

Research Article

Analgesic and Toxicity Studies of Aminoacetylenic Isoindoline-1,3-dione Derivatives

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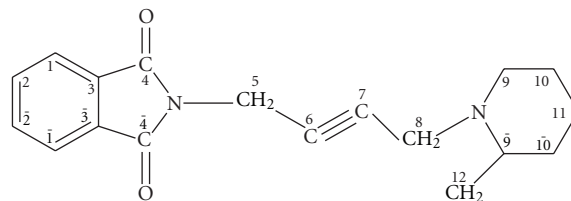
We have developed a series of aminoacetylenic isoindoline-1,3-dione compounds and showed their anti-inflammatory activities by reducing carrageenan-induced rat paw edema and modulating proinflammatory and anti-inflammatory cytokines. In the present study and due to efficacy reasons, we are exploring only two of these compounds, namely, ZM4 and ZM5, to reveal their analgesic activity and toxicity. Following oral administration, both compounds were effective in reducing significantly ($P < 0.05$ – 0.001) acetic acid-induced writhing behavior, hot plate latency test, and formalin-induced paw licking time as antinociceptive indicators in mice and rats, respectively. Regarding the toxicity, the acute (20, 50, and 150 mg/kg) and repeated oral administration (10, 20, and 50 mg/kg) of these compounds for ten days did not produce any mortality and the compounds were considered well tolerated. However, repeated oral administration of 50 mg/kg of both compounds induced erythropoiesis by means of increasing significantly red blood cells, hemoglobin, and packed cell volume. Moreover, these compounds did not induce gastric lesions in the stomach of experimental animals at the doses that exhibited analgesic and anti-inflammatory activity compared to indomethacin as a positive control. The results indicate that ZM4 and ZM5 possess potential analgesic activity while being preliminarily safe and have minimal ulcerogenic activity.

1. Introduction

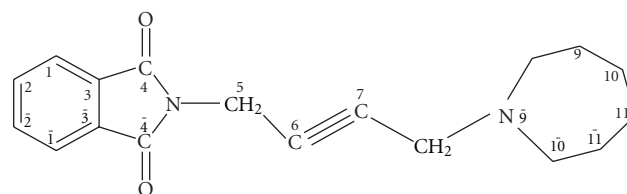
Cyclooxygenase (COX) plays an important role in the production of prostaglandins and the release of chemical pain mediators; therefore, inhibiting COX will reduce the painful response resulting from the prostaglandin cascade [1]. As inhibitors of COX enzyme, nonsteroidal anti-inflammatory drugs (NSAIDs) have been widely used to treat inflammation, mild-to-moderate pain, and fever. There are at least three isoforms of the COX enzyme: COX-1, COX-2, and COX-3 [2, 3]. COX-1 is expressed constitutively throughout the body and is important in maintaining vital functions such as glomerular filtration rate, platelet function, and gastric mucosal protection. COX-2, on the other hand, is undetectable in most normal functioning tissues [4]. However, the expression of COX-2 is induced as a response of inflammation whereas COX-3 was observed to be abundant in the cerebral cortex [2, 3]. All NSAIDs have a similar effect

on reducing pain [5, 6]. These include the selective NSAID or COX-2 inhibitor, Celecoxib, nonselective NSAIDs, such as ibuprofen and aspirin, and partially selective NSAIDs, such as meloxicam, nabumetone, and etodolac. Nevertheless, nonselective inhibition of COX enzyme can prevent the production of physiologically important prostaglandins which protect the gastric mucosa from damage by hydrochloric acid, maintain kidney function, and aggregate platelets when required [7]. For that, most of the current research is directed toward developing selective COX-2 inhibitors in order to minimize the side effects associated with the use of the nonselective NSAIDs. However, COX-2 inhibitors were associated with cardiovascular diseases that halted the possibility of their long-term use.

Therefore, we have developed new chemical compounds that incorporate isoindoline-1,3-dione and acetylenic derivatives to induce anti-inflammatory and analgesic activities, respectively [8–11] (Figure 1). In addition, we



(ZM4) N-[4-(2-Methylpiperidino-1-yl)-but-2-yn-1-yl] isoindoline-1,3-dione



(ZM5) N-[4-(2-Azepan-1-yl)-but-2-yn-1-yl] isoindoline-1,3-dione

FIGURE 1: Chemical structures of ZM4 and ZM5.

incorporated acetylenic group in order to increase the selective inhibition toward COX-2 isoform [12]. This unique combination represents a new series of compounds as potential anti-inflammatory agents as has been shown to reduce carrageenan-induced paw edema and structurally differs from the generally used drugs that contain acidic, enolic, sulfonamide, or sulfon groups which should exclude the direct insult on the gastrointestinal [11]. Of all the molecules reported in our previous studies, ZM4 and ZM5 were found to possess the best COX-2 inhibition activities, better in reducing carrageenan-inducing inflammation, and modulate proinflammatory and anti-inflammatory cytokines [11, 13, 14] and therefore were selected for further efficacy and safety investigations. The aim of the present study therefore is to study the analgesic activity of these compounds in addition to studying their toxicity and ulcerogenic effect on the stomach following single and repeated administration.

2. Materials and Methods

2.1. Drugs and Chemicals. The compounds, namely, ZM4 and ZM5 were synthesized and characterized as described by Al-Qaisi et al. [11]. Ibuprofen sodium and diclofenac sodium were kindly provided by the Jordanian Pharmaceutical Manufacturing Co. "JPM" (Naor, Jordan). Celecoxib as Celebrex of 200 mg capsules (Pfizer Inc, USA) and acetylsalicylic acid as Aspirin of 300 mg tablets (Bayer AG, Germany) were utilized in the experiment. Glacial acetic acid was purchased from Medex, UK. Formaldehyde solution (formalin) was obtained from Merck, Germany.

2.2. Animals. Sprague Dawley rats (180–280 g) and Balb/c mice (20–28 g) were obtained from Yarmouk University Animal House Unit (Irbid, Jordan) and housed in Petra University Animal Care Unit (Amman, Jordan). Animals were accommodated in a 12 hr light/dark cycle and a temperature of $20 \pm 2^\circ\text{C}$. All animals were acclimatized for at least 5

days prior to experiments with free access to standard diet and drinking water. Animal experiments were performed in compliance with FELASA guidelines (Federation of European Laboratory Animal Science Association) following protocols approval by the Ethical Committee of the Faculty of Pharmacy and Medical Sciences, Petra University, Jordan (Doc 4/2009).

2.3. Writhing Induction and Quantification. ZM compounds (25 and 50 mg/kg) dissolved in 0.1 N HCL followed by sonication at 40°C for 5 min and aspirin (200 mg/kg) dissolved in 0.9% saline as a reference positive control were administered orally 1 hour before intraperitoneal injection of 0.6% acetic acid solution (10 mL/kg) to 4 hours fasting mice. The mice were then kept individually in glass cages for observation, and the number of abdominal contractions (writhing movements) was counted for the next 20 min for each mouse. The data represent average of the total number of writhings observed.

2.4. Hot Plate Latency Test. Mice were divided into 4 groups as the following: Group 1, received saline solution (control); Group 2, received aspirin (200 mg/kg) as a positive control; Group 3 received ZM4 (20 mg/kg); Group 4 received ZM5 (20 mg/kg). Fifteen minutes after oral administration of the treatments, the mice were separately placed in a glass chamber mounted on a hot plate that was maintained at $52 \pm 0.5^\circ\text{C}$ as described elsewhere [15]. Initially, the mice that showed nociceptive responses within 12 seconds were only used for the subsequent experiments. The time between the placement of the mouse on the hot plate and the occurrence of the licking of hind paws was recorded as response latency. The nociceptive response was measured every 15 min over a 60 min period. The cut-off time was 45 seconds. Data are presented as mean % latency change calculated for each mouse by dividing the initial latency (before

treatment) by the latency determined at different time intervals posttreatment multiplied by 100.

2.5. Formalin-Induced Paw Licking. ZM compounds were prepared as mentioned earlier. Ibuprofen sodium, as a positive control, was dissolved in 0.9% saline followed by sonication [16]. Adult rats were pretreated orally as follows: Group 1, served as a negative control group receiving 0.9% saline solution (0.5 mL/g body weight); Group 2, ibuprofen (20 mg/kg) as a positive control; Group 3, received ZM4 (20 mg/kg); Group 4, received ZM5 (20 mg/kg). Thirty minutes after treatment, each rat was injected with 50 μ L of 2.5% solution of formalin subcutaneously under the plantar surface of the left hind paw. The injected rats were placed separately in mirror glass chambers for observation. The time spent for licking the injected paw was recorded, and the data were expressed as total licking time in the early phase (0–5 min) and the late phase (15–30 min) after formalin injection [17, 18].

2.6. Toxicity Studies

2.6.1. Acute Toxicity Testing of ZM4 and ZM5. Female Balb/c mice (20 \pm 2 g) were used for acute toxicity testing of the synthesized compounds. Oral and intraperitoneal routes of administration were investigated for ZM4 and ZM5 compounds at three dose levels (20, 50, and 150 mg/kg) and compared to control groups that received only vehicle. At the day of experiment, 4 hours fasting mice were grouped and administered freshly prepared compounds. The mice were continuously observed for 4 hours following administration to detect changes in the autonomic or behavioral responses and then monitored for any mortality for the following 14 days. In case of any death, an autopsy setup was prepared for gross organ inspection followed by histopathological investigation of any abnormal tissue.

2.6.2. Subacute Toxicity Testing of ZM4 and ZM5. Rats of both sexes were used for subacute toxicity testing following an oral route of administration of ZM4 and ZM5 at three dose levels (10, 20, and 50 mg/kg) and compared to control groups that received only vehicle. Rats were maintained in clean cages and had free access to food and water. The treatments were administered to the rats by oral gavages for ten days, and all rats were weighed daily. The animals were observed for clinical symptoms daily after 1 hour of treatment. At the end of the experimental period the rats were sacrificed and blood samples were obtained by cardiac puncture for hematological and serum biochemical analysis (only for control and 50 mg/kg). Autopsy was performed for all rats and the major organs; namely, liver, spleen, two kidneys, heart, and lungs were removed and accurately weighed.

2.6.3. Ulcerogenic Activity of the Synthesized Compounds. Rats were divided into 4 groups ($n = 5$) and received the following treatments: Group 1 received 4 mL/kg saline solution (control); Group 2 received indomethacin 20 mg/kg as a positive control; Group 3 received ZM4 (20 mg/kg); Group 4 received ZM5 (20 mg/kg). Fasting rats (16 hours) were orally

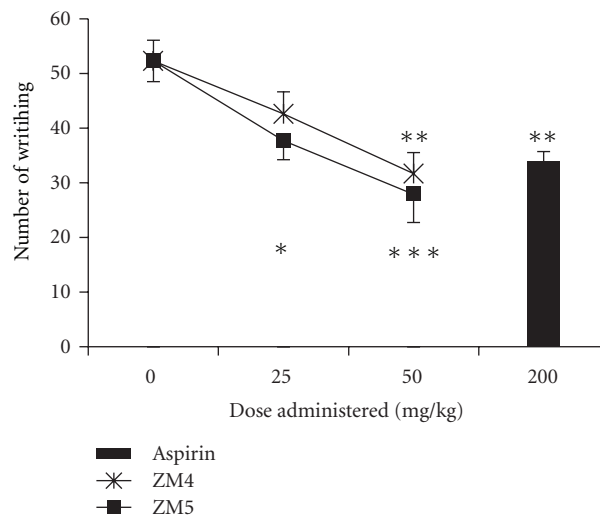


FIGURE 2: Reduction of acetic acid-induced writhing response in mice following treatment with aspirin, ZM4, and ZM5. Values are expressed as mean \pm S.E.M. ($n = 9$). Symbols represent statistical significance against 0 mg/kg dose as * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

administered the compounds, saline or indomethacin once daily for 3 consecutive days. On the fourth day the animals were sacrificed by an overdose of ether, and the stomach was quickly dissected, cut along the lesser curvature, and washed with saline. The gastric mucosa was examined using a hand held microscope (10x) for the presence of gastric irritation. Ulcers were scored using arbitrary scale where 0 = no lesion, 0.5 = hyperaemia, 1 = one or two slight lesions, 3 = very severe lesions, and 4 = mucosa full of lesions. Ulcer index (UI) was calculated as mean ulcer scores \pm SEM [19].

2.7. Statistical Analysis. Results are expressed as mean \pm SEM for each group. Data were assessed by one-way ANOVA, followed by one-tailed Dunnett's t -test (SPSS 17, USA). P value < 0.05 was considered significant.

3. Results

3.1. Writhing Test. A dose-dependent protective effect against writhing behavior was observed in mice treated with ZM4 and ZM5 (Figure 2). Significant reductions of writhing behavior were seen in mice treated with ZM4 (50 mg/kg) and ZM5 (25 and 50 mg/kg). The highest reduction was observed in mice treated with high dose of ZM5 ($P < 0.001$). Similarly, aspirin at 200 mg/kg significantly inhibited the writhing episodes ($P < 0.01$) but this reduction was not significantly different than ZM4 and ZM5 treated mice (Figure 2).

3.2. Hot Plate Test. The results of the hot plate test show that oral administration of aspirin (200 mg/kg), ZM4, and ZM5 (both at 20 mg/kg) produced a significant ($P < 0.05$) increase in latency times especially at 45–60 min after administration (Figure 3). It was noticed that ZM4 showed a fast onset of action and increased significantly the % of latency

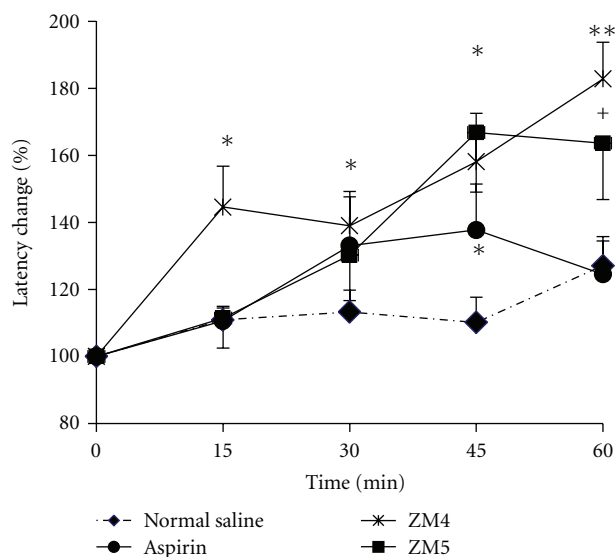


FIGURE 3: Percent of increase in latency periods of mice treated with normal saline (negative control), aspirin (positive control), ZM4, and ZM5. Treated mice were challenged on a hot plate every 15 min for 1 hour after oral treatment. Values are expressed as mean \pm S.E.M ($n = 9$). * $P < 0.05$, and ** $P < 0.01$ against normal saline and + $P < 0.05$ ZM4 and ZM5 against aspirin.

period after 15 min of administration. The effect of ZM4 continued to increase in a linear-like pattern during the testing period where it increased the latency period by 82% compared to the recorded initial latency periods at the end of 60 min testing time ($P < 0.01$). Both ZM4 and ZM5 showed more pronounced effects than aspirin (200 mg/kg). However, a statistically significant difference between the tested compounds and the positive control was detected only after 60 min of the oral administrations (Figure 3).

3.3. Formalin Test. Formalin administrations produce a typical pattern of two-phase pain response. The first phase (early, acute phase) starts immediately after administration of formalin and diminishes within 10 min whereas the second phase (late, tonic phase) starts at 15 min after formalin administration and lasts for 30 min after pain induction [17–19]. The first and the second phases of formalin test correspond to neurogenic and inflammatory pain, respectively [18, 20].

ZM4 at 20 mg/kg caused a significant antinociceptive effect by decreasing the licking time in both early and late phase ($P < 0.001$ and $P < 0.05$, resp.) similar to ibuprofen at 20 mg/kg (Figure 4). On the other hand, ZM5 caused a significant effect at early phase ($P < 0.001$) but not at the late phase ($P > 0.05$).

3.4. Toxicity Studies. No mortality was observed following the acute administration of 20–150 mg/kg of ZM4 and ZM5. In addition, animals did not show any change in the autonomic or behavioral responses during the observation period up to the 14th day of monitoring.

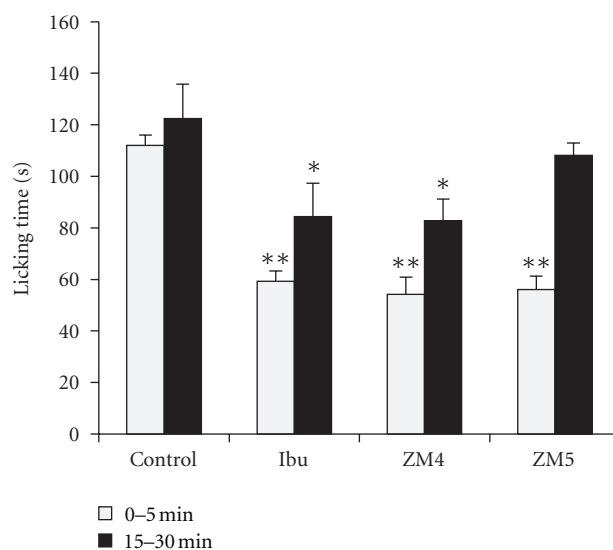


FIGURE 4: Nociceptive behavior of rats in the early phase (0–5 min) and late phase (15–30 min) after injection of formalin recorded as amount of time (s) spent licking the injected paw following treatment with ibuprofen, ZM4, and ZM5 (all at 20 mg/kg dose). Values are expressed as mean \pm S.E.M. ($n = 8$). Symbols represent statistical significance against control group as * $P < 0.05$ and ** $P < 0.001$.

Following a 10-day repeated administration, no significant effect of ZM4 and ZM5 on the animal weights was observed in comparison to control rats ($P > 0.05$). In addition, there was no significant effect of ZM4 and ZM5 at dose of 50 mg/kg on the liver, spleen, kidneys, and heart weights when compared to control. However, only the lungs' weight in female treated rats was significantly ($P < 0.01$) less than control animals, but no gross abnormalities were observed. As for the hematological parameters, ZM4 and ZM5 compounds at dose of 50 mg/kg markedly increased red blood cells (RBC), hemoglobin (Hb), and packed cell volume (PCV) values ($P < 0.01$ – 0.001) with a slight decrease in mean corpuscular volume (MCV), no change in the mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) of male or female rats. Furthermore, no significant change in total WBC or lymphocyte and monocyte counts was observed (Table 1). In addition, there was no significant change in clinical biochemistry values such as glucose, GOT, GPT, GGT, alkaline phosphates, urea, cholesterol, total protein, and albumin (Table 2), whereas a 10-day repeated administration of ZM5 (50 mg/kg) in females increased significantly creatinine and triglyceride serum levels ($P < 0.05$).

3.5. Ulcerogenic Activity of the Synthesized Compounds. The gastric effects of oral administration of ZM4 into rats were similar to normal saline administered control group (0.2 ± 0.1 UI for both treatments). However, oral administration of ZM5 into rats induced a minimal hyperemia (0.4 ± 0.1 UI) in comparison to severe full mucosal ulcers induced by 20 mg/kg indomethacin (3 ± 0.4 UI) (Figure 5).

TABLE 1: Rats hematological parameters following a 10-day administration of ZM4 and ZM5.

Parameter	Control		ZM4 50 mg/kg		ZM5 50 mg/kg	
	Males	Females	Males	Females	Males	Females
RBC ($\times 10^6/\mu\text{L}$)	5.5 \pm 0.2	5.4 \pm 0.3	6.2 \pm 0.1*	8.4 \pm 0.8*	9.0 \pm 0.1*	8.3 \pm 0.5*
HB (g/dL)	14.3 \pm 0.4	14.2 \pm 0.6	15.2 \pm 0.3	21.2 \pm 1.6**	21.5 \pm 0.2**	20.1 \pm 1.1**
PCV (%)	29.6 \pm 0.2	29.4 \pm 1.0	31 \pm 0.6*	42.9 \pm 3.3*	43.6 \pm 0.5*	40.5 \pm 2.3*
WBC ($\times 10^3/\mu\text{L}$)	10.5 \pm 1.3	6.3 \pm 0.2	9.2 \pm 1.0	7.9 \pm 1.2	8.1 \pm 1.5	6.0 \pm 0.8
Neutrophil (%)	9.8 \pm 1.9	11.7 \pm 1.2	12.9 \pm 1.3	15.1 \pm 1.7	15.9 \pm 1.7	16.3 \pm 3.4
Lymphocyte (%)	78 \pm 3.2	74.2 \pm 1.8	72.3 \pm 2.7	70.1 \pm 3.2	69.7 \pm 1.9	70.4 \pm 3.1
Monocyte (%)	12.2 \pm 1.9	14.1 \pm 1.2	14.8 \pm 1.5	14.9 \pm 1.6	14.4 \pm 0.5	13.3 \pm 1.1
Platelets ($\times 10^3/\mu\text{L}$)	663 \pm 138	780 \pm 96	904 \pm 182	679 \pm 105	399 \pm 52	395 \pm 46

* $P < 0.01$; ** $P < 0.001$.

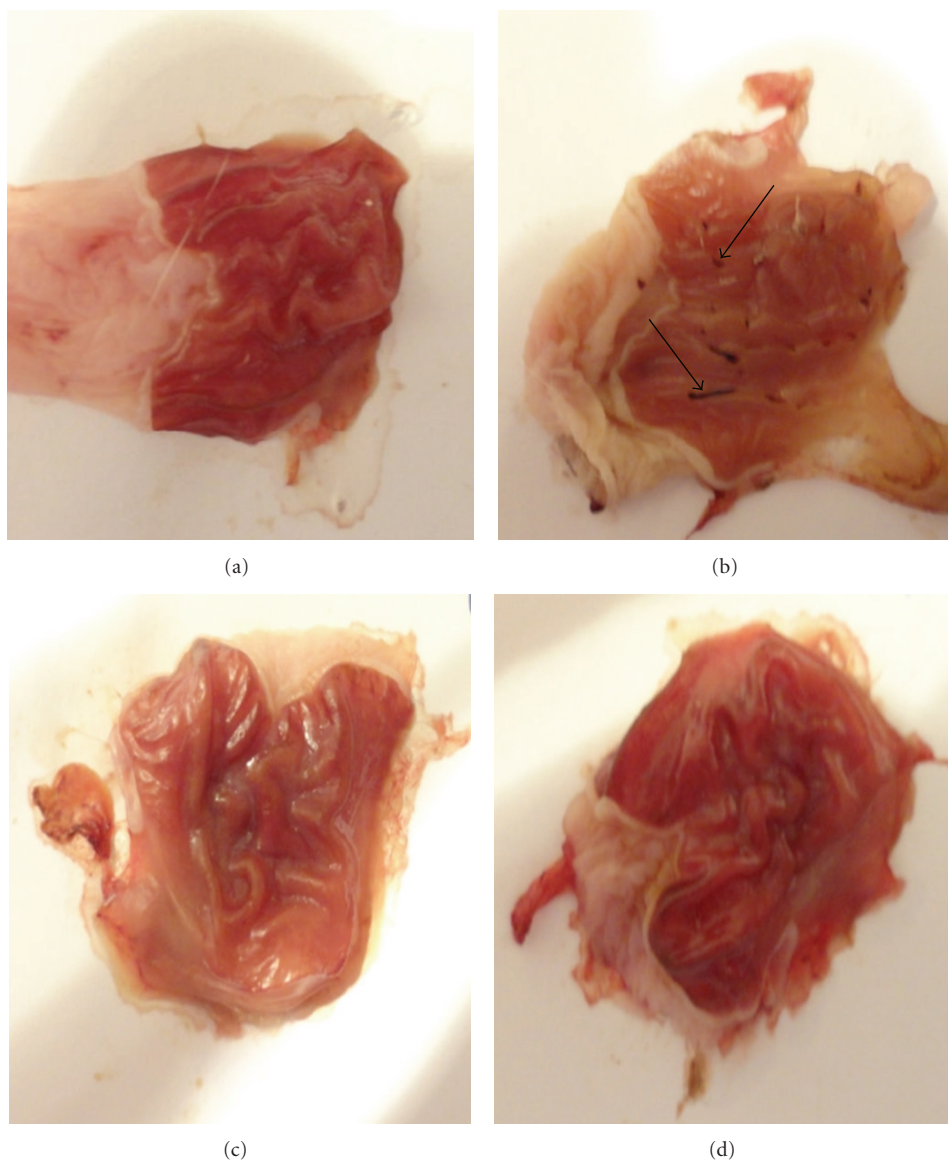


FIGURE 5: Photographs of open rat stomachs following 3-day administration of (a) normal saline, (b) indomethacin, (c) ZM4, and (d) ZM5. Arrows in photograph (b) indicate examples of full mucosal ulcers induced by indomethacin treatment.

TABLE 2: Rats serum biochemical levels following a 10-day administration of ZM4 and ZM5.

Parameters	Control	ZM4 50 mg/kg	ZM5 50 mg/kg
Cholesterol (mg/dL)	72 ± 9	86 ± 3	85 ± 5
Triglyceride (mg/dL)	61 ± 6	66 ± 7	92 ± 8*
Total protein (g/L)	67 ± 5	75 ± 2	77 ± 2
Albumin (g/L)	41 ± 1	42 ± 1	42 ± 1
GGT (IU/L)	0.6 ± 0.2	0.6 ± 0.1	0.9 ± 0.2
GPT (IU/L)	79 ± 8	73 ± 3	67 ± 5
GOT (IU/L)	125 ± 10	125 ± 6	126 ± 4
ALP (IU/L)	561 ± 68	527 ± 57	545 ± 78
Glucose (mg/dL)	170 ± 7	177 ± 7	177 ± 5
Urea (mg/dL)	46 ± 3	43 ± 2	40 ± 2
Creatinine (mg/dL)	0.2 ± 0.0	0.2 ± 0.0	0.4 ± 0.1*

* $P < 0.05$.

4. Discussion

Pain management is considered one of the major issues of healthcare systems worldwide. Analgesics used to treat nociceptive pain traditionally follow the World Health Organization ladder, stepping through paracetamol, NSAIDs, and finally opioids. However, neuropathic pain conditions respond better to antidepressant and anticonvulsant classes of medication [20]. The antinociceptive and anti-inflammatory effects of NSAIDs are mainly due to their common property of inhibiting COX enzymes involved in the formation of prostaglandins. Prostaglandins are potent hyperalgesic mediators which modulate multiple sites along the nociceptive pathway and enhance both transduction (peripheral sensitizing effect) and transmission (central sensitizing effect) of nociceptive information which in turn leads to normalization of the increased pain threshold associated with inflammation [21, 22]. A full inflammatory response, however, is sustained by prostanoids generated by both constitutive and inducible COX. The most prominent signs of acute inflammation due to prostaglandin-induced vasodilatation and increased blood vessel permeability, erythema, and edema have been shown to be inhibited by both non-selective traditional NSAIDs and selective COX-2 inhibitors through inhibition of prostaglandin synthesis [22].

Concerning analgesia, both peripherally and centrally mediated analgesic effects of our tested compounds were investigated in this study. The acetic acid-induced abdominal constriction, a behavior called writhing, generally elucidates peripheral analgesic activity [23]. Acetic acid, which is a common inducer of writhing syndrome in rodents [24, 25], causes algia by increasing the peritoneal fluid level of prostaglandins which in turn, excites the pain nerve endings. A dose-dependent reduction in the number of writhings was observed for ZM4 and ZM5 compounds at the high tested dose (50 mg/kg), and both compounds were equipotent to the effect of aspirin (200 mg/kg). Hot plate test was also performed to confirm the peripheral pain inhibition of the tested compounds as many previous studies proved the potential of using the hot plate test in investigating the peripheral analgesic activity of nonopioid analgesics such

as herbal drugs and NSAIDs [26]. Again, it was found that both ZM4 and ZM5 were superior in increasing the latency times compared to aspirin which reflects the ability of these compounds to reduce peripheral pain in rodents.

On the other hand, formalin test reveals both peripheral and central analgesic activities. It was previously reported that injecting formalin in rodent paws produces a typical pattern of two-phase pain response [17, 18]. The first phase (early, acute phase) starts immediately (0–5 min) after administration of formalin and corresponds to neurogenic pain while the second phase (late, tonic phase) usually starts 15 min after formalin administration and reflects inflammatory pain. The early phase has been proved to be sensitive to reversal by analgesics such as opioids and paracetamol while the second phase of response has classically been linked to inflammation as NSAIDs such as aspirin, ibuprofen, and ketoprofen that are active in reducing the associated behaviors [5, 6]. Since the nociception in formalin test is most likely produced via the COX-1 as well as COX-2 pathways in rat [27], ibuprofen rather than aspirin (irreversible COX-1) was chosen as a positive control in current formalin testing due to its nonselectivity in inhibiting COX enzyme. Previously, it was reported by Daud et al. [28] that ibuprofen can reduce significantly the licking time of rats injected with formalin in their paws. The reduction of pain response, that is, licking time, in both early and late phases after the administration of ZM4 was comparable to the effect of ibuprofen which indicates the ability of this compound to eliminate neurogenic and inflammatory pain. Conversely, ZM5 showed less pronounced activity in the late formalin phase ($P > 0.05$) which again reflects the superiority of ZM4 in reducing acute and chronic pain. Nevertheless, such conclusion should not reflect the less anti-inflammatory ability of ZM5 as we have recently reported that ZM5 showed comparable anti-inflammatory effects to diclofenac and celecoxib in rat paw edema test and enhances the production of transforming growth factor- β from T regulatory cells as compared with other tested compounds [11, 13, 14]. Therefore, it could be suggested that ZM5 has slow rate of absorption through gastrointestinal tract since it neither showed any anti-inflammatory effect during the first hour after carrageenan paw edema induction nor any significant reduction in the late phase of formalin test. On the other hand, the quick antinociceptive behavior and reduction of paw edema following oral administration of ZM4 indicate its quick absorption. Thus, pharmacokinetic studies are still warranted to confirm the rate and extent of oral absorption of ZM compounds.

Both single and repeated administration of high doses of ZM4 and ZM5 orally and intraperitoneally did not cause any mortality or detected behavioral changes including any impairment in motor function of the treated animals compared to the control. Repeated administration of high doses for ten days did not show any change in body weight or any change in most of the organ weights. Furthermore, the repeated administration did not show any significant change in lipid profiles except an unexplained rise in female triglyceride level that was not apparent in males. The kidney and liver function tests were reported normal except again the

unexplained rise in creatinine levels in females only, even though urea levels were not changed. However, increase in RBC, HB, and PCV values was noted indicating that these compounds could induce erythropoiesis. Further repeated studies are warranted to explain the effects and safety of ZM compounds on the respiration, blood gases, and the bone marrow to highlight their mechanism.

In conclusion, ZM4 and ZM5 were structured to exhibit analgesic activity as well as to have less adverse events. Recently, these compounds were shown to exhibit effect on enhancing anti-inflammatory and reducing proinflammatory cytokines from different population of spleen cells [14]. Such effects strengthen the use of such compounds, but more pharmacokinetic and pharmacodynamic studies are warranted.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Raghad Shakir performed the analgesia and toxicity study. Z. A. Muhi-eldeen designed the synthesis of the compounds. K. Z. Matalka participated in the statistical analysis and edited the paper. N. A. Qinna designed the experimental work, statistical analysis, and drafted the paper. All authors read and approved the final version of the paper.

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