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NOVEL ARYL/ALKYL AZOLE DERIVATIVES AS AN ANTI-NOCICEPTIVE AND ANTI-INFLAMMATORY DRUG CANDIDATES

YESIM KAYA-YASAR^{1,3*}, ELIF NUR BARUT¹, SECKIN ENGIN¹,
INCI SELIN DOGAN^{2,3} and SENA F. SEZEN^{1,3}

¹Department of Pharmacology, Faculty of Pharmacy,

²Department of Pharmaceutical Chemistry, Faculty of Pharmacy,

³Drug and Pharmaceutical Technology Application and Research Center,
Karadeniz Technical University, Trabzon, Turkey

Abstract: Novel aryl/alkylazole derivative compounds **C1** and **C2** were screened for *in vivo* anti-nociceptive and anti-inflammatory activities by using the hot plate test and formalin-induced hind paw edema test, respectively. The ability of these compounds to inhibit cyclooxygenase-1/2 (COX-1/2) and 5/15-lipoxygenase (5/15-LOX) were evaluated *in vitro* by using a colorimetric method. **C1** (30 and 50 mg/kg, i.p.) and **C2** (3 and 30 mg/kg, i.p.) increased the latency to withdrawal in the hot plate test, indicating an anti-nociceptive activity. **C1** (30 and 50 mg/kg) and **C2** (3, 30 and 50 mg) were able to decrease formalin-induced edema, indicating their anti-inflammatory properties. **C1** and **C2** exhibited inhibitory effects on the activity of COX(1-2) and 15-LOX. However, neither **C1** nor **C2** showed an inhibitory effect on 5-LOX. This study demonstrates that **C1** and **C2** have anti-nociceptive and anti-inflammatory activities, that are partially mediated by inhibition of COX and LOX enzymes. Our results suggest that **C1** and **C2**, novel aryl/alkylazole compounds, could serve as lead compounds to develop novel therapeutic options for the treatment of pain and inflammation.

Keywords: pain, inflammation, aryl/alkylazole, COX-LOX, formalin-induced hind paw edema test, hot plate test

Pain is defined as ‘an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage’ by the International Association for the Study of Pain (IASP) (1). Various dimensions of pain are used for classification, such as duration (acute versus chronic) or pathophysiology (neuropathic versus nociceptive). Acute pain commonly results from tissue damage or inflammation related to trauma, infection and hypoxia and serves a protective mechanism against harmful stimuli. On the other hand, it is estimated that over 1.5 billion people are suffered from chronic pain that is a significant health problem with a high medical, social and financial impact (2, 3). Non-steroidal anti-inflammatory drugs (NSAIDs), opioid derivatives like morphine, are currently available drugs to treat pain. NSAIDs exhibit antinociceptive and anti-inflammatory effects by suppressing the synthesis of prostanoids involved in pain and inflammatory response due to cyclooxygenase (COX) inhibition.

NSAIDs are less effective in some kind of painful conditions like neuropathic pain. Moreover, long-term use of NSAIDs is associated with serious side-effects such as gastric ulcers, nephropathy and congestive heart failure, which limit their clinical utility (4-6). Opioids derivatives are the most effective drugs for pain treatment. However, their use is limited by drug-induced tolerance, dependence and side effects such as respiratory depression and constipation (2). Recently, anticonvulsant or antidepressant drugs such as carbamazepine, gabapentin, pregabalin, amitriptyline, desipramine, duloxetine, venlafaxine have been used in the treatment of neuropathic type pain. But, their clinical use is limited due to their poor efficacy and several side-effects (3). Therefore, there is an ongoing need to develop novel anti-nociceptive and anti-inflammatory drugs with an improved efficacy and safety profile.

Aryl/alkyl azoles are a novel generation of anticonvulsant drugs including nafimidone and den-

* Corresponding author: e-mail: yesimyasarkaya@ktu.edu.tr

zimol. They decrease neuronal hyperexcitability by inhibiting voltage-dependent sodium channels (VDSC) like carbamazepine and phenytoin (7, 8). VDSCs are crucial for neuronal excitability and signaling in central and peripheral nervous systems. Expressions of several ion channels like sodium and calcium channels have been shown to be augmented in chronic inflammatory pain. A large body of evidence suggests VDSC as therapeutic targets for antinociceptive drugs (9). Suter et al. showed that rufinamide, a VDSC blocker, attenuated mechanical allodynia in a mouse model of diabetic neuropathy (10). Kamisli et al. also reported that nafimidone oxime ester derivatives are effective in reducing thermal hypoalgesia in diabetic mice (11).

Arachidonic acid (AA), a polyunsaturated fatty acid-containing 20-carbon, is released from membrane phospholipids by phospholipase A2 and it undergoes metabolism mediated by COX and lipoxygenases (LOX) (12, 13). AA is metabolized to PGs like PGD₂, PGE₂, PGF₂α, and PGI₂ by COX that is implicated in physiologic functions and pathological conditions. COX exists in two isoforms: a constitutive (COX-1) and an inducible form (COX-2). COX-1 catalyzes the formation of prostanoids which mediates gastrointestinal, renal and homeostatic functions, whereas COX-2 is involved in inflammation and cancer (14). COX-1 and COX-2 are inhibited by NSAIDs that are widely prescribed to treat inflammation and pain. However, NSAIDs are associated with gastrointestinal and renal effects due to COX-1 inhibition. Therefore, selective COX-2 inhibitors like the coxibs have gained attention in the treatment of inflammatory diseases. However, their clinical use was diminished due to severe cardiovascular toxicity (15). Thus there has been a growing interest in identifying new strategies to develop novel NSAIDs with improved efficacy and safety (16). Many studies have focused to develop effective compounds combining COX inhibitory effect with other mechanisms involved in inflammation and pain (17).

1-Phenyl-2-(1H-imidazol-1-yl) ethyl 2-propyl pentanoate hydrochloride (**C1**) and 1-(4-Chlorophenyl)-2-(1H-imidazol-1-yl) ethyl cyclohexane carboxylate hydrochloride (**C2**) synthesized by Dogan et al. are novel aryl/alkyl azole substituents derived from nafimidone and denzimol (Fig. 1A and 1B). It was reported that **C1** and **C2** have anticonvulsant and antimicrobial activity (18). The objective of the present study is to investigate the antinociceptive, anti-inflammatory effects of **C1** and **C2** on experimentally-induced pain and acute inflammation and to determine the inhibitory effect of these compounds on COX1-2 and 5-,15-LOX *in vitro*.

EXPERIMENTAL

Animals

Male Balb/c mice weighing 25-30 g were housed in plastic cages in a temperature-humidity controlled environment (22 ± 2°C and 60 ± 5%, respectively) with 12-hours light-dark cycle. Animals were allowed free access to food and water. The protocol was approved by the Karadeniz Technical University Animal Research Ethics Committee (Approval number: 2016/47). All animal procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals.

Chemicals

C1 and **C2** were synthesized by Dogan et al. based on the previously described methods (18). The following chemicals were used in the assays: diclofenac sodium (Sigma, USA) and morphine hydrochloride (Osel Pharma, Turkey). In addition, COX and LOX commercial assay kits (Cayman Chemical, USA) were used for *in vitro* enzyme inhibition studies. **C1** and **C2** were dissolved in the vehicle containing 1% dimethyl sulfoxide (DMSO) in saline and administered intraperitoneally (i.p.). Morphine hydrochloride was diluted in saline and administered subcutaneously (s.c.). Diclofenac sodium was dissolved in saline and administered i.p.

Figure 1. Chemical structures of **C1** (A) and **C2** (B).

Anti-nociceptive activity

The anti-nociceptive activity of **C1** and **C2** was evaluated by the hot-plate test (COMMAT Ltd, Ankara, Turkey) with some modifications of the method described previously (19). Mice were randomly divided into the following 8 groups: control (vehicle), **C1** (3; 30; 50 mg/kg), **C2** (3; 30; 50 mg/kg); morphine (10 mg/kg, positive control). Mice were placed on a metal surface maintained at $55 \pm 0.1^\circ\text{C}$. The time until the animals exhibit reactions such as licking hind paws or jumping was recorded as latency. Latency was used as an index of nociception and increase in latency was defined as anti-nociception. Latency values were presented as seconds (s). Latencies were measured before and at 15, 30, 45 and 60 min after the drug administration. Cut off time was set as 30 s to avoid tissue damage in mice.

Anti-inflammatory activity

The anti-inflammatory activity of **C1** and **C2** was evaluated by formalin-induced hind paw edema test with some modifications of the method described previously (20). Mice were randomly divided into the following 8 groups: control (vehicle), **C1** (3; 30; 50 mg/kg), **C2** (3; 30; 50 mg/kg); diclofenac (10 mg/kg, positive control). Intraplantar injection of 1% formalin (20 μL) was applied to the plantar surface of the right paws of the mice 30 min after drug administration to induce edema. Before and 30 min after the formalin injection, paw thickness and volume were measured with compass and plethysmometer, respectively. The amount of increase in edema thickness and volume was calculated subtracting the respective measurements before and 30 min after the formalin injection. Paw thickness was presented as millimeter (mm), whereas paw volume was presented as microliter (μL).

COX inhibitory activity

The ability of **C1** and **C2** to inhibit COX-1 (ovine) and COX-2 (human recombinant) was determined by using enzyme immunoassay (EIA) kit (Cayman Chemical Company, Item Number 701050), following the manufacturer instructions. **C1** (10^{-8} - 10^{-4} M), **C2** (10^{-8} - 10^{-4} M); diclofenac (10^{-8} - 10^{-4} M) and DMSO were used as positive and negative controls, respectively. The absorbance at 590 nm was read by using a microplate reader.

LOX inhibitory activity

The ability of **C1** and **C2** to inhibit 15-LO (soybean) and 5-LO (potato) using the LOX inhibitor screening assay kit (Cayman Chemical Company,

Item Number 760700), following the manufacturer instructions. **C1** (10^{-8} - 10^{-4} M), **C2** (10^{-8} - 10^{-4} M); nordihydroguaiaretic acid (NDGA) and DMSO were used as positive and negative controls, respectively. The absorbance at 490 nm was read by using a microplate reader.

Statistical analysis

All values were expressed as mean $\pm$ SEM. Maximal possible effect (MPE) values were calculated and represented as $[(\text{latency-baseline})/(\text{cut off time (30 min)-baseline})] \times 100$. Inhibitory effects of **C1** and **C2** on COX and LOX were presented as a maximal effect (E_{max}). IC_{50} was calculated as the negative logarithm of the concentration that causes 50% of the maximal inhibition.

The data were analyzed statistically by ANOVA followed by Bonferroni's test as post-hoc. Statistical analysis was carried out using GraphPad Prism version 5.01 (GraphPad Software Inc.). $P < 0.05$ is considered significant.

RESULTS

Anti-nociceptive activity

Administration of **C1** or **C2** dose-dependently increased the latency values in the hot plate test. As for **C1**, at 30 mg/kg (30; 60 min) and 50 mg/kg (15-60 min) doses, but not at 3 mg/kg dose increased the latency when compared to that of control; MPE values were calculated as, 18.41 ± 4.66 at 15 min; 28.79 ± 9.00 at 30 min; 21.19 ± 8.23 at 45 min; 24.75 ± 10.88 at 60 min for 30 mg/kg, 26.68 ± 4.75 at 15 min; 26.22 ± 13.73 at 30 min; 24.17 ± 6.37 at 45 min; 24.04 ± 12.43 at 60 min for 50 mg/kg. At the same time, the latency values were found similar to the morphine group at 30 mg/kg (30 min) and 50 mg/kg (15-45 min) doses in the **C1** treatment group. As for **C2**, increased latency values at 3 mg/kg (45 min) and 30 mg/kg (60 min) doses; MPE values were calculated as 17.65 ± 6.29 at 15 min; 21.03 ± 7.56 at 30 min; 34.20 ± 10.90 at 45 min; 12.58 ± 4.90 at 60 min for 3 mg/kg; 24.66 ± 3.78 at 15 min; 34.27 ± 8.51 at 30 min; 32.24 ± 4.19 at 45 min; 33.07 ± 7.22 at 60 min for 30 mg/kg. However **C2** did not alter latencies at 50 mg/kg dose. Furthermore, the latency values of **C2** (3 mg/kg) and morphine group were similar to each other.

Morphine (10 mg/kg) which was used as a positive control increased latency values on 15-45 min. compared with the control group. MPE values were elicited as 44.48 ± 6.37 at 15 min; 37.60 ± 5.61 at 30 min; 29.35 ± 4.47 at 45 min; 19.25 ± 5.15 at 60 min for morphine group (Fig. 2).

Anti-inflammatory activity

We performed the formalin-induced hind paw edema test to evaluate the anti-inflammatory activity of **C1** and **C2**. Intraplantar injection of 1% formalin caused a marked increase both in edema thickness and volume. **C1** at 3 mg/kg was not able to decrease edema thickness and volume. However, 30 and 50 mg/kg doses of **C1** significantly decreased edema thickness and volume compared with the control group, demonstrating that **C1** has a dose-dependent anti-inflammatory effect. **C2** at three doses tested significantly reduced edema thickness compared with the control group, suggesting a higher potency of **C2** compared to **C1**. (Fig. 3a and 3b).

COX inhibitory activity

To investigate a possible mechanism of action of anti-inflammatory activity, we examined the concentration-dependent inhibitory effect of **C1** and **C2** (10^{-8} - 10^{-3} M) on the COX-1 and COX-2 *in vitro*. **C1** inhibited COX-1 at the 10^{-7} - 10^{-4} M concentrations ($E_{max} = 68.69 \pm 0.76\%$); the inhibitory effect of **C1** was not in a concentration-dependent manner, we calculated IC_{50} value of **C1** for COX-1 as 4.56. In addition, **C2** inhibited COX-1 only at the 10^{-5} M ($E_{max} = 16.51 \pm 0.21\%$), IC_{50} value of **C2** could not calculated for COX-1. Diclofenac (10^{-8} - 10^{-3} M) displayed a concentration-dependent inhibitory effect on COX-1; E_{max} and IC_{50} values were 101.14 ± 0.26 and 7.26, respectively.

Figure 2. Effect of **C1**, **C2** and morphine on the latency in hot plate test. Data are expressed as mean $\pm$ SEM (n = 7-12). *p < 0.05, **p < 0.01, ***p < 0.001 compared with control, #p < 0.01 compared with morphine.

Figure 3. Effect of **C1**, **C2** and diclofenac on edema thickness (A) and edema volume (B) in formalin-induced hind paw edema test. Data are expressed as mean $\pm$ SEM (n = 5-12). *p < 0.05, **p < 0.01, ***p < 0.001 compared with control.

Table 1. *In vitro* COX-1 and COX-2 enzyme inhibition data.

COX-1	0.1 μ M	1 μ M	10 μ M	100 μ M	IC ₅₀ (μ M)
C1	66.12 $\pm$ 0.38	68.69 $\pm$ 0.76	55.70 $\pm$ 0.62	32.76 $\pm$ 0.52	4.56
C2	-	-	16.51 $\pm$ 0.21	-	-
Diclofenac	64.18 $\pm$ 0.46	88.32 $\pm$ 0.83	101.14 $\pm$ 0.26	96.46 $\pm$ 0.97	7.26
COX-2					
C1	60.09 $\pm$ 0.23	13.78 $\pm$ 0.33	16.72 $\pm$ 0.90	17.16 $\pm$ 0.34	5.88
C2	31.33 $\pm$ 1.39	42.18 $\pm$ 0.43	44.21 $\pm$ 0.42	-	7.25
Diclofenac	60.01 $\pm$ 0.22	91.91 $\pm$ 0.51	92.24 $\pm$ 0.93	94.42 $\pm$ 0.81	6.99

C1 inhibited COX-2 at 10^{-7} - 10^{-4} M concentrations. $E_{\max}$ and IC₅₀ values were calculated as 60.09 $\pm$ 0.23 and 5.88, respectively. The inhibitory effect of **C1** on COX-2 was not concentration-dependent. Furthermore, **C2** showed a concentration-independent inhibitory effect on COX-2; $E_{\max}$ and IC₅₀ values were 44.21 $\pm$ 0.42 and 7.25, respectively. Diclofenac (10^{-8} - 10^{-3} M) inhibited COX-2 enzyme; the inhibition was concentration-dependent. $E_{\max}$ and IC₅₀ values were calculated as 94.42 $\pm$ 0.81 and 6.99, respectively (Table 1).

LOX inhibitory activity

The concentration-dependent inhibitory effect of **C1** and **C2** (10^{-8} - 10^{-3} M) were investigated on the 15- and 5-LOX *in vitro*. **C1** and **C2** inhibited 15-LO enzyme at only 10^{-4} M, $E_{\max}$ values were calculated for **C1** and **C2** as 18.80 $\pm$ 2.67 and 3.97 $\pm$ 0.05, respectively. NDGA which was used as positive control inhibited 15-LO ($E_{\max}$ = 89.06 $\pm$ 9.18). Neither **C1** nor **C2** showed an inhibitory effect on 5-LO.

DISCUSSION AND CONCLUSION

In this study, we have demonstrated that **C1** and **C2**, aryl-alkyl azole derivatives, have anti-nociceptive effect in hot plate test and presented an anti-inflammatory effect on acute edema formalin-induced hind paw edema test. Moreover, these effects of **C1** and **C2** were partially related to COX and LOX inhibition. We used a hot plate test to investigate the anti-nociceptive effects of **C1** and **C2**. According to our results, **C1** (30 and 50 mg/kg) and **C2** (3 and 30 mg/kg) increased the latency in the hot plate test, indicating anti-nociceptive activity. Moreover, the onset of the anti-nociceptive activity of **C2** was faster than **C1**. The time delay of the onset of the **C1** effect may be due to the active metabolites of **C1**.

We used the formalin-induced hind paw edema test to evaluate the anti-nociceptive activity of **C1** and **C2**. In this test, **C1** and **C2** decreased edema thickness and volume, indicating their anti-inflammatory properties. **C2** exerted anti-inflammatory effect at 3 mg/kg which is lower than the effective dose of **C1**, suggesting higher potency of **C2**. Furthermore, we performed *in vitro* enzyme inhibition studies to investigate whether COX and LOX inhibition underly the mechanism of anti-nociceptive and anti-inflammatory activities of **C1** and **C2**. Both compounds inhibited COX-1 and COX-2, in a concentration-independent manner. Although the inhibitory potency of **C1** on COX-1 and COX-2 enzymes was higher than that of **C2**; the selectivity of **C2** against COX-2 was greater than **C1**. In addition, **C1** and **C2** slightly inhibited 15-LOX; the enzyme inhibitory potency of **C1** was higher than **C2**. These results suggest that inhibition of COX-1, COX-2 and partially 15-LOX involved in the anti-inflammatory activity of **C1**. Also, the anti-inflammatory activity of **C2** may be related to its inhibitory effects on COX-1 and COX-2 enzyme inhibition. It has been suggested that several compounds containing azole substituents are anti-inflammatory by inhibiting COX. These novel compounds appear to be promising in the discovery of new analgesic drugs (21). Lamie et. al. demonstrated that the 4-chloro benzoxazole derivative, one of the compounds derived from 1,2-diaryl-4-substituted-benziliden-5(4H)-imidazolon, is an inhibitor of COX-2 and 5-LOX activity. It has been also reported that this compound has greater selectivity against COX-2 when compared to COX-1 (22). Other studies reported that substituted 1,3,4-oxadiazole/tiadiazole and 1,2,4 triazole compounds have anti-inflammatory activities (23-26). Similarly, Sowmya et. al. showed that methoxy-asetamido pyrrolyl oxazole exerted anti-inflammatory activity in a rat model of carrageenin-induced edema test (27). Synthesis of

novel compounds that contain thiazolyl-carbonyl-tiyosemikarbazid thiazolyl-azole moiety has been reported to exhibit antioxidant and anti-inflammatory activities by inhibiting inducible nitric oxide synthase in an experimental model of inflammation (28).

Our present study demonstrated that novel aryl-alkyl azole derivatives **C1** and **C2** have anti-nociceptive and anti-inflammatory activities in mice, which is partially due to their COX and LOX inhibitory properties. To our knowledge, this is the first *in vivo* and *in vitro* study demonstrating that these compounds could serve as potential lead compounds in the development of novel anti-nociceptive and anti-inflammatory drugs. Future studies will be important to further clarify these effects in other models of pain and inflammation to understand underlying mechanisms of actions.

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Conflict of interest

There is no conflict of interest

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