the formalin-induced paw-licking test, distinguishing between the early phase (**A**) and the late phase (**B**). Each column portrays the mean ± SEM derived from a carefully selected cohort of seven mice. Statistical analyses were conducted via a one-way ANOVA followed by Dunnett's post hoc test. Statistical significance was indicated by **** ($p < 0.0001$) and * ($p < 0.05$) versus vehicle control.

and 240 mg/kg resulted in antinociception percentages of 22.5%, 48.5%, and 76%, respectively (Figure 2A). Similarly, in the late phase, MCBA exhibited reductions in response latency, with percentages of antinociception recorded at 79.2% for 60 mg/kg, 90.2% for 120 mg/kg, and 95.5% for 240 mg/kg (Figure 2B). In a comparative analysis, the peripherally-acting antinociceptive drug, diclofenac sodium (DCF), exhibited significant ($p < 0.0001$) inhibition only in the latency of second phase nociception recorded at 77.7%.

As depicted in Figure 3, the impact of MCBA becomes obvious, including a pronounced and statistically significant inhibition of glutamate-induced nociception ($p < 0.0001$) observed at all dose levels. This inhibitory effect is particularly noteworthy with a substantial 97.5% reduction at a dose of 240 mg/kg; the reductions were 67% and 82% at 60 mg/kg and 120 mg/kg, respectively, *vs*. the vehicle control group. The administered positive control agent, DCF, at a dose of 20 mg/kg, also demonstrated appreciable efficacy with a 60% inhibition compared to the control mice.

Employing the PMA-induced nociception paradigm, MCBA further demonstrated a conspicuous suppression of PMA-induced paw licking in mice (depicted in Figure 4). The oral administration of MCBA at 240 mg/kg yielded a substantial inhibition of 93.5% compared to the vehicle control group. At 60 mg/kg and 120 mg/kg, the reductions were 41.7% and 84%, respectively. Furthermore, the positive control, DCF administered at 20 mg/kg, exhibited a significant 63.9% inhibition against PMA-induced nociception.

The impact of MCBA on capsaicin-induced nociception in mice is presented in Figure 5. A substantial and statistically significant reduction ($p < 0.01$) in capsaicin-induced neurogenic pain response is demonstrated upon oral administration of MCBA at 120 mg/kg and 240 mg/kg. Importantly, MCBA at 240 mg/kg and 120 mg/kg exhibited significant decreases of 39.5% and 20%, respectively, in the paw-licking response *vs*. the vehicle control group. Moreover, the positive control agent, CAPZ (0.17 mmol/kg), demonstrated a robust inhibition of 58.5% in comparison to the control group, thus reaffirming its potent antinociceptive activity.

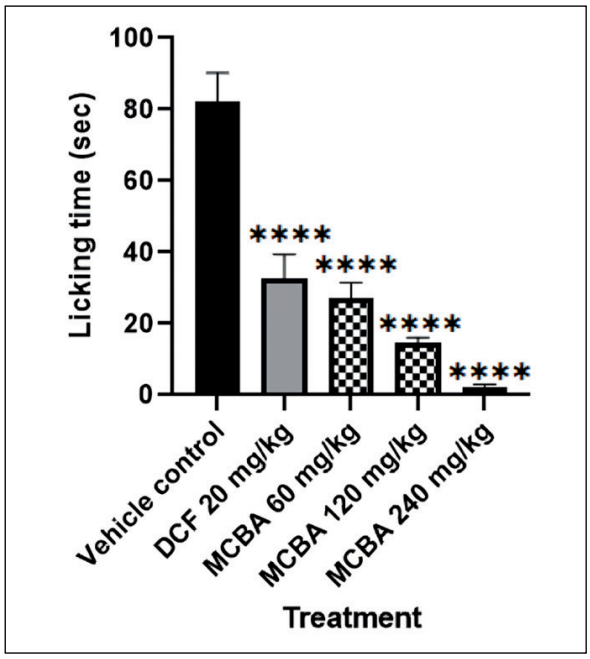

Figure 3. MCBA's impact on the glutamate-induced paw licking test in Mice. Each column represents the average ± SEM derived from a group of seven mice. Statistical analysis was carried out using a one-way ANOVA followed by followed by Dunnett's post hoc test. The level of significance is denoted by **** ($p < 0.0001$) in comparison to the vehicle control.

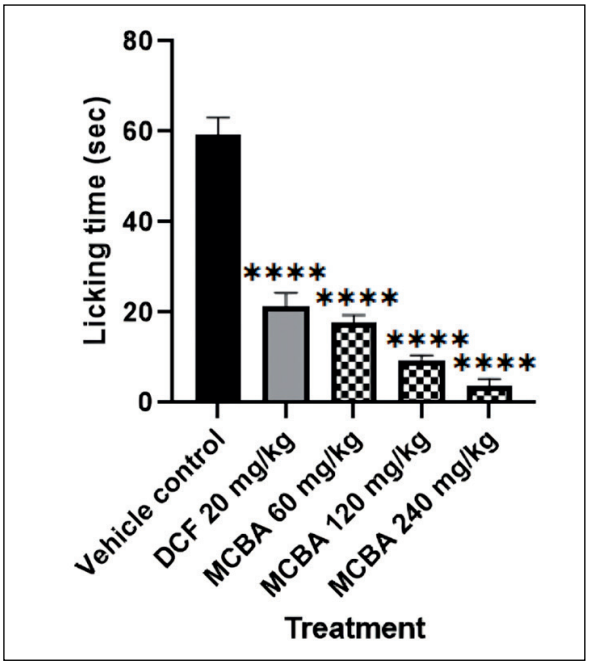

Figure 4. MCBA's effect on PMA-induced nociception in mice. Each column represents the average ± SEM derived from a group of seven mice. Statistical analysis was carried out using a one-way ANOVA by Dunnett's post hoc test. The level of significance is denoted by **** ($p < 0.0001$) compared to the vehicle control group.

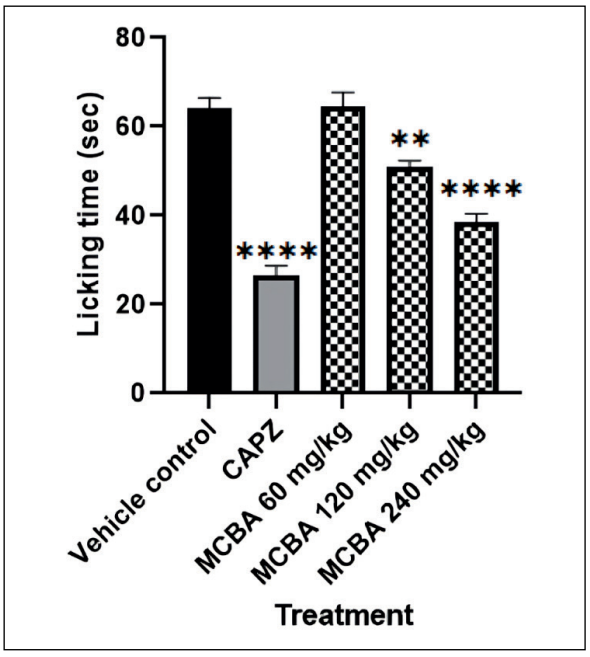

Figure 5. MCBA's effect on capsaicin-induced nociception in mice. Each column represents the average ± SEM derived from a group of seven mice. Statistical analysis was carried out using a one-way ANOVA followed by Dunnett's post hoc test. The level of significance is denoted by ** ($p < 0.01$) and **** ($p < 0.0001$), indicating significant differences from the vehicle control.

The evaluation of MCBA's central antinociceptive potential extended to thermal-induced nociception through the hot plate test, as illustrated in Figure 6. At the administered dosage of 120 and 240 mg/kg, MCBA demonstrated a marked capacity to substantially prolong response latency ($p < 0.001$) in contrast to the vehicle control. Correspondingly, the well-acknowledged analgesic standard, MOR, exhibited more profound antinociceptive efficacy ($p < 0.0001$) than MCBA at the test doses.

Figure 7 displays the outcomes of the acetic acid-induced writhing test. Notably, administration of both MCBA and DCF led to significant suppression of abdominal constriction, exhibiting inhibition percentages of 88.3% and 64.3%, respectively. However, the antinociceptive effectiveness of MCBA was significantly diminished ($p < 0.0001$) when challenged with receptor antagonists, specifically caffeine, atropine, and yohimbine, as well as the ATP-sensitive potassium channel blocker, glibenclamide. On the other hand, pretreatment with naloxone and ondansetron failed to inhibit antinociceptive activity. Intriguingly, the antagonist of dopaminergic receptors, haloperidol, potentiated the locomotor abnormalities associated with MCBA treatment.

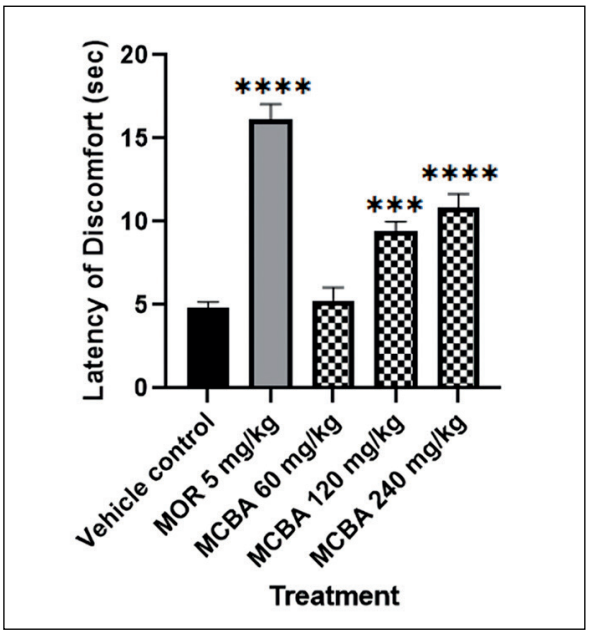

Figure 6. MCBA's antinociceptive effect in the hot plate test in mice. Each column represents the average ± SEM derived from a group of seven mice. Statistical analysis was carried out using a one-way ANOVA followed by Dunnett's post-hoc test. The level of significance is denoted by *** ($p < 0.001$) and **** ($p < 0.0001$), indicating a significant difference from the vehicle control.

Discussion

Motor impairments can introduce confounding variables into studies that assess antinociception. To ascertain that the observed analgesic effect is not influenced by motor deficits, we examined MCBA's impact on the actophotometer test – a conventional model for evaluating motor coordination and locomotor activity. Administration of MCBA at doses ranging from 120 to 240 mg/kg *via* oral route did not yield a significant reduction in locomotor activity, thus implying that the antinociceptive effects of MCBA observed in this investigation are unlikely to stem from peripheral neuromuscular blockade or sedation induction. While the administration of MCBA at a dose of 240 mg/kg demonstrated a dose that was tolerated to the maximum, there was an increased occurrence of salivation and urination *vs.* untreated mice or mice that received the vehicle. This observation, combined with dose-dependent motor impairment, prompts consideration of the potential for enhanced activation of the parasympathetic division within the autonomic nervous system – especially considering MCBA's dopaminergic antagonist properties[35,36]. The fact that coadministration of haloperidol potentiated the motor deficit, as described in the results section, suggests a synergistic interaction between MCBA and haloperidol in affecting the dopaminergic pathways within the autonomic nervous system. Thus, gaining a profound understanding of the complex interplay between MCBA and its combined impact on dopamine signaling is crucial for fully grasping the mechanisms that underlie its clinical implications.

Previous research[37] has firmly established benzoxazole derivatives as a significant category of compounds with distinct mechanistic attributes. Certain members of this derivative group can efficiently retard the release of proinflammatory cytokines from mast cells. Additionally, specific benzoxazole derivatives showcase a dual functionality encompassing selective COX-2 inhibition and heightened anti-inflammatory effectiveness, thus distinguishing themselves from conventional NSAIDs such as celecoxib and diclofenac[38]. Similarly, this study's findings underline MCBA's superiority in terms of analgesic properties compared to the well-established diclofenac, a distinction supported by diverse animal models. Notably, MCBA's analgesic effects were found to be dose-dependent, with increasing efficacy at higher doses. This implies that MCBA holds promise as a potential therapeutic option for pain management, potentially outperforming existing drugs in terms of efficacy. Furthermore, the investigation's outcomes expound upon MCBA's analgesic mechanism, thus revealing a multifaceted interaction that extends across diverse nociception test models encompassing both chemical (acetic acid and formalin-induced) and thermal (hot plate-induced) stimuli. This intricate engagement involves a myriad of receptors and molecular pathways within the physiological context, transcending a mere reliance on COX enzyme inhibition. This intricate profile underscores MCBA's analgesic effectiveness as being orchestrated through a nuanced convergence of pathways, thus encompassing both central and peripheral systems and auguring a more all-encompassing and sophisticated spectrum of pain management strategies.

The formalin test, also known as the formalin-induced paw-licking test, offers a valuable approach to unraveling the intricate dynamics of nociception encompassing both peripheral and central aspects. This method proves its worth by administering formalin into the intraplantar (i.p.) region of mice's hind paws, thus setting in motion

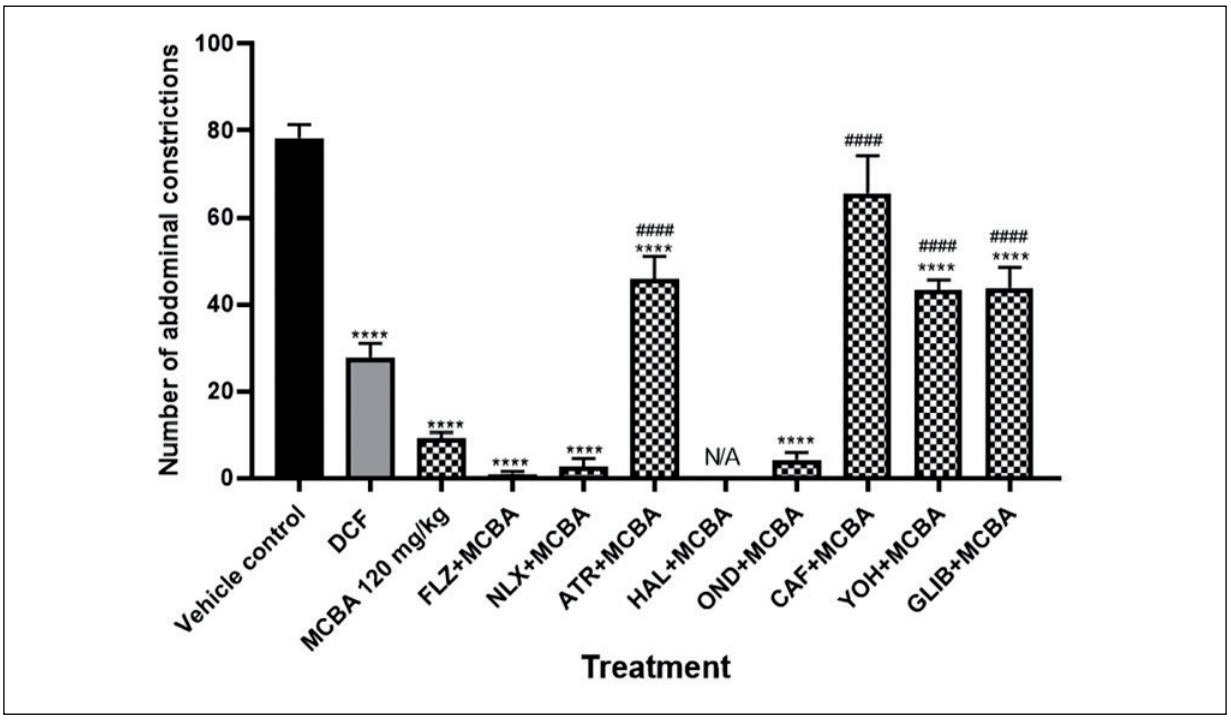

Figure 7. The involvement of various opioid and nonopioid receptor antagonists, along with an ATP-sensitive potassium channel blocker in MCBA-mediated antinociception within the context of the acetic acid-induced abdominal constriction test in mice. Flumazenil [(FLZ); 2 mg/kg, i.p.], naloxone [(Nal); 1.5 mg/kg, i.p.], atropine [(ATR); 10 mg/kg, i.p.], haloperidol [(HAL); 0.2 mg/kg; i.p.], ondansetron [(OND); 0.5 mg/kg, i.p.], caffeine [(CAF); 3 mg/kg, i.p.], yohimbine [(YOH); 0.2 mg/kg, i.p.], and glibenclamide [(GLIB); 10 mg/kg, i.p.] administered prior to either vehicle (10 mL/kg, p.o.) or MCBA (240 mg/kg, p.o.). Each column presents the mean ± SEM derived from a cohort of seven mice. Statistical analysis involved a one-way ANOVA followed by Dunnett's post hoc test. The level of statistical significance is indicated as $^{****}p < 0.0001$ in comparison to the control group and as $^{\#\#\#\#}p < 0.0001$ in contrast to the 120 mg/kg MCBA-treated group. "N/A" indicates that a particular comparison is not applicable.

a captivating sequence of nociceptive reactions characterized by two distinct phases: early/first and late/second phases. The early phase occurs immediately post-formalin injection and owes its origin to the direct interaction between formalin and nociceptors. This phase persists for approximately 5 minutes, during which nociceptors experience robust activation. This initial phase often termed the neurogenic phase, is notably associated with pain not driven by inflammation, earning it the title of non-inflammatory-mediated pain. It hinges on the direct chemical stimulation of nociceptors, particularly those engaged through Aδ fibers situated in central nociceptive primary afferent terminals[39]. Conversely, the late phase unfolds with a distinct storyline emerging 15 and 60 min after formal administration[40]. This phase is shaped by a sophisticated interplay between peripheral inflammation and spinal cord processes[41]. Within this timeframe, activation of dorsal horn neurons comes into play; they have a pivotal role in the generation of nociceptive behavior. This intricate interplay ultimately results in intensified nociceptor activation facilitated by the heightened input from C fibers[39,42]. Within the context of the current investigation, the capacity of MCBA to effectively mitigate both phases of nociceptive responses observed in the formalin test offers compelling evidence of its intrinsic resemblance to centrally acting analgesics.

Accumulated insights garnered over recent decades provide robust support for the pivotal role that the excitatory amino acid glutamate assumes in orchestrating the intricate modulation of nociceptive processing[43]. Notably, glutamate and its cognate receptors exhibit precise distributions spanning cerebral, spinal, and peripheral domains that intricately intertwine with pain perception and the propagation of pain signals[44,45]. The activation of glutamate receptors within discreet cerebral regions appears inherently inclined towards promoting nociceptive responses – a

phenomenon underscored by their prominence in locales such as the thalamus and trigeminal nucleus[46]. Furthermore, the inhibition of glutamate release or the antagonism of glutamate receptors, whether in the spinal cord or periphery, elicits a discernible dampening effect on both acute and chronic pain states in various animal models[47]. In the context of animal experimentation, the nociception prompted by the intraplantar administration of glutamate appears to materialize through an intricate interplay. This intricate process involves the activation of both NMDA and non-NMDA receptors, with the underlying mechanism intricately dependent on the stimulation of the l-arginine-nitric oxide pathway[48]. Moreover, the emergence of glutamate-induced paw edema seems predominantly orchestrated through the modulation of non-NMDA ionotropic glutamate receptors, culminating in the subsequent release of nitric oxide[48]. In this regard, the current investigation's observation that MCBA inhibited the glutamate-induced licking response suggests its potential as an agent capable of intervening in glutamate-mediated nociceptive signaling, potentially contributing to its efficacy in pain management.

The well-established role of TRPV1 in pain processing highlights its susceptibility to modulation by a range of stimuli, including capsaicin (the pungent compound in chili peppers), elevated temperatures exceeding 43°C, and protons at low pH levels[49]. TRPV1 activation within nociceptive neurons, its primary site of expression, initiates a cascade of events leading to the release of neuropeptides and transmitters[50]. This process ultimately generates action potentials that ascend to higher areas of the CNS that are frequently interpreted as pain perception. Additionally, TRPV1 activation triggers the peripheral release of pro-inflammatory compounds, thus potentially sensitizing neighboring neurons to various physical, thermal, or chemical stimuli[51]. Given these multifaceted roles, alongside its paradoxical capacity to induce analgesia upon sustained activation, TRPV1 has emerged as a promising target for pharmacological intervention in pain management[52]. The utilization of the capsaicin-induced licking test holds significant prominence within the realm of pain research. These are intricately entwined with the complex modulatory effects stemming from TRPV1 activation[53]. By administering capsaicin, a selective agonist of the TRPV1 receptor, this test effectively induces nociceptive responses in experimental animals. The resulting licking behavior offers a comprehensive glimpse into the complex amalgamation of nociceptive signaling pathways activated by TRPV1, thus embodying a convergence of peripheral and central elements within pain processing[30]. In light of these insights, the findings here indicating MCBA's inhibition of capsaicin-induced licking behavior hold profound implications. This observation suggests that MCBA may exert its analgesic effect by interfering with or modulating the TRPV1-mediated nociceptive pathways, further highlighting its potential as a novel therapeutic candidate for pain management.

Protein kinase C (PKC) is a class of serine/threonine kinases that play a central role in coordinating cell communication through phosphorylation of target proteins[54]. This multifaceted group has profound effects on a wide range of intracellular mechanisms, including the complex field of pain perception, which is achieved by influencing ion channels and neurotransmitter release[55]. Their presence spans different cellular domains. In the nervous system, their prevalence in neurons and glial cells is closely linked to processes such as plasticity of the brain synapses, neurotransmitters, and complex pain modulation[56]. As an illustration, the effects of PKC extend to receptors such as TRPV1 and NMDA, thus reshaping their responsiveness to stimuli such as heat and chemicals, a dynamic that shapes pain sensitivity[57-59]. Consistent with these findings, the present investigation highlighted MCBA's ability to counteract the pain-inducing effects induced by PMA, a PKC-activating agent. This demonstration highlights the potential of MCBA to interfere with pathways regulated by PKC modulation. The hot plate test, utilized in animal pain models, leverages polysynaptic reflexes initiated at the spinal level and modulated by supraspinal centers[60]. These reflexes are triggered in response to thermal heat stimuli, thus eliciting pain-related behaviors like jumping and paw licking[33]. Serving as an acute pain model, the hot plate paradigm induces pain through heat-mediated tissue damage and ensuing inflammation, thus leading to the release of peripheral mediators. Historically, a hot plate test has been employed to assess the effectiveness of centrally acting substances, primarily opioids and related compounds[31]. However, recent research[61,62] has brought attention to the central antinociceptive effects exhibited by certain NSAIDs, including piroxicam, zaltoprofen, and diclofenac sodium, in attenuating hot plate-induced nociception. Corre-

spondingly, the current investigation underscores MCBA's ability to inhibit hot plate-induced nociception, thus aligning with its potential to centrally modulate pain perception. This finding is consistent with observations from the formalin test reinforcing MCBA's central effects.

The acetic acid-induced abdominal writhing test serves as a valuable screening tool for the evaluation of analgesic agents. Abdominal writhing is characterized by stretch, hind leg extension, abdomen contraction leading to contact with the floor, or trunk twisting in mice. The constriction caused by acetic acid represents a nonselective antinociceptive model because acetic acid indirectly prompts the release of endogenous mediators that activate nociceptive neurons responsive to nonsteroidal anti-inflammatory drugs, narcotics, and other centrally acting compounds[63]. The intricate regulation of acetic acid-induced nociception involves receptor systems such as adenosine, adrenergic, cholinergic, dopaminergic, serotonergic, GABAergic, as well as potassium channels[64,65].

The outcomes of the current investigation demonstrated that prior administration of caffeine (an adenosine receptor antagonist), atropine (a competitive antagonist of muscarinic acetylcholine receptors), yohimbine, alpha 2 adrenergic receptor antagonist, and glibenclamide (ATP-sensitive potassium channel blocker) resulted in a significant reversal of acetic acid-induced nociception in mice. However, pre-treatment with naloxone, flumazenil, and ondansetron did not exhibit the same effect in reversing acetic acid-induced nociception. These findings imply the plausible engagement of adenosine, muscarinic, adrenergic, and potassium channel pathways in the modulation of nociceptive responses, whereas the absence of reversal observed with naloxone, flumazenil, and ondansetron suggests the non-involvement of opioid, GABAergic, and serotonin receptors in the antinociceptive effects observed.

Adenosine receptors exert multifaceted influences on pain transmission at both peripheral and spinal levels. Activation of adenosine A1 receptors in peripheral nerve terminals of rodents diminishes cyclic AMP levels, thus inducing antinociception, while adenosine A receptors in the spinal cord confer antinociceptive effects across acute nociceptive, inflammatory, and neuropathic pain states with indications of spinal adenosine A2 receptor involvement in pain modulation[66]. Therefore, the reversal of the antinociceptive effect of MCBA by caffeine, which exhibits a relatively equal affinity for both A1 and A2A receptors[67] offers potential evidence for the participation of adenosine A1 and A2 receptors in MCBA's antinociceptive effect.

The emerging body of research[68,69] indicates that cholinergic interneurons located in the spinal cord hold a pivotal role in governing pain modulation. These interneurons release a neurotransmitter called acetylcholine, which engages with specific muscarinic receptors known as mAChRs, thereby orchestrating the control of glutamate release from primary afferent nerves. Furthermore, the activation of distinct muscarinic receptors, particularly the M2 and M4 subtypes, within the second-order spinal neurons triggers a complex series of events involving specialized potassium channels. Through the opening of these channels, potassium ions exit the cell and ultimately reduce their excitability. This intricate mechanism acts to lessen the intensity of pain signals akin to decreasing the "volume" of pain messages traveling through the spinal cord.

Additionally, a compilation of studies[70] underscores that direct activation of cholinergic receptors or the extension of endogenous acetylcholine's effects achieved through inhibiting acetylcholine esterase using substances like donepezil that can mitigate pain both in rodents and humans. Conversely, inhibiting muscarinic cholinergic receptors heightens sensitivity to pain. Hence, the observed reversal of MCBA's antinociceptive effects upon pretreatment with atropine – a muscarinic subtype non-specific antagonist – provides compelling evidence suggesting the engagement of the cholinergic system in MCBA's ability to alleviate nociception. In addition, the observed side effects of elevated salivation and augmented urination align with the typical manifestations associated with cholinesterase inhibitors[71], thus reinforcing the notion of MCBA's influence on muscarinic system control. These findings highlight the potential of MCBA as a promising avenue for addressing conditions such as xerostomia and Alzheimer's disease, where targeted manipulation of the muscarinic system holds promise for therapeutic intervention[72,73].

The pivotal role of potassium channels in the regulation of neuronal excitability and the propagation of action potentials has been substantiated through a wealth of empirical research[74,75]. Particularly, ATP-sensitive potassium channels manifest a complex and multifaceted role that transcends their traditional canonical function associated with insulin secretion, encompassing

a nuanced influence within the intricate domain of pain modulation. Notably, the downregulation of ATP-sensitive potassium channels in response to hyperglycemic conditions augments the sensitivity of nociceptive pathways, while the strategic pharmacological induction of these channels through agents such as diazoxide yields discernible analgesic effects across both central and peripheral nervous systems[76]. Moreover, a recent study by Clement et al[77] suggests that directing therapeutic attention towards specific ATP-sensitive potassium channel subtypes, particularly Kir6.1/SUR2B, holds potential as a promising pathway for migraine treatment. Additionally, Zhu et al[78] have demonstrated the efficacy of ATP-sensitive potassium channel opener treatment for postoperative pain in animals, which is attributed to the activation of the JNK/MCP-1 pathway in astrocytes.

The role of Alpha-2 adrenergic receptors in the modulation of nociception has been well-reported[79,80]. These receptors are distributed throughout the nervous system, including the spinal cord, brainstem, and higher brain centers[81]. Activation of alpha-2 adrenergic receptors can lead to the inhibition of pain transmission by reducing the release of neurotransmitters involved in pain signaling, such as substance P and glutamate[80,82,83]. This can result in the attenuation of pain signals being transmitted from peripheral nerves to the central nervous system. In this regard, medications that target alpha-2 adrenergic receptors, such as clonidine and dexmedetomidine, are used in pain management strategies, especially in certain types of acute and chronic pain conditions[84]. Similarly, the inhibition of MCBA's antinociceptive effect by pretreatment with yohimbine, an alpha 2-receptor antagonist, suggests a crucial involvement of noradrenergic pathways in mediating MCBA's pain-relieving effects. This finding highlights the complexity of MCBA's mechanism of action, potentially entailing complicated interactions with diverse modulation systems. The results indicate the significance of alpha 2 receptors and the noradrenergic system in contributing to the overall antinociceptive efficacy of MCBA, thus potentially offering valuable insights for the development of targeted pain management strategies and emphasizing the need for further research into these complex interactions.

While the present study provides insights into the antinociceptive mechanisms, it does not comprehensively explore all potential pathways or receptors affected by MCBA. The duration of observation is limited and is hindered by a comprehensive understanding of the compound's long-term implications and sustained efficacy. Furthermore, the study does not definitively clarify the cholinergic effects of MCBA, leaving unanswered questions about whether these effects stem from direct cholinergic agonism or cholinesterase inhibition. Addressing these limitations in future research will enhance the applicability and robustness of the study's findings.

Conclusions

This study has elucidated the multifaceted antinociceptive mechanisms prompted by MCBA operating both within the central and the peripheral nervous system. These encompass the inhibition of nociceptive pathways mediated by PKC, glutamate, and TRPV1 along with activation of adenosinergic, muscarinic-cholinergic, and alph2 adrenergic receptors, as well as the facilitation of ATP-sensitive potassium channel function.

Ethics Approval

The protocol concerning the use of animals was granted approved by the Scientific Research Ethics Committee of Isra University under reference number SREC/21/12/018.

Conflict of Interest

The authors declare that there are no conceivable conflicts of interest in connection to the research, authorship, and/or publication of this manuscript.

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Data Availability

The datasets produced or analyzed in the context of the present study are at the disposal of interested parties and can be obtained from the corresponding author upon submitting a reasonable request.

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