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Research article

RMD86, a thiophene derivative, promotes antinociceptive and antipyretic activities in mice

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ABSTRACT

Treatment of pain and fever remains an important challenge for modern medicine. Non-steroidal anti-inflammatory drugs (NSAIDs) are the pharmacological options most often used, but their frequent use exposes the patient to serious side effects and dangerous drug interactions. In this context, thiophene derivatives are promising therapeutic alternatives. In this study, we evaluated the *in vivo* and *in silico* antinociceptive and antipyretic properties of **RMD86**, a thiophene derivative. At 100 mg/kg, **RMD86** induced no significant changes in the motor coordination of mice in the Rotarod test. At 25, 50, and 100 mg/kg **RMD86** significantly reduced the number of abdominal contortions induced by acetic acid (antinociceptive activity) in mice when compared to the control. In the formalin test, for the first phase, there was a reduction in licking times at doses of 50 and 100 mg/kg. In the second phase, reduction occurred at all doses. In the hot plate test, **RMD86** (at 100 mg/kg) increased latency time in the first 30 min. For antipyretic activity, **RMD86,** when compared to the reference drug acetaminophen (250 mg/kg), significantly reduced pyrexia at 30, 60, and 120 min, at dosages of 25, 50 and 100 mg/kg. Molecular docking studies revealed that **RMD86** presents a greater number of interactions and lower energy values than both the co-crystallized ligand and the reference drug (meloxicam) against COX-1 and COX-2 isoenzymes. The results give evidence of the analgesic and antipyretic properties like NSAIDs suggesting its potential for pain therapy.

1. Introduction

Pain is characterized as an unpleasant sensory and/or emotional experience associated with either potential or real tissue damage. A painful process involves not only transduction of the nociceptive stimulus but also cognitive and emotional processing by the brain, which makes it sufficiently complex to produce differing behavioral responses [1, 2, 3, 4].

Pain can also be associated with other processes such as inflammation and fever. Inflammation can be described as the release of a variety of chemical mediators, and as cells that activate and recruit other cells to the site of inflammation. Fever is a physiological reaction in response to inflammatory pyrogenic molecules [5, 6, 7, 8].

It is estimated that about 30% of the world population suffers from some form of chronic pain, and these patients require health services five times more often than the rest of the population. For this reason, pain is considered a public health problem [9, 10].

Medicines such as painkillers and non-steroidal anti-inflammatory drugs (NSAIDs) are among the main pharmacological options used to relieve pain. In chronic diseases such as arthritis, osteoarthritis, and gout, the use of these drugs, being only for symptomatic relief, does not reverse inflammatory conditions, this represents limitations in their usage [11, 12]. In chronic inflammatory diseases, the continued use of NSAIDs exposes patients to side effects including undesirable gastrointestinal tract, renal, cardiovascular, and central nervous system outcomes. They can also cause serious drug interactions [11, 13, 14, 15].

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Given this scenario, many research groups seek to develop new drug candidates with more pronounced anti-inflammatory and antipyretic properties, but with fewer adverse effects. Among such new therapeutic alternatives, drugs containing a thiophenic ring in their main structure maintain a prominent position. The commercial NSAIDs tinoridine (used for the treatment of pain and inflammation) and tiaprofenic acid (used especially for treatment of arthritic pain) both contain a thiophenic ring (Figure 1).

Given its potential to become a new therapeutic alternative, investigation of the therapeutic potential of a new thiophene derivative (analogous to tinoridine) as a commercial anti-inflammatory is both important and promising. Despite the structural similarity between RMD86 and tinoridine, the main differential between the two molecules is: the aromatic ring substitution, which can reduce the toxicity throughout the body, specifically the pulmonary system, central and peripheral nervous system, gastrointestinal, cardiovascular, renal, hepatic, dermal, and hematological systems [16]. The ester group at C-3 was maintained, thus maintaining the prodrug characteristic of the new compound. This is also interesting for reducing the typical gastrointestinal effects of classical NSAIDs promoted by the presence of the carboxylic acid group (–COOH), however, without resulting in loss of anti-inflammatory potency [17].

There are already reports in the literature of thiophenic derivatives with analgesic activities, such as a group of thiophenes that have the Transient Receptor Potential Vanilloid Type-1 (TRPV1) modulation mechanism [18]. Tests such as hot plate and tail-flick with these derivatives showed that they were able to promote antinociception at the peripheral and central levels [19]. Other products of this class with antinociceptive and anti-inflammatory activity with a predisposition to bind to enzymes such as COX-2 have also been described in the literature [20].

In the Drug Design Discovery process, the use of Computer-Aided Drug Design techniques is extremely important, helping to predict interactions between drug candidates and their potential pharmacological targets. The virtual screening process, also known as molecular docking, allows optimization and targeting in pharmacological tests, reducing the number of animals used in experiments, and assisting to elucidate probable mechanisms of action for drug candidates [21, 22].

Therefore, it may be interesting to study a new compound called RMD86 belonging to the class of thiophene derivatives but with a chemical structure similar to that of tinoridine and probably with low toxicity due to a change in the original structure [23].

In this context, the aim of this work was to evaluate the antinociceptive and antipyretic activities of the thiophene derivative **RMD86** in nociception and pyrexia models *in vivo*, as well as to investigate (based on molecular docking studies) some of its possible targets.

2. Materials and methods

2.1. Drugs and reagents

Glacial acetic acid (Reagen - Brasil); Morphine hydrochloride (Merck – E. U. A.); Ethanol (IPeFarM/UFPB – Brasil); Formaldehyde 37% (Vetec – Brasil); **RMD86** (UEPB - Brasil); Tween 80 (Vetec – Brasil); Diazepam (Cristália – Brasil); Acetaminophen (Medley - Brasil); Yeast (*Saccharomyces cerevisiae*, Saf do Brasil Produtos Alimentícios Ltd, Brazil).

2.2. Compound RMD86

RMD86 (Figure 2) was synthetically obtained through the procedure previously described [23] followed by de-protection of the primary amine using potassium carbonate (K_2CO_3). Nuclear Magnetic Resonance (NMR) data confirm the structure of **RMD86**. *6-(6-Bromohexanoyl)-2-amino-4,5,6,7-tetrahydro-thieno[2,3-c]pyridine-3-carboxylic ethyl ester* **(RMD86):** $C_{16}H_{22}BrF_3N_2O_3S$. ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.05 (bs, 2H), 4.69 (bs, 3H), 4.51 (s, 1H), 4.38 (s, 0.5H), 4.27 (q, 2H, $J = 7.0$ Hz), 3.79 (t, $J = 5.5$ Hz, 1H), 3.64 (t, $J = 5.5$ Hz, 1H), 3.41 (t, 2H, $J = 7.0$ Hz), 2.81–2.87 (m, 2H), 2.34–2.43 (m, 2H), 1.86–1.92 (m, 2H), 1.63–1.72 (m, 2H), 1.46–1.53 (m, 2H), 1.33 (q, 3H, $J = 7.0$ Hz). ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.3, 162.4, 132.7, 130.5, 114.0, 104.9, 59.6, 44.2, 43.1, 40.8, 39.3, 33.6, 33.0, 32.5, 28.0, 27.8, 24.4, 24.2, 14.5.

2.3. Animals

Male albino Swiss mice (*Mus musculus*), male (30–35 g), were obtained from the Dr. Thomas George vivarium of the Pharmaceuticals and Medicines Research Institute (IPeFarM), for the Postgraduate Program in Natural Products and Bioactive Synthetics (PPgPNSB), at the Federal University of Paraiba (UFPB). The animals were randomly housed in cages containing six animals each, with free access to food and water, and kept on a 12-hour/12-hour light-dark cycle, with the light phase starting at 6:00 am. All procedures were previously approved by the Ethics Committee on the Use of Animals (CEUA), of the Federal University of Paraíba (Certificate No. 3220051018) and this study was carried out in accordance with the internationally accepted principles for proper use of laboratory animals the International Council for Laboratory Animal Science (ICLAS).

2.4. Pharmacological tests

2.4.1. Rotarod test

The animals were pre-selected 24 h before the experiment to eliminate those unable to remain on the device's rotating bar (Insight ©, Ribeirão Preto, SP, Brazil) for a period of 60s (7 rpm). The animals were then divided into three groups (n = 6), being treated 30 min before the test with either vehicle, RMD86 (100 mg/kg, i.p.), or diazepam (4 mg/kg, i.p.). Each mouse was timed on the rotating bar for a maximum of 3 min, with observations occurring at 30, 60, and 120 min after the initial treatments [24].

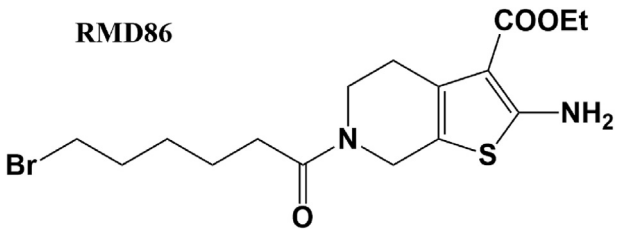
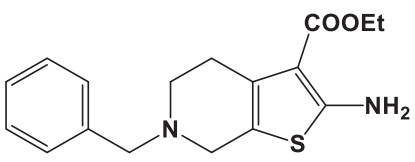
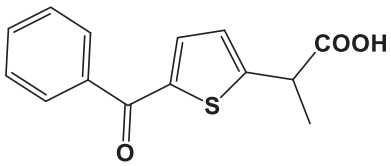


Figure 2. Chemical structure of **RMD86**.



Tinoridine



Tiaprofenic acid

Figure 1. Structures of commercial NSAIDs containing a thiophene ring (tinoridine and tiaprofenic acid).

2.4.2. Acetic acid induced abdominal contortions

This study was performed in accordance with Koster (1959) [25]. Mice (n = 6, per group) were pre-treated with RMD86 (25, 50, and 100 mg/kg, i.p.), morphine (10 mg/kg, i.p.), and vehicle (Tween-80, 5%, i.p.). After 1 h, the mice received 1% acetic acid (i.p.), which caused contortions characterized by extension of the hind limbs and contraction of the abdominal muscles. After 5 min of acetic acid administration, the number of abdominal contortions was counted over a period of 15 min.

2.4.3. Formalin-induced nociception

As already described in the literature [26], nociception was induced by administration of 20 μl of 2% formalin in the subplantar region of the right hind leg this generates an intense nociceptive stimulus through direct activation of nociceptors [27]. The mice (n = 6, per group) previously received the respective treatments of RMD (25, 50, and 100 mg/kg, i.p.), morphine (10 mg/kg, i.p.), and vehicle, at 30 min before formalin administration. They were then placed individually in mirrored chambers (25 × 25 × 25 cm), and the time (in seconds) spent licking their paw was immediately recorded. Nociception was quantified from 0-5min (neurogenic first phase), and from 15-30min (inflammatory second phase) after formalin administration.

2.4.4. Hot plate test

The animals were pre-selected. Those with a response time (to pain) of less than 10 s when placed on a metallic surface heated to 55 ± 1 °C (Insight ©, Ribeirão Preto, SP, Brazil) [28] were considered fit. The selected mice were pre-treated with vehicle, RMD86 (25, 50 and 100 mg/kg, i.p.), or morphine at 10 mg/kg (i.p.). and were then placed on the hot plate at 30, 60, and 120 min after pretreatment. The parameter recorded was latency time to jumping or licking the hind legs. To minimize destruction of the animal's paw tissue, the time on the plate was not allowed to exceed 30s.

2.4.5. Antipyretic activity

Hyperthermia was induced by subcutaneous injection of 20% beer yeast. 24 hours after this fever-inducing agent injection, the animals' body temperature was checked with a digital thermometer (Omron® - model MC-720). The animals were then treated with vehicle, RMD86 (25, 50, and 100 mg/kg, i.p.), or acetaminophen at 250 mg/kg (v.o.). Temperatures were measured at times 0, 30, 60, 120 and 180 min after administration of the compounds [29, 30].

2.5. In silico studies

2.5.1. Molecular docking

Molecular docking was performed using the Molegro Virtual Docker (MVD) program. The complete, three-dimensional X-ray crystallography structures for murine cyclooxygenase were downloaded from the protein data bank (PDB - www.rcsb.org/pdb/), for COX-1 (PDB ID: 4O1Z) and COX-2 (PDB ID: 4M11). These variants were chosen for molecular docking due to the absence of the human enzymes. The structures were co-crystallized with the ligand meloxicam (MXM). All of the water molecules were deleted from the enzyme structure, and the enzyme and ligands were prepared using the default parameter settings in the same software. The docking procedure was performed using a 15 Å radius GRID, and a 0.30 resolution, covering the ligand binding site for the COX-1 and COX-2 structures. The MolDock Score [GRID] algorithm was used as the score function, and the search algorithm was MolDock [31]. Root-mean-square deviation (RMSD) of docked ligands with respect to the corresponding ligand in the original PDB structure was performed and was computed to evaluate the quality of the inverse-docking procedure.

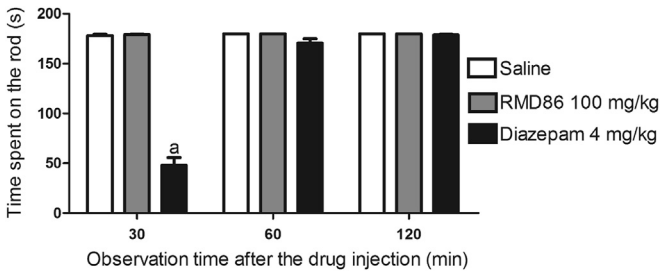


Figure 3. Representative graph of the effect of treatment with RMD86 (100 mg/kg) and diazepam (4 mg/kg) on the animal's ability to remain on the rotating bar. Data presented as mean ± standard error of six animals. Analyzed, by "one-way" ANOVA followed by Tukey's test with [a] <0.05 as compared to the control group.

2.6. Statistical analysis

The results were analyzed using one-way Analysis of Variance (ANOVA) followed by the Tukey test (for parametric measurements), and Kruskal-Wallis, followed by Dunn's test (for non-parametric measurements). The values obtained were expressed as mean ± standard error of the mean (S.E.M.), and the results were considered significant at $p < 0.05$.

3. Results

3.1. Pharmacological tests

3.1.1. Rotarod test

Treatment with RMD86 at a dose of 100 mg/kg did not induce significant motor coordination changes in the mice, assessing the animals ability to remain on the rotating bar at observation times of 30 (178.4 ± 1.1 s), 60 (180.0 ± 0.0 s), and 120 (180.0 ± 0.0 s) minutes from the initial treatments, these as compared to the control group at the same observation times (178.4 ± 1.1 s; 180.0 ± 0.0 s; 180.0 ± 0.0 s) (Figure 3).

3.1.2. Abdominal contortions induced by acetic acid

Treatment with **RMD86** at doses of 25 mg/kg (24 ± 2; $p < 0.05$), 50 mg/kg (12 ± 2; $p < 0.05$), and 100 mg/kg (3 ± 1; $p < 0.05$) significantly reduced the number of abdominal contortions induced by acetic acid in mice when compared to the control (36 ± 2) (Figure 4).

3.1.3. Formalin-induced nociception

Treatments with **RMD86** at doses of 50 (36.1 ± 3.7 s; $p < 0.05$) and 100 (22.7 ± 3.5 s; $p < 0.05$) mg/kg were able to significantly reduce licking time in the first phase of the formalin test when compared to the control (71.5 ± 3 s; $p < 0.05$). The dose of **RMD86** at 25 mg/kg was unable to reduce the parameter evaluated. The standard drug morphine at a dose of 10 mg/kg (21.3 ± 2 s; $p < 0.05$) was able to significantly reduce the parameter. In this same graph, it can be seen that **RMD86** at doses of 50 mg/kg and 100 mg/kg did not present significant differences when compared to the group treated with morphine (Figure 5).

In the second phase of the test, treatment with **RMD86** was able to significantly reduce the licking time at doses of 25 (79 ± 15.1 s; $p < 0.05$), 50 (38.8 ± 7.3 s; $p < 0, 05$), and 100 (11.1 ± 2.2 s; $p < 0.05$) mg/kg as compared to the controls (240.9 ± 27.9 s). The same was observed for morphine at a dose of 10 (11.7 ± 1.9 s; $p < 0.05$) mg/kg. As in the first phase, the groups treated with **RMD86** at doses of 50 mg/kg and 100 mg/kg did not present significant differences when compared to the group treated with morphine (Figure 6).

3.1.4. Hot plate test

Treatment with **RMD86** at a dose of 100 mg/kg (9.4 ± 0.4 s; $p < 0.05$) was able to significantly increase latency in the hot plate test when compared to the control (3.8 ± 0.1 s) within 30 min. The same

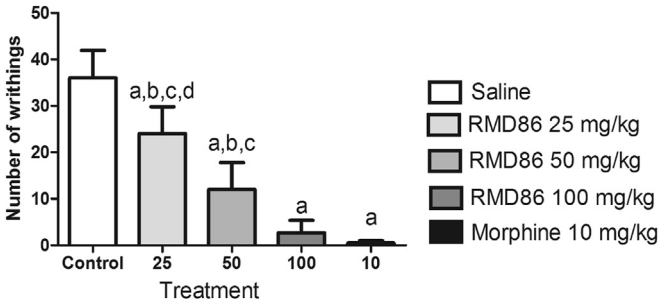


Figure 4. Representative graph of the effect of treatment with **RMD86** (25, 50, and 100 mg/kg), and morphine (10 mg/kg) for evaluation of antinociceptive potential by testing abdominal contortions induced by acetic acid. Data presented as mean ± standard error of the mean of six animals, analyzed using "one-way" ANOVA followed by Tukey's test with [a]p <0.05 as compared to the control group; with [b]p <0.05 as compared to the morphine group, and [c]p <0.05 as compared to the RMD86 group (100 mg/kg). [d]p <0.05 as compared to the **RMD86** group (50 mg/kg).

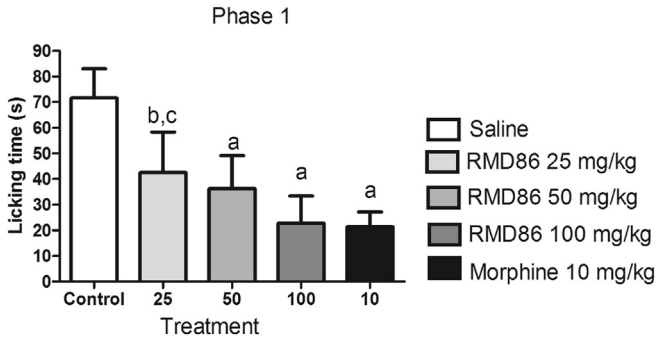


Figure 5. Representative graph of the effect of treatment with **RMD86** (25, 50, and 100 mg/kg), and morphine (10 mg/kg) in the first stage of the formalin test in mice. Data presented as mean ± standard error of the mean for six animals analyzed by "one-way" ANOVA followed by Tukey's test. [a]p <0.05 as compared to the control group. [b]p <0.05 as compared to the morphine group.

was observed for morphine at a dose of 10 mg/kg (15 ± 0.1 s; p < 0.05).

In evaluations performed at 60 and 120 min, **RMD86** was unable to significantly increase latency times when compared to the control group. The standard drug was able to increase this parameter significantly as compared to the control at the times mentioned (Figure 7).

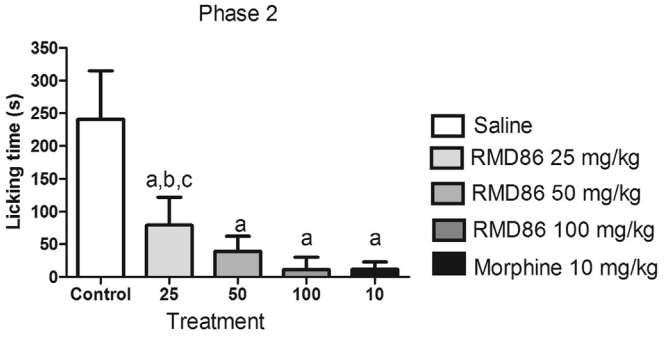


Figure 6. Representative graph of the effect of treatment with **RMD86** (25, 50, and 100 mg/kg) and morphine (10 mg/kg) in the second stage of the formalin test in mice. Data presented as mean ± standard error of the mean for six animals analyzed by "one-way" ANOVA followed by Tukey's test. [a]p <0.05 when compared to the control group. [b]p <0.05 when compared to the morphine group.

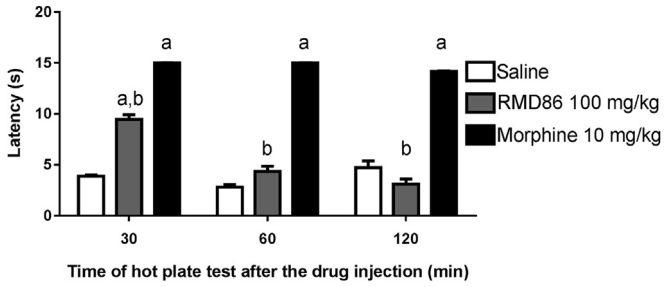


Figure 7. Representative graph of the effect of treatment with **RMD86** (100 mg/kg) and morphine (10 mg/kg) in the Hot Plate Test in mice. Data presented as mean ± standard error of the mean for six animals analyzed by "one-way" ANOVA followed by Tukey's test. [a]p <0.05 as compared to the control group. [b]p <0.05 as compared to the morphine group.

3.1.5. Antipyretic activity

Treatment with **RMD86** at doses of 25, 50, and 100 mg/kg significantly reduced pyrexia at 30, 60, and 120 min in mice compared to the controls, but did not reduce pyrexia at 180 min (Figure 8).

3.2. Molecular docking

To gain insight into the interaction modes of RMD86, acetaminophen, and meloxicam (co-crystallized ligand) against COX-1 and COX-2 isoenzymes (Figure 9), molecular docking experiments were performed using X-ray crystal structure data for COX-1 (PDB ID: 4O1Z) and COX-2 (PDB ID: 4M11). The average energy (MolDock Score) associated with these intermolecular interactions obtained upon docking for all compounds within the COX-1 and COX-2 active site is summarized in Table 1.

The docking results revealed for RMD86, a MolDock Score of less than -106.93 (for COX-1), and -110.84 (for COX-2), while for the meloxicam standard reference it was -88.71 (for COX-1) and -94.77 (for COX-2), and for the reference drug (acetaminophen) it was -48.67 (for COX-1), and -47.38 (for COX-2). The best docking pose orientations for meloxicam, RMD86, and acetaminophen (in COX-1 and COX-2) are shown in Figures 10 and 11.

For COX-1, it was observed that meloxicam exhibited three steric interactions: bonds with Ala527, Try387 and Val116, and one hydrogen bond with Ser530. **RMD86** displayed six steric interaction bonds: with the same amino acid residue Ala527, and in other interactions with Val349, Leu359, Ile345, Leu534, Met113. Acetaminophen presented one hydrogen bond with Ser530.

For COX-2 it was observed that meloxicam exhibited three steric interaction bonds: with Ala527, Try387 and Ser530, while **RMD86** displayed four steric interaction bonds: with the same amino acid Ala527 and in other interactions with Val349, Leu534 and Ile345, acetaminophen did not interact. In both enzyme analyses, the docking presented an

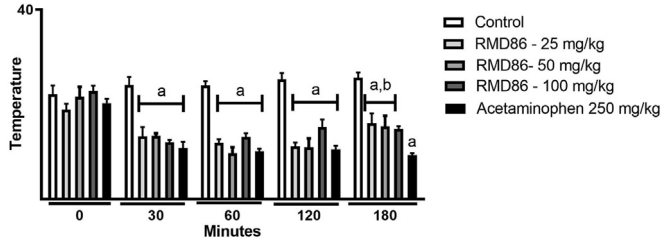


Figure 8. Graphical representation of the effect of treatment with **RMD86** (25, 50, and 100 mg/kg) and acetaminophen (250 mg/kg), for evaluation of antipyretic potential in a pyrexia model caused by beer yeast. Data presented as mean ± standard error of the mean for six animals analyzed by "one-way" ANOVA followed by Tukey's test. [a]p <0.05 compared to the control group. [b]p <0.05 compared to the acetaminophen group.

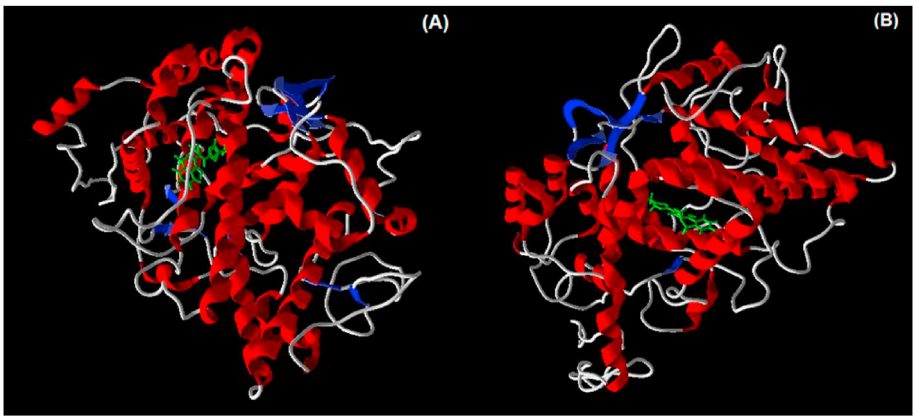


Figure 9. The superimposed structure of COX-1 (A) and COX-2 (B) modeled with the meloxicam template (green).

Table 1. MolDock energies, interaction type, and respective PDB structure residues for COX-1 and COX-2.

Name	**COX-1** (4O1Z)		
	MolDock Score	**Interaction type**	**Residues**
Meloxicam	-88.71	Steric	Val116, Ala527, Trp387
		H-Bond	Ser530
RMD86	-106.93	Steric	Ala527, 2(Val349), 2(Leu359), 2 (Ile345), Leu534, Met113
Acetaminophen	-48.67	H-Bond	Ser530
Name	**COX-2** (4M11)		
	MolDock Score	**Interaction type**	**Residues**
Meloxicam	-94.77	Steric	2 (Ala527), Trp387, Ser530
RMD86	-110.84	Steric	2 (Val349), Ala527, Ile345, 2 (Leu534)
Acetaminophen	-47.38	H-Bond	None

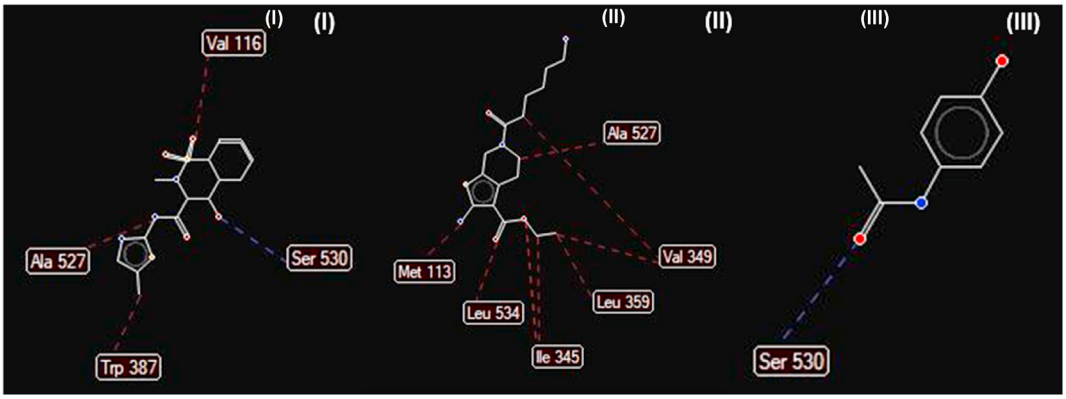


Figure 10. Interaction of meloxicam (I), RMD86 (II), and acetaminophen (III) with COX-1 amino acid residues.

amino acid Ala527 bond in common, which may be an important residue for biological activity response.

4. Discussion

Due to their enormous pharmacological potential as medicinal drugs and diagnostic agents, heterocyclic compounds such as thiophene derivatives, have been attracting the attention of many researchers. An increasing number of thiophene derivatives have become drug candidates; used in clinical research and in development of pharmaceutical products [32, 33].

Investigation of the antinociceptive and antipyretic potential of RMD86 initiated with the rotarod test, which is used to evaluate whether a drug influences animal motor activity. The test consists of assessing the ability of the mouse to balance itself on a bar that rotates at a constant speed [34].

Several drugs able to reduce the animals time of stay on the revolving bar have already been described in the literature; including central nervous system (CNS) depressants (for example: neuroleptics, benzodiazepines, barbiturates and ethanol), and muscle relaxants [35, 36].

In assessing the neurotoxic potential of **RMD86**, it was observed that there were no changes in the length of stay on the rotating bar as compared to animals in the control group. It can thus be inferred that **RMD86** presents no CNS depressant or relaxant effects. The data corroborate other studies investigating the *in vivo* and *in vitro* neuroprotective potential of thiophene derivatives [37, 38].

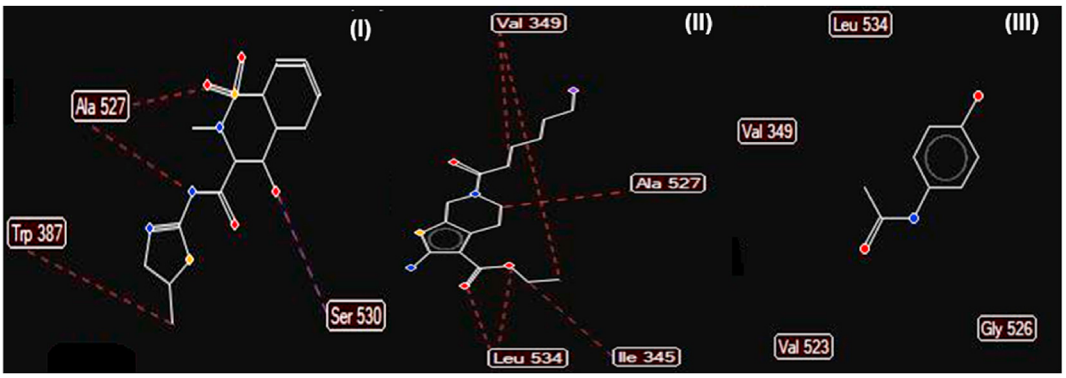


Figure 11. Interaction of MXM (I), **RMD86** (II), and acetaminophen (III) with the COX-2 amino acid residues.

Regarding the standard drug used (diazepam 4 mg/kg), a muscle relaxant effect that lasts until the first 30 min of the test can be observed due to the fact that the maximum effect of diazepam occurs after about 60 min of parenteral administration. Therefore, having been administered 30 min before the start of the test, a reduction in effect is expected after 60 min [39].

It is important to note that the solvent Tween-80 was used in the concentration of 5% to enhance the solubilization of the product. Some researchers have evaluated the behavioral effects of vehicles of various compounds used to dissolve drugs and they observed that tween-80 could cause a reduction in locomotor activity only at a dose of 32% [40]. In addition, another study in rats was found that tween-80 could cause intestinal toxicity only at doses above 10%. Therefore, a 5% dose can be considered safe for animal use [41].

The antinociceptive activity of **RMD86** was assessed using chemical nociception models, abdominal contortions testing - induced by acetic acid, and the formalin test. Hot plate thermal nociception testing was also used.

Abdominal contortions induced by acetic acid, (considered a classic model of visceral pain and used as a screening tool to assess analgesic and anti-inflammatory activity) [42], was employed in assessing the antinociceptive potential of RMD86. At 25, 50, and 100 mg/kg, RMD86 significantly reduced the number of abdominal contortions. Other studies performed with heterocyclic derivatives have presented similar results in the same test, demonstrating the potential of this group of compounds [17, 43].

Although the results were suggestive of RMD86's anti-nociceptive effect, due to the low specificity presented by the test, it was not possible to determine whether antinociception was central or peripheral. We therefore employed a more specific nociception model; the formalin test.

In this model, two phases of nociceptive behavior can be observed and seem to involve different stimuli. The first (neurogenic) phase begins immediately after formalin injection, extending for the first 5 min, and results from direct chemical nociceptor stimulation, mainly from C fibers [17]. The second (inflammatory) phase occurs mainly due to spinal cord stimulation, after sensitization of both nociceptors and central neurons, with release of serotonin, histamine, prostaglandins (PGE2), nitric oxide (NO), excitatory amino acids (glutamate and aspartate), and bradykinin [44, 45].

Treatment with **RMD86** promoted a significant inhibition of nociceptive behavior in both phases of the formalin test. Studies with thiophenes performed by Oliveira et al, 2009 [46] have similarly demonstrated the antinociceptive potential of this derivative class.

The fact that RMD86 showed a significant reduction in paw licking time in the inflammatory phase of the test, even at its lowest dose (25 mg/kg), can be explained by its structural similarity with the anti-inflammatory tinoridine. To prove the anti-inflammatory profile of this product, tests such as carrageenan-induced peritonitis should be performed. It has been shown that thiophene derivatives with structures similar to RMD86, that is, in addition to being part of this class, these products have the 2-amino-thiophene ring, have antinociceptive and anti-inflammatory activities [47].

In another more detailed study [48], it was seen that other 2-amino--thiophene derivatives were able to increase cytokines closely linked to inflammation such as TNF-α and IL-12 *in vitro*. In addition, other derivatives of this have been shown to be able to reduce inflammatory cytokines with IL-8 [49].

Taking into account the promising results observed in the formalin test, the thermal nociception assay (hot plate) was performed to identify whether RMD86 presents central anti-nociception mechanisms.

The hot plate test is aimed at drugs that modulate supraspinal nociception, such as opioid analgesics [50, 51] peripherally acting analgesics (NSAIDs) are ineffective for this test [52, 53].

Although a potent antinociceptive effect was observed for abdominal contortions induced by acetic acid, and in both phases of the formalin test, **RMD86** only managed to reduce the nociceptive response time during the first 30 min of testing. In comparison, Wen et al, (2011) [54] analyzing another thiophene derivative, found that the compound increased latency time to 2 h in the hot plate test, being even more effective than morphine which presented antinociceptive effect for approximately 1 h. Then, using pharmacological analysis, it was found that the thiophene derivative presented signs of mechanisms involving opioid receptors.

Observing that **RMD86** presents antinociceptive, and possibly anti-inflammatory effects, and based on reports of other heterocyclic compounds in the thiophene group, a study was performed to assess the antipyretic potential of the compound.

For this, the yeast-induced pyrexia model was used, and **RMD86** presented antipyretic activity at 30–120 min. This result is in accordance with another study performed by El-Sharkawy & AlBratty (2018) [55], where thiophene derivatives demonstrated antinociceptive and antipyretic activities lasting up to 4 h.

While acetaminophen, a standard drug, maintained its antipyretic activity for up to 3 h after oral administration. The administration of acetaminophen orally was due to its use in the clinic, as it can be found in the form of tablets, effervescent tablets, suspension, powder to prepare oral liquid medicine (sachets) and rectal suppositories. When administered orally, clinical effect of acetaminophen appears after 30 min. In addition, can be safely used in the digestive tract; on one hand due to its non-acidic chemical structure (unlike acidic NSAIDs gathering in the gastric epithelial cells) and on the other hand, due to a weak impact on COX-1 [56].

After demonstrating that RMD86 presents *in vivo* antinociceptive and antipyretic effects, a molecular docking study was performed on two enzymes greatly involved in inflammatory processes, being also targets of the principal NSAID classes, COX-1 and COX-2. This was performed to assess the potential of RMD86 to interact with these enzymes. In this study, the

interaction energy values were compared, together with the interactions with the amino acid residues in the best orientation poses, with the anti-inflammatory and co-crystallized ligand meloxicam. Acetaminophen was included as an additional control drug. For COX-1 meloxicam presented only three sites of interaction with amino acids while RMD86 presented six, one of which was with the same amino acid residue (Ala527) as with the co-crystallized ligand. The results for COX-2 demonstrated that while meloxicam presented three sites of interaction, RMD86 presented four. The greater number of interaction sites resulted in lower binding energy values, suggesting that RMD86 is potentially a better enzyme inhibitor than either the co-crystallized ligand or the reference drug.

In *in vitro* and *in vivo* studies [57] it was demonstrated that a group of thiophene derivatives inhibits the enzyme COX-2. The same authors performed a molecular docking assay using the most promising molecules from their *in vitro* results; these compounds presented more binding sites with the enzyme than Celecoxib.

Recent studies has been performed with several groups of molecules, including compounds with thiophene rings. *In vitro* and *in vivo* studies revealed potent anti-inflammatory activity via inhibition of the COX-2 enzyme. To confirm the results, further *in silico* testing was performed, which again demonstrated the potential of thiophene derivatives to inhibit this enzyme [58].

RMD86, being a thiophene derivative and presenting (as a drug candidate) several binding sites with COX-1 and COX-2 enzymes, clearly suggests that inhibition of these same targets is its possible mechanism of action.

By means of *in vivo* tests, the present study has demonstrated the antinociceptive and antipyretic activities of the thiophene derivative RMD86. This drug candidate presented no *in vivo* evidence of neurotoxicity, and the *in silico* results suggest that its likely mechanism of action is inhibition of the cyclooxygenase isoforms (COX-1 and COX-2), as is also observed for the majority of NSAIDs.

In addition, previous meloxicam - COX-2 interaction studies have revealed that modulations due to subtle changes around Phe518, give rise to preferential inhibition of COX-2 over COX-1 [59], as can be seen in Figure 10, with formation of a steric bond composed by the RMD86 methylene group with the Ile45 residue (instead of Val434), which may suggest a predisposition for this compound to bind more strongly with COX-2 than with COX-1, and thus reduce the appearance of side effects, involving the gastrointestinal system for example [60].

The data observed in this study provided evidence that the tested product has central and peripheral antinociceptive activity, as well as antipyretic activity. In addition, *in silico* tests, demonstrated a predisposition to inhibit inflammatory enzymes such as COX-2 and a moderate affinity with COX-1. Therefore, this product can be considered a new option for the development of new analgesic agents, however further studies to determine the mechanism *in vivo* must be performed.

Declarations

Author contribution statement

R.N. de Almeida: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

R.M.D. da Cruz: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

R.M. Braga, H.H.N. de Andrade, A.B. Monteiro, I.S. Luna and R.M.D. Cruz: Performed the experiments.

M.T. Scotti and F.J.B. Mendonça-Junior: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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