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Research Article

2´,3,3,5´-Tetramethyl-4´-nitro-2´H-1,3´-bipyrazole exerts antinociceptive effect using various nociception models

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Abstract

Background: 2′,3,3,5′-Tetramethyl-4′-nitro-2′H-1,3′-bipyrazole (TMNB) is a novel bipyrazole compound that exhibited antidiabetic and anti-inflammatory properties. However, its analgesic effect has not been investigated. This study aimed to assess the antinociceptive activity of TMNB using different nociception mouse models.

Methods: TMNB doses (50, 100, 150, and 200 μg/kg) were assessed in mice using the acetic acid-induced writhing test, hot plate test, and formalin-induced paw licking assay. The effects were compared to those of mice treated with acetylsalicylic acid or morphine in the presence or absence of naloxone. Capsaicin- and glutamate-induced paw-licking tests were also used to evaluate the involvement of the vanilloid and glutamatergic systems, respectively.

Results: TMNB produced significant dose-dependent inhibition of nociceptive behavior in the acetic acid-induced writhing test, showing 66% inhibition at a dose of 200 μg/kg. TMNB also caused a significant increase in the latency period in response to the hot plate test (68.2% at 200 μg/kg), and significantly inhibited both the neurogenic and inflammatory phases in the formalin-induced paw-licking test. Naloxone significantly reverses the effect of TMNB in both the hot plate test and formalin-induced paw-licking test. Moreover, TMNB significantly inhibited the neurogenic nociception induced by intraplantar injections of glutamate and capsaicin (53% and 77.1%, respectively at a dose of 200 μg/kg).

Conclusion: TMNB possesses antinociceptive activity in mice that is mediated through both central and peripheral pathways.

Keywords

2′,3,3,5′-Tetramethyl-4′-nitro-2′H-1,3′-bipyrazole, nociception, naloxone, vanilloid, glutamate

Introduction

Pain is a sensation or feeling that is uncomfortable and caused by injury to body tissues or associated with damage (Walters and Williams 2019). It is widely accepted in the scientific community that pain, regardless of whether it is caused by actual or potential tissue damage, is a protective mechanism for the organism (Tracey 2017; Walters 2018). Nociceptive pain is one of the two main types of pain that results from damaging stimuli to body tissues (Tracey 2017; Walters 2018). This type of pain usually occurs when nociceptors, specialized sensory receptors, are activated by harsh mechanical stimuli, strong cold, intense heat, or a range of chemical stimuli. In recent decades, there has been significant interest in understanding the underlying mechanisms of pain (Vardeh et al. 2016). The neural pathways involved in pain have been extensively researched, including the neurotransmitter systems, receptors, ion channels, and neuromodulators involved (Yam et al. 2018; Hermanns and Printing: Ridderprint BV 2021).

Non-steroidal anti-inflammatory drugs (NSAIDs) are considered one of the most frequently used therapeutics that can be used to treat inflammatory-related pain. Although NSAIDs, their long-term use is associated with several significant adverse effects such as bleeding, peptic ulcers, and gastrointestinal lesions (Wang et al. 2014).

Likewise, opioids are another major class of analgesics used in patients with severe pain which is prescribed for short-term post-operative/neurogenic pain. Opioids have significant side effects such as tolerance, respiratory depression, and constipation. Therefore, alternative therapeutic agents with mild action and fewer side effects are receiving increasing attention (Wang et al. 2014).

Azoles are monocyclic heteroarenes that contain one nitrogen atom and at least one non-carbon atom, such as nitrogen, oxygen, or sulfur, in their ring structure. They belong to a class of nitrogen-heterocycles and are significant compounds in the pharmaceutical and agrochemical industries (Kumar Bhaumik et al. 2021). Pyrazoles are one example of azoles. The Knorr pyrazole synthesis reaction, a widely recognized chemical reaction, was employed in 1883 to produce the first pyrazole compound, which showed antipyretic bioactivity (Ruffell et al. 2021). Pyrazole synthesis involves combining a 1,3-dicarbonyl compound with hydrazine through condensation (Dadiboyena and Nefzi 2011).

Pyrazole groups are present in various pharmacological agents, including antimicrobials, hypoglycemic agents, and anti-inflammatory agents, due to their well-established biological properties that result in analgesic, anti-inflammatory, and anti-depressant effects (El-Sayed et al. 2012). Bipyrazoles, a specific type of pyrazole compound, have also demonstrated advantageous therapeutic effects in the treatment of infectious diseases, inflammatory conditions, and certain cancers, and have shown anti-diabetic effects (Akhramez et al. 2022).

A novel bipyrazole compound called 2′,3,3,5′-Tetramethyl-4′-nitro-2′H-1,3′-bipyrazole (TMNB) was synthesized through the classical Knorr pyrazole synthesis method. This involved combining 0.17 grams (1 mmol) of 5-hydrazino-1,3-dimethyl-4-nitro-1H-pyrazole with 0.11 grams (1.1 mmol) of acetyl-acetone. The structure of this compound was confirmed using various analytical techniques, including infrared (IR) spectroscopy, HNMR, CNMR, and mass spectrometry (Salameh et al. 2020).

The biological properties of TMNB, specifically its anticancer activity, have only been investigated in human MCF-7 breast cancer cells and human K562 chronic myelogenous leukemia cells (Salameh et al. 2020). Additionally, TMNB has exhibited potential antidiabetic and anti-inflammatory properties (Alqudah et al. 2023).

Despite the demonstrated antidiabetic, anti-inflammatory, and anticancer properties of TMNB, its potential antinociceptive effects have not been investigated. Therefore, the goal of this study was to explore the antinociceptive activity of TMNB on adult male mice using various nociception models.

Methods

Experimental animals

Adult male Swiss albino mice weighing between 24–28 g, which were bred and raised at the Animal House Unit of the Hashemite University, were utilized for a study. The mice were kept under controlled temperature conditions of 21 ± 1 °C and a 12-hour light/dark schedule with the lights being on between 0600 and 1800 hours. The mice were given free access to food and water. To reduce stress, the mice were moved to the testing area one hour before the experiments to acclimate to the laboratory conditions. During the animal tests, two trained observers who were unaware of the experimental design monitored the mice to prevent bias. The tests were conducted in a soundproofed room.

Acetic acid-induced writhing test

In this experiment, six groups of mice were treated intraperitoneally (i.p.), as described previously (Koster et al. 1959). The treatments were as follows: the first group (vehicle control) received a solution of 5% DMSO and 95% distilled water. TMNB was prepared in the same vehicle solution, and then administered to groups two to five in doses of 50, 100, 150, and 200 µg/kg, respectively. The sixth group received a dose of 100 mg/kg of acetylsalicylic acid (A5376, Sigma-Aldrich, Merck, USA). The doses of TMNB were determined based on previous pilot experiments. After 60 minutes, acetic acid 0.6% (A6283, Sigma Aldrich, Merck) was administered to each mouse in a dose of 10 mL/kg of body weight, following the vehicle, TMNB, or acetylsalicylic acid (ASA) treatments. In this experiment, the researchers recorded and counted complete writhing in mice for 30 minutes after they were treated with acetic acid. Writhing was defined as body

elongation, abdominal contraction, pelvis-ending twisting, and/or trunk twisting associated with limb extension. The percentage of inhibition of writhing (PIW) was calculated using the following formula: % Inhibition = [(C - T) / C] × 100 Where C is the number of writhing episodes in the control group and T is the number of writhing episodes in the treated group.

Hot plate test

In this test, the treatment was given to eight groups of mice during the test. The vehicle control group, group one, was administered 5% DMSO in distilled water. Groups two to five received TMNB doses of 50, 150, 150, and 200 µg/kg, respectively. Group seven was given Morphine (M8777, Sigma Aldrich, Merck) dissolved in sterile saline and administered i.p. at a dose of 5 mg/kg. To test for the involvement of the opioidergic system, groups six and eight received naloxone hydrochloride, a non-selective opioid receptor antagonist, at a dose of 5 mg/kg i.p., 15 minutes before treatment with TMNB (200 µg/kg) and morphine (5 mg/kg i.p.), respectively (Zakaria et al. 2016). All treatments were administered i.p. 60 minutes before exposing the mice to testing with the analgesiometer hot plate (55 ± 5 °C). To measure the reaction time, the time interval between when the animal was placed on the hot plate and when it began paw licking was recorded. The reaction time was measured twice: once before treatment and again 60 minutes after treatment. The results were presented as the percentage increase in baseline, calculated using the formula: Percentage increase in baseline = ((A–B) / B) × 100. In this formula, a represents the reaction time after treatment, and B represents the reaction time before treatment.

Formalin-induced paw licking test

The first group (referred to as the vehicle control) was given distilled water containing 5% DMSO. The second to fifth groups received 50, 100, 150, and 200 µg/kg of TMNB, respectively. The sixth group was treated with morphine (5 mg/kg), and the seventh group received ASA (100 mg/kg). To investigate the involvement of the opioidergic system (Tjølsen et al. 1992), we conducted the following experiment: The nonselective opioid receptor antagonist, naloxone hydrochloride (5 mg/kg, i.p.), was administered 15 minutes before administering TMNB (200 µg/kg) to group eight or morphine (5 mg/kg) to group nine. All treatments were given through intraperitoneal injection. After 60 minutes, 20 µL of 2.5% formalin (Sigma Aldrich, Merck, purity 37%, 103999) was injected into the subplantar region of the right hind paw to induce pain. The nociceptive response was measured by recording the time spent by each mouse licking the formalin injection site. The licking times were recorded in two phases: the first phase was 0–5 minutes after formalin injection, representing the early (neurogenic) phase, and the second phase was 15–30 minutes after formalin injection, representing the late (inflammatory) phase. To calculate the percentage of licking inhibition, we used the following formula: Percentage of inhibition of licking (PIL) = [(Licking time (control) – Licking time (treatment)) / Licking time (control)] × 100.

Capsaicin-induced paw licking test

The purpose of this test was to evaluate the antinociceptive effect of TMNB on the vanilloid receptor, also known as the Transient Receptor Potential Vanilloid type-1 (TRPV1). As previously mentioned six groups of mice (n=6 mice/group) were treated through intraperitoneal injection as follows: The first group (the vehicle control) received distilled water with 5% DMSO, while groups two to five received TMNB at doses of 50, 100, 150, or 200 µg/kg, respectively. Group six was given the TRPV1 receptor antagonist, capsazepine (211280, Sigma-Aldrich, Merck), at a dose of 0.17 mmol/kg through intraperitoneal injection. Sixty minutes after this treatment, 20 µL (1.6 µmol/paw) of capsaicin (211275, Sigma Aldrich, Merck) was injected into the right hind paw of each mouse through the intraplantar route. The nociceptive response to capsaicin was measured as the time each mouse spent licking or biting the injection site, and these measurements were recorded 0–5 minutes after the injection of capsaicin.

Glutamate-induced paw licking test

This test was conducted to evaluate the antinociceptive effects of TMNB through the glutamatergic receptors. The experiment was conducted on five groups of mice, each consisting of six mice. The first group was given 5% DMSO intraperitoneally (i.p.) as vehicle control, while the remaining four groups received varying doses of TMNB (50, 100, 150, and 200 µg/kg) i.p. After 60 minutes, 20 µL of glutamate (1446600, Sigma-Aldrich, Merck) was injected into the ventral surface of the right hind paw of each animal. The mice were then observed for 15 minutes, and the time each mouse spent licking and/or biting the glutamate injection site was recorded (28, 30).

Statistical analysis

The Prism 5 software from GraphPad Software in the USA was used for data analysis. All analyzed parameters were tested for the normality of the data using Kolmogorov-Smirnov test. The data are presented as the mean ± standard error of the mean (SEM). Differences between groups were analyzed using one-way ANOVA, followed by Tukey's posthoc test. Statistical significance was set at $P<0.05$.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Results

TMNB reduced writhing induced by acetic acid

The intraperitoneal administration of TMNB at doses of 100, 150, and 200 µg/kg resulted in significant reductions (P<0.001) in the number of acetic acid-induced writhing episodes in treated mice as compared to the control group (as shown in Fig. 1). Moreover, the effect of TMNB was found to be dose-dependent (P<0.05). The group receiving the highest dose of TMNB showed a reduction of about 66% in the percentage of writhing inhibition compared to the control group (P<0.001). Conversely, the reduction in nociceptive behavior caused by the reference drug, ASA (100 mg/kg), was approximately 71% in comparison to the control group (Fig. 1).

TMNB increased latency time in the hot plate test

Administration of TMNB at doses of 50, 100, 150, and 200 µg/kg resulted in a significant (P<0.001) increase in the time it took for the animals to lick their posterior paw when placed on a hot plate (Fig. 2). Furthermore, the effect of TMNB was found to be dependent on the dosage (P<0.05). Among all the doses, the highest dose of TMNB (200 µg/kg) produced the most significant reduction in pain compared to the control group. The TMNB-treated group (200 µg/kg) showed a 68.2% increase in baseline latency time, which was statistically significant (P<0.001). In comparison, morphine, which was used as a reference drug, led to a greater increase in latency time (95.1%).

To investigate the antinociceptive mechanism of TMNB, we administered naloxone (an opioid antagonist) to mice 15 minutes before administering either 200 µg/kg of TMNB or 5 mg/kg of morphine, followed by subjecting the mice to the hot plate test. Treatment with naloxone significantly blocked the morphine-induced increase in latency time (P<0.001) compared to morphine treatment alone. Similarly, naloxone significantly blocked the increase in latency time induced by TMNB (200 µg/kg) (P<0.001) compared to TMNB treatment alone (Fig. 2).

TMNB reduced licking time in both the early and late phases after formalin

In this study, mice were given different doses of TMNB (100, 150, and 200 µg/kg) and were observed for reductions in licking times after formalin injection. The results showed that all doses of TMNB caused significant reductions in both early and late phases of licking times (Fig. 3) in a similar pattern. Morphine, which was used as a reference drug, also produced a significant reduction in both phases. However, acetylsalicylic acid did not show any significant effect on early phase licking time (Fig. 3A), but it did produce a significant reduction in the late phase (P<0.001, Fig. 3B).

To investigate the role of the opioidergic system in the pain-relieving effects of TMNB and morphine, mice were given naloxone 15 minutes before receiving either TMNB (200 µg/kg) or morphine (5 mg/kg) before being subjected to the formalin-induced paw-licking test. The results showed that when naloxone was given before morphine, it significantly increased the licking time in both early and late phases compared to the group that received only morphine (P<0.001). In contrast, when naloxone was given before TMNB, the licking times were significantly reduced in both early and late phases to the same degree (P<0.001),

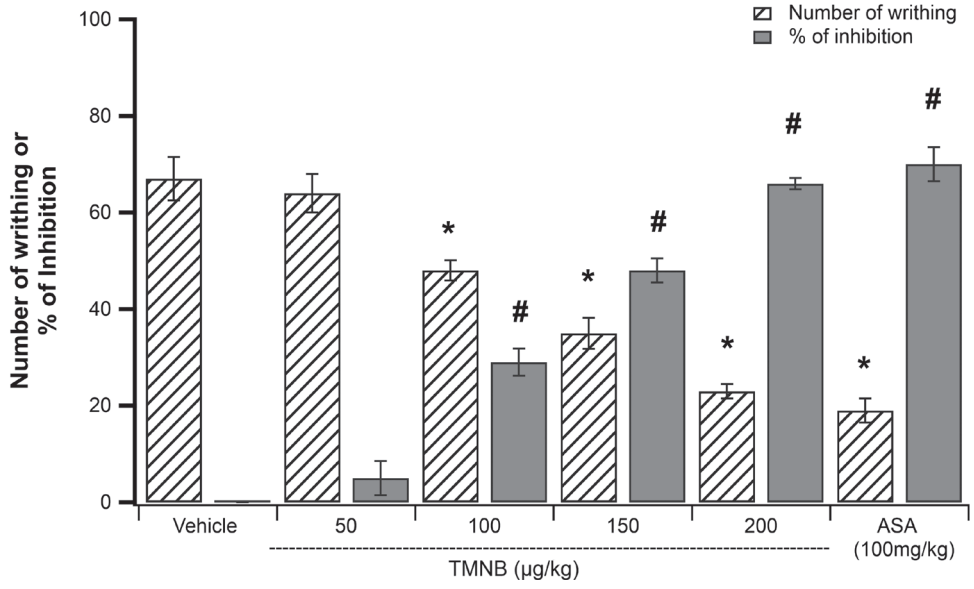

Figure 1. Effect of TMNB on writhing in mice induced by 0.6% acetic acid. Six mice were included in the study and were injected with either 5% DMSO (vehicle), TMNB (at doses of 50, 100, 150, 200 µg/kg i.p.), or acetylsalicylic acid (ASA, 100 mg/kg). V *P<0.001 a significant reduction in the number of writhing compared to the vehicle. Similarly, the percent of inhibition caused by TMNB was also significantly different from the vehicle, with #P<0.001.

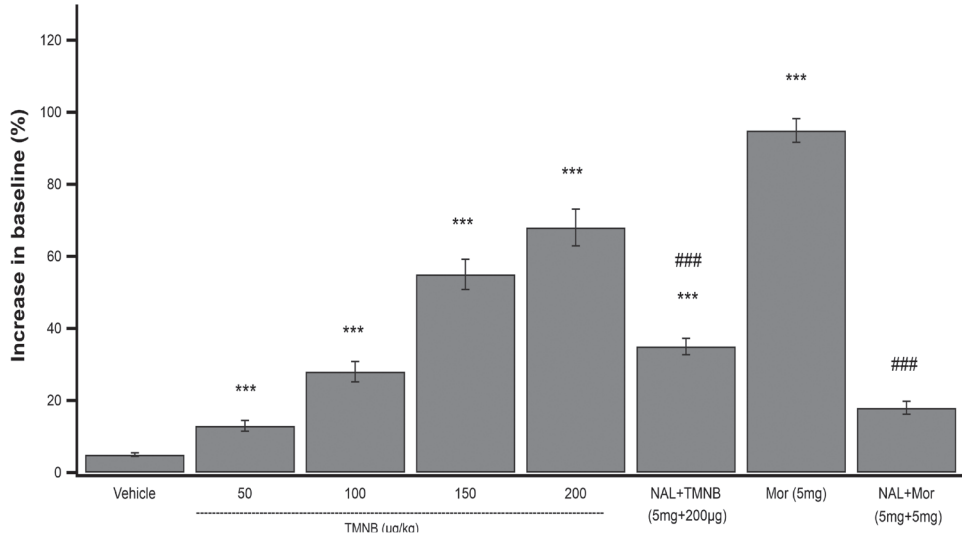

Figure 2. Effect of TMNB on mice (n=6) in the hot plate test. The statistical analysis showed that TMNB caused a significant difference compared to the vehicle (5% DMSO) with ***P<0.001. Additionally, Nal blocked the effect of 200 µg/kg TMNB and 5 mg/kg morphine with ###P<0.001. (NAL: naloxone, Mor: morphine). $ denotes the difference between Nal + Mor compared to Mor alone.

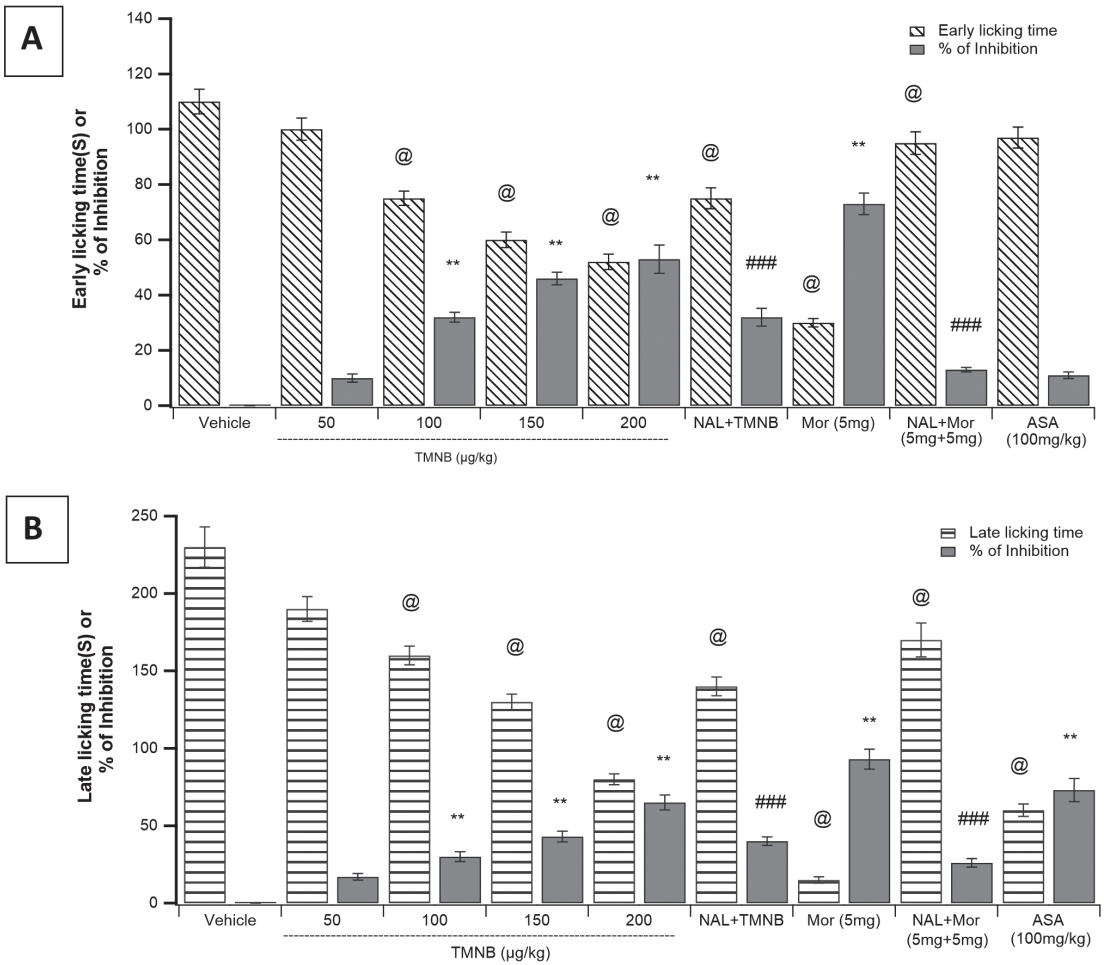

Figure 3. The impact of TMNB on early (**A**) and late (**B**) phases of 2.5% formalin-induced paw-licking behavior in mice (n=6). The mice were given different injections: 5% DMSO (vehicle), TMNB (50, 100, 150, and 200 µg/kg, i.p), morphine (Mor) (5 mg/kg, i.p), or acetylsalicylic acid (ASA, 100 mg/kg, i.p). Naloxone (NAL, 5 mg/kg, i.p.) was administered 15 minutes before the injection with TMNB (200 µg/kg) or Mor (5 mg/kg, i.p.). **P<0.001 indicates a significant difference in the percentage of inhibition compared to the vehicle; @ P<0.001 indicates a significant difference in licking time compared to the vehicle. # # #P<0.001 indicates a significant difference between TMNB + Nal compared toTMNB (200 µg/kg) alone; $ indicates a significant difference between Nal + Mor compared to Morphine alone.

indicating that the antinociceptive effects of TMNB are mediated by a mechanism of action of opioidergic system.

Licking time was reduced with TMNB treatment after capsaicin injection

This experiment was conducted to examine the antinociceptive effect of TMNB on the vanilloid system. Administration of TMNB at doses of 50, 100, 150, and 200 μg/kg significantly (P<0.01) reduced the licking time in mice (Fig. 4). The effect of TMNB was found to be dependent on the dosage. Moreover, treatment with capsazepine, a capsaicin antagonist, significantly (P<0.01) reduced the licking time in mice, with a percentage reduction of 77.1% (Fig. 4).

TMNB reduced paw licking time after glutamate injection

The administration of TMNB at doses of 100, 150, and 200 μg/kg resulted in a significant reduction (P<0.001) in the paw-licking time of mice injected with glutamate (Fig. 5). The antinociceptive effect of TMNB on glutamate injection was found to be dependent on the dosage (P<0.05).

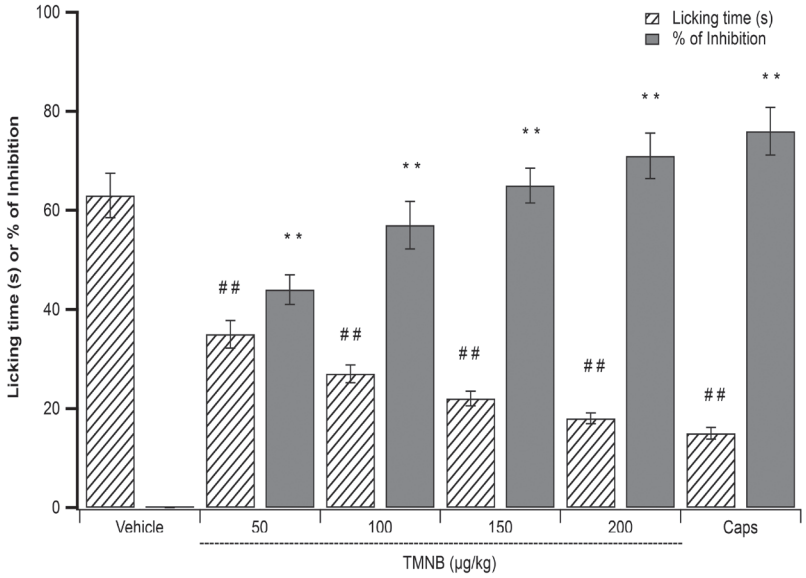

Figure 4. Effect of TMNB on capsaicin-induced paw licking in mice. The animals were divided into five groups, and each group contained six male mice. The mice were injected with either 5% DMSO (vehicle), TMNB at doses of 50, 100, 150, and 200 μg/kg (i.p.), or the capsaicin antagonist, capsazepine (Capsz, 0.17 mmol/kg, i.p.). After 60 minutes, the mice were challenged with capsaicin, and the licking time was recorded. **P<0.01 indicating a significant reduction of the percent of inhibition compared to the control group. ##P<0.01 indicating a significant reduction in licking time compared to the control group.

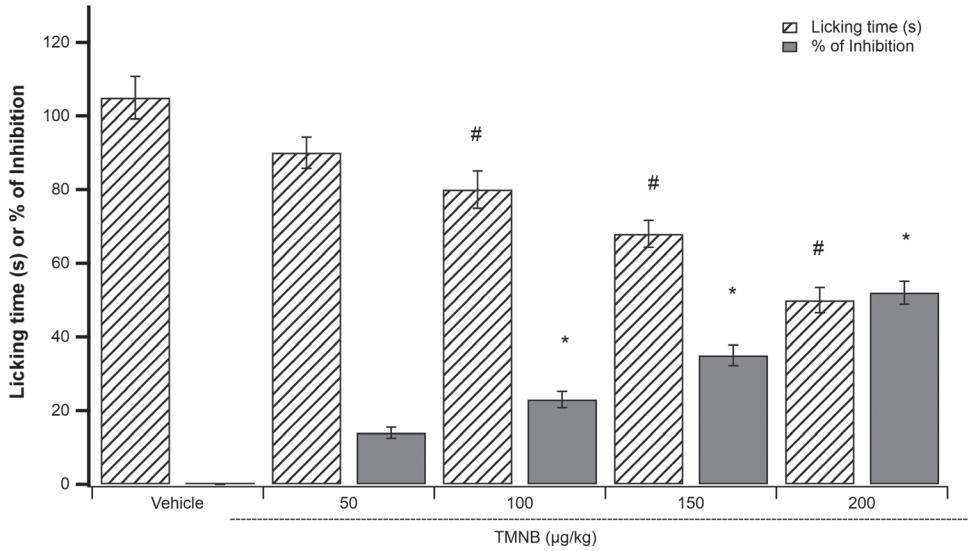

Figure 5. Effect of TMNB on glutamate-induced paw licking in mice. The animals were divided into five groups, and each group contained six male mice. The mice were injected intraperitoneally with either 5% DMSO (vehicle) or TMNB at doses of 50, 100, 150, and 200 μg/kg. After 60 minutes, 20 μL of glutamate was intraplantarly administered into the right hind paw, and the licking time was recorded. *P<0.001 indicating a significant reduction of the percent of inhibition compared to the control group. #P<0.001 indicating a significant reduction in licking time compared to the control group.

Discussion

The findings of this study indicate that TMNB has antinociceptive effects, which have not been previously demonstrated. The results show that TMNB inhibits the nociceptive effects of acetic acid, which is a well-known inducer of pain. Acetic acid-induced writhing test, which works by directly activating a non-selective ion channel in peripheral nociceptive fibers (Wang et al. 2014). Additionally, acetic acid can indirectly stimulate nociceptors by releasing several autacoid mediators, including serotonin, histamine, bradykinin, and substance P, and increasing levels of prostaglandins (E2 and F2). Acetic acid also triggers the production of lipoxygenase, which further activates peripheral nociceptive neurons (Wang et al. 2014). The nociceptive effects of acetic acid are attributed to the release of nitric oxide (NO) and inflammatory cytokines such as TNF-α, IL-1β, and IL-8 from peritoneal mast cells and macrophages into the peritoneum (Larson et al. 2000). This, in turn, activates peripheral nociception receptors (Ribeiro et al. 2000). According to Le Bars et al. (2001), the sensation of nociceptive pain was experienced rapidly following the intraperitoneal (i.p.) injection of acetic acid (Le Bars et al. 2001). This study observed that acetic acid-induced physical responses in the form of writhing. The reflex mechanism involves the conversion of arachidonic acid into eicosanoids, which are crucial mediators of pain, by lipoxygenases (LOX) and cyclooxygenases (COX). Eicosanoids stimulate the release of prostaglandins and other mediators from the peritoneal cavity. In addition, eicosanoids produced by COX and LOX cause hyperalgesia by sensitizing the peripheral pain neurons (Ricciotti and Fitzgerald 2011; Jang et al. 2020). Nonsteroidal anti-inflammatory drugs (NSAIDs) like ASA have been found to inhibit the synthesis of prostaglandins by blocking the activity of COX and reducing the body's response to pain (Meek et al. 2010). In this study, it is possible that TMNB inhibited acetic acid-induced nociception by suppressing the peripheral levels of COX and LOX. This would have indirectly reduced the production of pain mediators, such as prostaglandins, suggesting that TMNB has a peripheral antinociceptive effect. However, caution should be taken as other non-analgesic agents, including antihistamines, anticholinergic agents, and muscle relaxants, may produce false positive results in the acetic acid-induced writhing test (Sani et al. 2012). Therefore, the study utilized other methods, such as formalin and hot plate tests, to determine whether the antinociceptive effects of TMNB are centrally or peripherally mediated.

The hot plate test is a method used to evaluate the supraspinal and spinal biological properties of novel drugs, without inputs from peripheral nociception neurons. hot plate test, in which paw licking and jumping behavior in response to hot plate stimulation occurs at the supraspinal level. The duration of the licking and jumping behavior can be used to assess animal responses. In this test, the latency time of mice can only be prolonged by centrally acting opioid-like drugs and not by peripherally acting drugs. A drug or substance that increases the latency of mice feeling discomfort on the hot plate indicates centrally mediated activity, similar to that of opioids (Lavich et al. 2005; Abdul Rahim et al. 2016; Vidal-Torres et al. 2019). In this study, the results showed that TMNB prolonged the latency of mice in feeling discomfort on the hot plate, indicating the centrally mediated antinociceptive activity of TMNB. Based on the results of the acetic acid-induced writhing test and the hot plate test, it is suggested that TMNB may exert both central and peripheral antinociceptive activity.

We conducted the formalin-induced paw-licking test to confirm the antinociceptive activity of TMNB at both central and peripheral levels. The formalin-induced paw-licking has two distinct phases of nociceptive behavior that occur after formalin injection. The first phase begins immediately after injection and lasts for approximately 5 minutes which characterized by a direct effect of formalin on nociceptors, and prostaglandin does not have a role in this phase (Hunskaar and Hole 1987; Mehanna et al. 2018), while the second phase occurs 15–30 minutes after injection and is caused by the release of inflammatory mediators, such as histamine, prostaglandins, and bradykinins (Mohammad-Zadeh et al. 2014; Dehkordi et al. 2017; Balali Dehkordi et al. 2019). Our findings demonstrate that TMNB effectively suppresses nociceptive responses in both phases, indicating its potential as a centrally-acting analgesic agent. This is significant because opioids, which are centrally-acting agents, are known to inhibit both phases, while drugs that act peripherally only inhibit the second phase (e.g., NSAIDs). Furthermore, our study shows that pre-treatment with the non-selective opioid antagonist, naloxone, significantly reduced the antinociceptive effect of morphine and TMNB. These results suggest that TMNB mediating its action through the opioidergic system.

To investigate whether TMNB can modulate pain reception through vanilloid receptors and/or the glutamatergic system, we conducted capsaicin-induced and glutamate-induced paw-licking tests. Capsaicin activates TRPV1 receptors, which causes the influx of Ca2+ and Na+ (mostly Ca2+), and activates C- or Aδ-fibers in afferent neurons, leading to neurogenic pain. This test induces both hyperalgesia and analgesia (Tominaga 2007; O'Neill et al. 2012). In addition, capsaicin is known to induce the release of pro-inflammatory peripheral mediators such as neuropeptides, neurokinins, nitric oxide, and excitatory amino acids like glutamate and aspartate. Additionally, capsaicin can transduce nociceptive pain from vanilloid receptors to the spinal cord (Caterina and Julius 2001). Inflammatory mediators can also activate and sensitize vanilloid receptors, leading to an amplification of the inflammatory mediator levels and creating a loop that potentiates nociception (Cortright and Szallasi 2004; Tominaga 2007)

Our findings showed that TMNB had a significant inhibitory effect on capsaicin-induced paw licking, as evidenced by the results in Fig. 5. This inhibition was comparable to the effect produced by capsazepine, a well-known TRPV antagonist. These results suggest that TMNB has the ability to interfere with the transmission of nociception through vanilloid receptors and block the release or activity of the inflammatory agents induced by capsaicin. Furthermore, the observation that TMNB can ameliorate the writhing responses and the inflammatory responses in the second

phase of the formalin test is consistent with the notion that TMNB can interfere with the activity of inflammatory mediators. The fact that capsazepine also reduced the licking time in our experiments confirms the validity of the capsaicin-induced pain model and supports the efficacy of our treatment with TMNB. To investigate the ability of TMNB to interfere with glutamate-mediated nociceptive transmission, we conducted a glutamate-induced paw-licking test. In this test, glutamate injection induced licking behavior in mice, but this behavior was significantly reduced by different concentrations of TMNB. This test is similar to the late phase of the formalin test, which is known to be inhibited by glutamate receptor antagonists such as MPEP or CPCCOE (Bhave et al. 2001; Neugebauer 2002). Interestingly, this effect was limited to the late phase of the formalin test and did not affect the neurogenic early phase

Glutamate-induced paw-licking test is another test that is used in pain research. Glutamate is an important excitatory neurotransmitter present at high levels in the central nervous system, and its activation leads to nociceptive transmission. Glutamate receptors are distributed in both the central and peripheral nervous systems (Neugebauer 2002). Our study demonstrated that TMNB has the ability to inhibit glutamate-induced nociceptive responses, suggesting that it can modulate pain transmission mediated by the glutamatergic system. This effect may be due to TMNB's interaction with glutamate receptors or its ability to interfere with the release of NO, which is known to play a role in pain signaling. However, further investigations are required to fully understand the role of NO and its downstream signaling in the antinociceptive activity of TMNB.

Conclusions

Our results show that the TMNB has antinociceptive effects through various physiological pathways in both the peripheral and central nervous systems. Specifically, it acts as an opioid receptor agonist and is capable of inhibiting the vanilloid and glutamatergic receptor systems.

Ethics approval

All animal procedures were approved by the Animal Research Ethics Committee at the Hashemite University (IRB number:HU[[48/2021) and were in accordance with the guidelines of the U.S. National Institutes of Health on the use and care of laboratory animals and with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines (https://arriveguidelines.org).

Conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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Author contribution

AA and YB contributed to the conception and design of the study AA, EYQ, MAW, and KAS carried out animal experiments and acquisition of data. YB & OG analyzed and interpreted data. AA & OG prepared the primary draft of the manuscript.

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