



Original Article

Evaluation of In-Vivo Anti-Inflammatory Activity of Methyl 2-(5-Butyl-6-Thioxo-1, 3, 5-Thiadiazinan-3yl), Butanoate

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ABSTRACT

Inflammation is the body's way of fending off damage. Nonetheless, continuous and increasing inflammation may lead to the development of some chronic illnesses. It is still needed to evaluate new and safe anti-inflammatory therapies because existing medications have major side effects. **Objective:** To examine a novel compound's anti-inflammatory properties of methyl 2-(5-butyl-6-thioxo-1, 3, 5-thiadiazinan-3yl), butanoate (MBTTB) in albino mice models. **Method:** To test the anti-inflammatory effects in vivo, carrageenan-induced paw edema in albino mice of both sexes was utilized, and inflammation was measured by using Digital Plethysmometer. After extraction of carrageenan induced paw edematous, tissue level of inflammatory mediators like TNF- α , IL-1 β , NF- κ β , and COX-2 was ascertained by Western Blot analysis. **Result:** Based on *in-vivo* anti-inflammatory effects, compound MBTTB was shown to exhibit a maximum percentage inhibition of 16.7% after 1 hour, which is comparable to the standard drugs 12.8%. Following three hours, compound MBTTB showed the highest percentage inhibition of 23.2%, while the standard was 14.5%. Compound MBTTB showed the highest percentage inhibition after five hours (26.2 %), while the standard was 15.4%. The expression of many mediators' proteins, including TNF- α , IL-1 β , NF- κ β , and COX-2, was ascertained. The computational analysis was carried out to forecast the level of decline of various mediators, including Tumor Necrosis Factor- α , (TNF) Interleukin-1 β , NF- κ β , and COX-2. **Conclusion:** When considered as a whole, our current research indicates that MBTTB is a viable option for preventing inflammation in mice that has been caused by carrageenan in edematous paw.

INTRODUCTION

The body uses inflammation as a reflexive mechanism in response to harmful situations such microbial infections, tissue damage, antibody binding to antigens, and other unpleasant circumstances [1-5]. The body releases a variety of bioactive mediators at the site of damage during this reaction, including, interleukin-1 (IL-1 β), Nuclear Factor Kappa-Light-Chain -Enhancer, and Tumor Necrosis Factor (TNF- α) [6-11]. These mediators help the body by inducing an inflammatory response through a variety of processes in overcoming negative triggers and ensuring repairing. Long-term, severe inflammation, however, does

not go away and is a significant cause of morbidity and death [12, 13]. One of the various approaches employed to reduce the frequency and incidence of Distinctive gastrointestinal (GI) side effects of anti-inflammatory medications issues is increasing cyclooxygenase-2 (COX-2) specific Non-steroidal anti-inflammatory drugs (NSAID) prescriptions. Because specific inhibitors of COX-2 lower the recognized indicators of pain and inflammation, the COX-2 enzyme is being targeted for the treatment of several illnesses. Furthermore, it is known that lipoxygenases (LOXs) metabolize arachidonic acid,

producing a mixture of leukotrienes (LTs), which are powerful inflammatory mediators [14, 15]. A few NSAIDs and corticosteroids are currently used to treat inflammatory conditions that might be acute or chronic. However, due to the harmful side effects, prolonged use is not advised [16, 17]. Therefore, it is important to find better and more recent anti-inflammatory medications that have minimal to no side effects. The core of medicinal chemistry is the creation and research of novel therapeutic agents. The cyclic dithiocarbamates Tetrahydro-2H-1,3,5-thiadiazine-2-thiones (THTTs) are bioactive heterocycles that have attracted attention recently due to reports of their antibacterial, antiatherosclerosis neuroprotective, anticancer, and anti-inflammatory properties. Thiadiazinethione derivatives' superior lipid solubility and straightforward enzymatic hydrolysis have led to considerable research on them as prodrugs with antibacterial activity. These substances, which include milneb and sulbentine, have been shown to have antifungal properties. The majority of them have been described as strong cell cycle disruptors in the field of cancer research. The anti-nociceptive and anti-inflammatory activity of 5-amino-3-phenyl-1,3,5-thiadiazine-2-thione have been established. It has been discovered that diacetic acid (2,2'-(2-thioxo-1,3,5-thiadiazinane3,5-diyl) glycine linked thiadiazinethione derivative is an alternate pain reducing and anti-inflammatory medication with fewer ulcer causing effect than aspirin. The actions are linked to these species because they are known to divide into Dithiocarbamates and isothiocyanates in the biosystem, both of which have therapeutic benefits in both acute and chronic inflammatory diseases. Researchers have also become interested in certain fused ring triazolothiadiazine heterocycles because of their common usage as analgesics and anti-inflammatory drugs. In keeping with our pursuit of novel bioactive agents, we have directed our efforts toward the design and synthesis of new thiadiazinethione analogues, which we will then explore for their possible applications as antinociceptive and anti-inflammatory drugs as well as antagonist of COX-2 and 5-LOX, which may result in higher strength and preference having less harmful complications. This current study also concerns the bioavailability of two active scaffolds (dithiocarbonates and isothiocyanates) that enable the fair regulation of several targets as opposed to a single ligand. Furthermore, the carboxylic acid functionality of our proposed molecules is comparable to that of NSAIDs that are therapeutically efficacious, such as aspirin, diclofenac, and ibuprofen [16]. All examined compounds showed low toxicity in mice, as the Effective Dose (ED50) is larger than 1,000 mg/kg and the fatal dose of thione derivatives in mice is greater than 5000-10,000 mg/kg. All studied thione

derivative compounds resulted minimal toxic effect in these animals, with median effective dose (ED50) >1,000 mg/kg and lethal dosages >5,000-10,000 mg/kg [18]. Considering this perspective, MBTTB a derivative of cyclic dithiocarbamates, THTTs was considered a potential opponent of inflammation [19].

METHODS

Mice and the Treatment Detail

Albino mice of either gender, three to five weeks' old were employed in this research study. They were housed in an animal home with a 12 h light/12 h dark cycle, maintained at 22.0 ± 2.0 °C, and limitless availability of food and water. The trial protocols were approved by Khyber Medical University's Basic Medical Sciences Research and Bioethics Committee vide a reference number KMU/IBMS/IRBE/7thmeeting/2023/1209-6 and activities were carried out in compliance with Declaration of Helsinki in 1964. The mice were randomly divided into six groups (n = 6 mice). 1. Mice treated with saline: Control (Ctrl), 2. Mice treated with car, 3. Mice treated with Car + Diclofenac sodium (Car+ Diclofenac sodium 50 mg/kg), 4. (Car + MBTTB 10 mg/ kg), 5. (Car+ MBTTB 50 mg/kg) and 6. (Car + MBTTB 100 mg/kg). The mice underwent Intraperitoneal Car Injections for a consecutive 15 days at a dose of 10 ml/kg each day, Saline (0.9% solution) was given intraperitoneal (I.P.) to control mice once a day for 15 days, while MBTTB was diluted with distilled water and supplied Intraperitoneal every day for fifteen days as shown in table 1.

Table 1: Animals grouping and drug dose description

Animal Groups	Dose Description
Group-1 (control)	Normal Saline
Group-2	Carrageenan 10 ml/kg
Group-3	Diclofenac Sodium 50 mg/kg + Carrageenan 10 ml/kg
Group-4	MBTTB 10 mg/kg + Carrageenan 10 ml/kg
Group-5	MBTTB 50 mg/kg + Carrageenan 10 ml/kg
Group-6	MBTTB 100 mg/kg + Carrageenan 10 ml/kg

Behavioral Experiment: Carrageenan-Induced Model

To evaluate the properties that reduce inflammation of MBTTB, mice were given a phlogestic substance (carrageenan) to induce paw edema. Carrageenan-induced paw edema occurs in two phases. Bradykinin, histamine, cytokines, and serotonin are released during the initial stage of edema, while prostaglandins are secreted during the subsequent stage. Important inflammatory mediators that lead to vasodilation and increased vascular permeability include histamine and serotonin. In mice with Paw edema caused by phlogestic agent, the anti-inflammatory properties of MBTTB were investigated. The experiment began with two hours of no feeding for the mice. Vehicle (10 ml/kg), diclofenac (50 mg/kg, I.P.), and

compound extract (10, 50, and 100 mg/kg) were given to the mice (i.p.). The paw's sub plantar area received 0.1 milliliter of a 1% solution, which is the phlogistic agent. The edema was assessed using a digital plethysmometer at 1, 3, and 5 hours following the injection of the phlogistic substance. Six mice of either sex were distributed into six groups (n=6) percent inhibition was measured using following equation

$$\% \text{inhibition} = (C - T/C) \times 100$$

Where T and C represent the relative increases in paw volume in the test and control treatment groups.

An analysis using a western blot was conducted. To put it briefly, the Bio-Rad protein assay solution was used to quantify the paw homogenates. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to fractionate the homogenates (30 µg protein) utilizing a Bio-Rad Tetra cell (15 percent; Bio-Rad, Hercules, CA, USA). Following transfer, the membranes were incubated with primary antibodies and blocked in 5% skimmed milk (or BSA). for 12 hours at 4 °C, and when secondary antibodies conjugated with horseradish peroxidase reacted, cross-reacting proteins were detected using Enhanced chemiluminescence (ECL). Santa Cruz Biotechnology was the source of the primary antibodies, which included mouse-derived ones like NF-κB, TNF-α, IL-1β, and cox-2, among others (Santa Cruz, CA, USA). Following the application of membrane-derived secondary antibodies, the manufacturer's instructions were followed for the use of Bio-ECL Rad's detection reagent for visualization. ImageJ software was used to analyze the bands using densitometry (ImageJ-win 64 1.8). The density values were calculated in arbitrary units (A.U.s) with respect to the untreated control.

Statistics were analyzed using One Way ANOVA, and GraphPad Prism was used to carry out Dunnett's post hoc analysis. Using ImageJ, the density of the Western blot data was calculated. The data were presented as mean ± SEM.

RESULTS

Behavioral Activity

Carrageenan-Induced Inflammatory Model

Using an anti-inflammatory assay, the MBTTB's effectiveness in resistance to the swelling brought on by carrageenan injections into the supplanter region of the tested mice's hind paw was assessed (Table 2). One hour before to injecting carrageenan, the compounds were injected intraperitoneally, and the mice with edema in hind paw were observed one, three, and five hours after the phlogistic agent administration. Time-related changes linked to inflammation generated by carrageenan were significantly reduced by the MBTTB used (p < 0.001). Following one hour (P < 0.001), three hours (P < 0.001), and five hours (P < 0.01) of testing the MBTTB, the 100 mg/kg

dose was more successful in reducing paw edema. The standard dosage of 50 mg/kg of diclofenac also effectively decreased inflammation (P < 0.001). Percent inhibition value showed that the MBTTB was assessed as having more potency in the third and fifth hours (P < 0.001) (Table 2).

Table 2: Anti-Inflammatory Effect of MBTTB on Paw Edema Induced by Carrageenan in Mice

Treatment group	Dose (mg/kg)	1 st h		3 rd h		5 th h	
		Volume (ml)	Inhibition (%)	Volume (ml)	Inhibition (%)	Volume (ml)	Inhibition (%)
N Saline	10	0.1775	---	0.1725	---	0.1625	---
Diclofenac	50	0.165	12.8 ± 0.06	0.162	14.5** ± 0.04	0.152	15.4*** ± 0.004
MBTTB	10	0.177	5.2 ± 0.006	0.152	11.6* ± 0.002	0.145	13.9** ± 0.005
	50	0.172	12.6 ± 0.004	0.137	20.3*** ± 0.002	0.127	21.6*** ± 0.002
	100	0.1725	16.7 ± 0.004	0.132	23.2*** ± 0.005	0.12	26.2*** ± 0.005

The data, which are shown as mean ± SEM (n = 6), is subjected to one-way ANOVA and then the Dunnet post hoc test using GraphPad Prism. The results show that *** = p ≤ 0.001, ** = p ≤ 0.01, and * = p ≤ 0.05.

Western Blot Analysis

The anti-inflammatory efficacy of MBTTB in the carrageenan-induced paw edema activity in mice was further examined by assessing the levels of pro-inflammatory cytokines TNF-α, IL-1β, NF-κβ, and COX2. GPDH (Gly-Asp-Pro-His) was used as positive control. This was done using western blot analysis. Results of the assessment indicated that the chemical significantly (P < 0.01) decreased TNF-α, IL-1β, NF-κβ, and COX2 compared to the vehicle group given carrageenan. As the conventional positive control, 50 mg/kg of diclofenac sodium reduced (P < 0.001) the expression of TNF-α, IL-1β, NF-κβ, and COX2 as shown in (Figure 1). The results of the western blotting demonstrate that, in comparison to mice administered with vehicle, the MBTTB at a dose of 10, 50, 100 mg/kg considerably reduced the level of TNF-α, IL-1β, NF-κβ, and cox2 proteins (p < 0.01, p < 0.001).

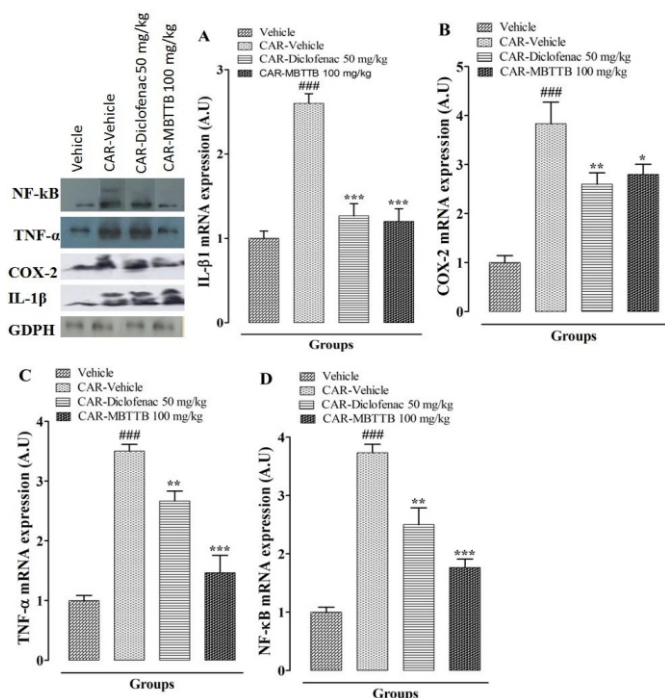


Figure 1: Effect of MBTTB on the mRNA level of IL-1 β (A), COX-2 (B), TNF- α (C), and NF- κ β (D) in the inflamed hind paws of mice. GPDH was used as the loading control.

The relative arbitrary unit (A.U.) is used to display the results. The bars show the mean expression in A.U. \pm SEM.

$P < 0.001$ in relation to the group that received vehicle treatment.

*** $P < 0.001$ in comparison to the Car + Vehicle group.

DISCUSSION

Numerous disorders, including rheumatoid arthritis, autoimmune diseases, inflammatory bowel disease, and malignancies, are associated with inflammatory processes. Corticosteroids, NSAIDs, and some other anti-inflammatory drugs are the current drugs that can stop and slow the growth of inflammation, but using them chronically comes with additional side effects. As such, encouraging novel entities that target inflammation remains a difficult endeavor. Thus, the current investigation was carried out to find a novel and superior anti-inflammatory drug. The newly synthesized compound MBTTB was assessed using an inflammatory model as a potential anti-inflammatory agent. For a very long time, the carrageenan-induced inflammatory model has been the most extensively used model for evaluating the anti-inflammatory qualities of numerous possible therapeutic compounds. Inflammatory mediators, prostaglandins, and the release of lysosomal enzymes were the two phases of the carrageenan-induced inflammatory reactions. The application of compound (100 mg/kg) as a pretreatment significantly and comparably reduced the paw edema and different stages of acute and chronic inflammation caused by the use of the aforementioned compound. When

compound (100 mg/kg) was applied as a pretreatment, the different stages of acute and chronic inflammation and paw edema brought on by the use of the previously described phlogistic agent were markedly and comparably reduced. All inflammatory models showed that the MBTTB had a notable inhibitory effect on the models of inflammation. It was verified in this study that the compound's systemic (I.P.) administration significantly reduced the amount of inflammation in mice's acute and chronic inflammation models brought on by carrageenan [6]. The effectiveness of the anti-inflammatory agent *in vivo* was evaluated utilizing the conventional mice paw edema model caused by carrageenan. The administration of carrageenan typically results in a biphasic event. Histamine, bradykinin, and serotonin are released during the first phase, which lasts for one to two hours. Prostaglandins and a number of cytokines, including IL- β , IL-6, IL-10, and TNF- α , are produced during the 2nd stage. Here, we examined the impact of MBTTB on paw edema caused by carrageenan. The findings showed that the synthesized chemical effectively reduced the paw edema by likely suppressing the inflammatory mediators at every stage of the inflammation [7, 20]. Additionally, the results of the western blotting demonstrate that at doses of 10, 50, and 100 mg/kg, MBTTB significantly downregulates IL-1 β , IL-6, and TNF- α proteins expression in mice relative to mice given a vehicle. Based on the findings of the western blot analysis, the application of carrageenan markedly increased the expression of inflammatory mediators in paw tissue, that were then greatly decreased by MBTTB. The paw edema was effectively reduced by the MBTTB, according to the data, most likely by the reduction of inflammatory mediators at every stage of the inflammation, alternatively, as an antiphlogistic. The paw edema was effectively reduced by the MBTTB according to the data, most likely by the reduction of inflammatory mediators at every stage of the inflammation alternatively as an antiphlogistic [7].

CONCLUSIONS

The administration of MBTTB treated mice decreased the level of mediators of inflammation like IL-1 β , NF- κ β , and TNF- α and COX2 as in relation to carrageenan treated mice. The results of the study indicate that MBTTB may be useful in the treatment of inflammation because it reduced the levels of many mediators of inflammation, including COX2, NF- κ β , TNF- α , and IL-1 β .

Authors Contribution

Conceptualization: UM

Methodology: HB, RU

Formal analysis: SA, AR

Writing, review and editing: ZS

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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