ACS OMEGA

http://pubs.acs.org/journal/acsodf

Article

Involvement of the Opioidergic Mechanism in the Analgesic Potential of a Novel Indazolone Derivative: Efficacy in the Management of Pain, Neuropathy, and Inflammation Using *In Vivo* and *In Silico* Approaches

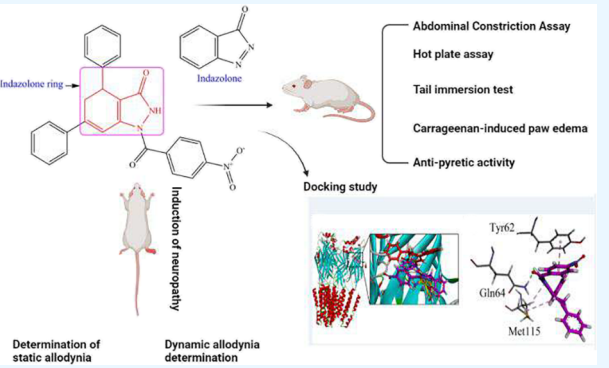
Qarib Ullah, Zarshad Ali, Umer Rashid, Gowhar Ali,* Nisar Ahmad, Rasool Khan, Sami Ullah, Muhammad Ayaz,* and H C Ananda Murthy*

Cite This: *ACS Omega* 2023, 8, 22809−22819

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: Indazolones possess interesting pharmacological activities. The search for indazole and indazolone-containing nuclei as drugs is an important research area of medicinal chemistry. The current work aims to evaluate a novel indazolone derivative against *in vivo* and *in silico* targets of pain, neuropathy, and inflammation. An indazolone derivative (ID) was synthesized and characterized using advanced spectroscopic techniques. Well-established animal models of abdominal constriction, hot plate, tail immersion, carrageenan paw edema, and Brewer's yeast-induced pyrexia were employed for evaluating the potential of the ID at different doses (20−60 mg kg^{-1}). Nonselective GABA antagonists, opioid antagonist naloxone (NLX) and pentylenetetrazole (PTZ), were employed to assess the potential role of GABAergic and opioidergic processes. The antineuropathic potential of the drug was evaluated using a vincristine-induced neuropathic pain model. *In silico* studies were performed to assess any possible interactions of the ID with pain target sites like cyclooxygenases (COX-I/II), $GABA_A$, and opioid receptors. This study revealed that the selected ID (doses of 20−60 mg kg^{-1}) efficiently hampered chemically and thermally induced nociceptive responses, producing significant anti-inflammatory and antipyretic effects. These effects produced by the ID were dose-dependent (i.e., 20−60 mg kg^{-1} and p range of 0.001−0.01) and significant in comparison to standards ($p < 0.001$). Antagonistic studies with NLX (1.0 mg kg^{-1}) and PTZ (15.0 mg kg^{-1}) revealed the involvement of the opioidergic mechanism rather than the GABAergic mechanism. The ID showed promising anti-static allodynia effects as well. *In silico* studies revealed preferential binding interactions of the ID with cyclooxygenases (COX-I/II), $GABA_A$, and opioid receptors. According to the results of the current investigation, the ID may serve in the future as a therapeutic agent for the treatment of pyrexia, chemotherapy-induced neuropathic pain, and nociceptive inflammatory pain.



INTRODUCTION

Since ancient times, clinical management of pain and inflammation to restore the health of an individual is the main objective of all health care professionals including drug discovery researchers.[1] Etiologically, pain may be categorized as nociceptive, inflammatory, and neuropathic in nature.[2,3] New drug discovery with the desired pharmacological contour is a huge challenge in the area of clinical investigation.[4] Pakistan is a third world country with inadequate facilities for health. In Pakistan, new drug development has been a derelict field of investigation. Hence, this state requires projects of new drug development that comprise research groups of diverse expertise.[5]

Literature reports show that nitrogen-containing indazolone nuclei are the integral parts of several therapeutically important drugs. Development of new drugs with indazolones and indazoles is an extremely crucial area of medicinal field research.[6] Literature reports have shown that nitrogen-containing indazolone and indazole nuclei are the integral part of a number of therapeutically important drugs.[7,8] Interesting pharmacological activities have been reported for a chemically important class of compounds characterized as indazolones. Some interesting pharmacological activities of therapeutic importance include antinociceptive, anti-inflammatory, antipyretic, antineoplastic, and antimicrobial (antibacte-

Received: March 14, 2023
Accepted: May 26, 2023
Published: June 12, 2023

rial/antifungal).[9−13] Currently, copious investigational preparations have been conducted by scientists to control the pain syndrome; however, therapies are not completely satisfactory.[14,15] The compound under study is newly synthesized with a series of compounds, which are screened for preliminary behavioral tests. The basis of selected *in vivo* activities was the efficacy in preliminary behavioral tests at 20−60 mg kg^{-1} doses, which were selected based on the up and down method of dose selection. There are no previously reported pharmacological activities for this compound; however, the basic nucleus of this compound possesses several interesting pharmacological activities as mentioned above. We expect that our selected indazolone, i.e., 1-(4-nitrobenzoyl)-4,6-diphenyl-1,2,4,5-tetrahydroindazol-3-one (Figure 1), may be one of the potential candidates for pain management.

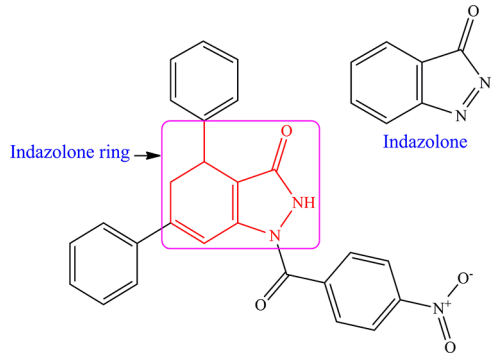


Figure 1. Structures of indazolone and its derivative.

Presently, mild to moderate pain and inflammation are managed with an extensively used class of analgesics characterized as NSAIDs (nonsteroidal anti-inflammatory drugs), while narcotic analgesics (e.g., morphine) are given for severe pain (like cancer pain, bullet pain, bone injury, etc.) that cannot be controlled with classical NSAIDs.[16] Although non-narcotic analgesics (e.g., NSAIDs) and narcotic analgesics (opioids) are generally prescribed for the management of mild to moderately severe painful conditions, these therapies have serious limitations as well. For example, there are well documented pieces of evidence that continuous use of NSAIDs displays several toxicity such as GIT ulceration, bleeding, perforation, cardiovascular disorders, and analgesic nephropathy, while a major problem with narcotic analgesics is the development of drug dependence (physical and psychological) and drug tolerance.[17,18] Hence, across the globe, extensive search is in progress in the area of pain syndrome.

MATERIALS AND METHODS

Chemicals and Drugs. Brewer's yeast was acquired from Merck (Germany), and vincristine was from Pharmedic Laboratories Pakistan. Diclofenac sodium from Suzhou Chem (China), glacial acetic acid from Pancreac (Spain), gabapentin from Lowitt Pharmaceuticals (Peshawar), tramadol from Searle (Pakistan), normal saline from Otsuka Lasbella (Pakistan), paracetamol from Afine Chemicals (China), and lambda carrageenan, naloxone, and pentylenetetrazole from Sigma-Aldrich (USA) were used in this study.

Chemical Synthesis of the ID (1-(4-Nitrobenzolyl)-4,6-diphenyle-1,2,4,5-tetrahydroindazol-3-one). The indazolone derivative (ID) was synthesized by mixing 10 mmol of ethyl-2-oxo-4,6-diphenylcyclohex-3-enecarboxylate and 10 mmol of 4-nitrobenzohydrazide in 1:1 ratio, and the mixture was refluxed in 25 mL of ethanol for 10 h. Glacial acetic acid (1 mL) was added as a catalyst. The resultant solid product was cooled, filtered, and purified by recrystallization from ethanol. ^{1}H and ^{13}C NMR, IR, and mass spectrophotometry were used for the characterization of the compound (Figure 2). This compound was synthesized by Dr. Rasool Khan of Institute of Chemical Sciences, University of Peshawar (a co-author in this manuscript). Actually, a series of compounds were synthesized in this institute and then we selected this promising compound for our selected pharmacological studies.

Animals. Mice (Balb-c, weighing 18−30 g) were used in these studies and were given regular laboratory diet and water. The temperature (22 ± 2 °C) was maintained by a reversible air condition system with a 12:12 h dark and light period. The Animals Scientific Procedures Act (1986) of U.K. and the protocols of Research Ethical Committee, Pharmacy Department, Peshawar University, Pakistan (Approval no. 12/EC-17/Pharm.) were followed as experimental guidelines.

Biological Studies. *Acute Toxicity.* I.P. injections of the ID at dosages of 20, 40, 80, and 160 mg kg^{-1} were administered to the rats (n = 6 per group). The mortality rate and any abnormality in behavior were observed for 72 h of the treatment.[19]

Abdominal Constriction Assay. The mice (male or female) having weights of 18−22 g were fasted for 2 h before the experiments began. Acetic acid solution (1%) was administered i.p. to induce the writhing reflex in the mice.[20,21] The number of abdominal constrictions that occurred during 20 min was counted following the acetic acid injection. Animals were assigned into five groups of six at random. The mice in group 1 were administered with normal saline, while groups 2, 3, 4, and 5 received diclofenac sodium (50 mg kg^{-1}) and 20, 40, and 60 mg kg^{-1} ID, respectively. The pain score in this model was calculated as a mean number of writhes for all groups.

Hot Plate Assay. In this experiment, Balb-c mice of either sex, weighing 18 to 22 g, were used. With n = 6 in each group,

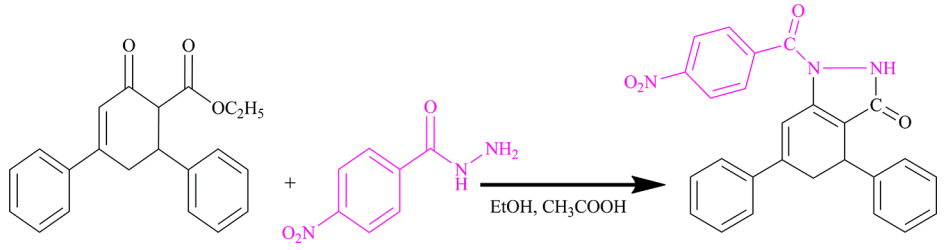


Figure 2. Scheme of synthesis for the indazolone derivative (ID).

https://doi.org/10.1021/acsomega.3c01717
ACS Omega 2023, 8, 22809−22819

the animals were divided into five groups at random. Group 1 animals were treated after pretesting with normal saline, while groups 2, 3, 4, and 5 received tramadol (30 mg kg^{-1}) and ID (20−60 mg kg^{-1} i.p.). The temperature of the hot plate analgesiometer was set at 54 ± 0.1 °C. The latency time for each animal was determined at 30 to 90 min intervals after treatment.[22,23] To have a clue of the underlying antinociceptive mechanisms, separate groups of animals were injected with naloxone (NLX, 1 mg kg^{-1}) subcutaneously (s.c.) or pentylenetetrazole (PTZ, 10 mg kg^{-1}, i.p.) 10 or 30 min before the treatment, respectively. The response of animals was noted at 30, 60, and 90 min intervals.[20,24]

Test for Tail Immersion. The tail immersion test was conducted as previously reported elsewhere to assess the analgesic effect of the ID.[25] Male albino (Balb-c) mice of 18−22 g were used. The lower segment of the tail (5 cm) of mice was dipped in a beaker containing hot water at 55 ± 0.5 °C.[26] With a 10 s time limit, the tail's withdrawal time from the water was measured in s. The withdrawal time was determined before and after i.p. injection of vehicle (group 1), tramadol (30 mg kg^{-1}, group 2), and the test compound at 20−60 mg kg^{-1} (groups 3 to 5) at 30, 60, and 90 min intervals. The pain score was calculated as the mean of tail withdrawal latencies (seconds).

Model for Carrageenan-Induced Paw Edema. The carrageenan-induced paw edema mice model was utilized to evaluate ID's anti-inflammatory efficacy.[19,27] The right hind paw of mice was injected subcutaneously to produce inflammation of 1% lambda-carrageenan (0.1 mL). The mice were injected with 10 mL kg^{-1} (group 1), 50 mg kg^{-1} diclofenac sodium (group 2), and the test compound (20, 40, and 60 mg kg^{-1}, i.p., groups 3 to 5) 1 h before injecting carrageenan. The edema was measured at 0−5 h using a digital plethysmometer. The change in paw edema was calculated, and the anti-inflammatory potential was calculated as [%-inhibition = (1 − Δtest/Δvehicle) × 100].

Antipyretic Activity. Mice (Balb-c, 30−35 g) were used in this study. The mice were divided into five groups; vehicle (10 mL kg^{-1}), paracetamol (40 mg kg^{-1}), and the test drug (20, 40, and 60 mg kg^{-1}). A digital thermometer was used to measure the mice's rectal temperature. Pyrexia was caused by injection (s.c.) of 20% Brewer's yeast solution into the neck of the mice after overnight fasting.[28] After 24 h, the rectal temperature was determined and only the mice whose rectal temperature increased by 0.3−0.50 °C were selected for further study. The respective treatments were administered to the chosen animals. Afterward, the temperature was monitored through 0.5−1.5 h of treatment.[29]

Induction of Neuropathy. The anticancer drug vincristine (0.1 mg kg^{-1} once a day, i.p.) was given over the course of 10 days to treat the onset of peripheral neuropathic pain.

Determination of Static Allodynia. Applying several von Frey hairs (0.4, 0.6, 1, 2, 4, 6, 8, 10, and 15 g), starting with a 2.0 g filament, vertically to the dorsal side of the right hind paw for 6 s or until the animal demonstrated a withdrawing response (lifted the paw), was used to assess static allodynia. The study only included animals that responded to forces of 3.63 g or less, with 15 g being the cutoff force.[30,31]

Determination of Dynamic Allodynia. A cotton bud was used to test for dynamic allodynia, and it was gently stroked across the mouse's hind paw dorsal surface. The lifting or licking reaction of the paw was considered as a positive response, and the time span between the application of the stimulus and the subsequent lifting or licking behavior was noted as "paw withdrawal latency". The time of 15 s was considered as the cutoff time.[30]

Docking Study. To predict the probable interactions of the ID with the target sites of the opioid receptors (mu, delta, and kappa), $GABA_A$, and cyclooxygenase (COX-I/II), molecular docking studies were conducted in accordance with previous protocols.[17−20] Protein Data Bank (PDB ID 1ACl and 1POI) was used to obtain the 3D crystal structures of the target receptor and enzyme protein complexes. After adding hydrogen atoms to the structure, MOPAC 7.0 was used to reduce their energy. Using Site Finder, the model was put through a thorough conformational search with default settings and an RMS gradient of 0.00001 kcal/mol. The site discovery tool of MOE was used to choose the active site of the receptor and enzymes.

Before docking the indazolone derivative, the protocol of docking was validated using a re-dock method. Native ligands were re-docked into the binding sites of the target enzymes by using different methods (Placement) and scoring functions (London dG, Affinity dG, ASE). The method has a root mean square value less than 2.0 Å. After docking studies, poses with the lowest binding energy value (S in kcal/mol) and good RMSD were chosen for further investigations. The same process was followed when docking the ID. The binding interactions and binding pattern analyses were then performed using the generated ligand-receptors/enzyme complex model.[32−35]

Statistical Analysis. For the data analysis, Graph Pad Prism (version 5) was used. The significance level was considered at $p < 0.050$.

RESULTS AND DISCUSSION

Characterization of ID (1-(4-Nitrobenzolyl)-4,6-diphenyle-1,2,4,5-tetrahydroindazol-3-one). Yellow solid, Yield = 70%, m.p. = 189−190 °C; R_f = 0.54 ethyl acetate/*n*-hexane (1:1); IR (KBr) υ max cm^{-1}: 3355 (N-H), 3076 (Ar-H), 1688 (C=O), 2868 (aliphatic C-H); ^{1}H NMR (400 MHz, CDCl3) δ: 11.05 (s, 1H, N-H), 7.2−8 (m, 14H, Ar-H), 3.2 (t, 1H, J = 3.4, C-4), 3.1 (d, 2H, J = 2.1, C-5), and 3.24 (s, 1H, C-7), ^{13}C NMR (100 MHz, CDCl3) δ: 170.0 (C=O), 125−130 (Ar-CH), 116 (C-6), 112.0 (C-4), and 142.39 (C-5) EI-MS; m/z (rel. int. %): 437 (M+); CHN Anal. Calcd: C, 71.39; H, 4.38; N, 9.61. Found: C, 71.24; H, 4.28; N, 9.51.

Acute Toxicity. The animal behavior and mortality were observed for 72 h after the administration of the ID. No mortality was observed in the test animals at the tested dose range at 20−160 mg kg^{-1}.

Antinociceptive Assays. *Writhing Assay.* The compound demonstrated a dose-dependent action in the acetic acid-induced writhing test in comparison to the vehicle-treated mice after the administration of the ID. The compound considerably reduced the painful reaction caused by acid in a dose-dependent manner at 20 mg kg^{-1} (*p = 0.040), 40 mg kg^{-1} (**p = 0.003), and 60 mg kg^{-1} (***p < 0.001) as compared to the vehicle-treated animals. Diclofenac sodium (50 mg kg^{-1}) produced a significant antinociceptive activity in this model as well (p < 0.001) (Figure 3).

Hot Plate Assay. A remarkable antinociceptive response was observed in mice on the hot plate test at 20 mg kg^{-1} [*p = 0.020 (60 min), *p = 0.040 (90 min)], 40 mg kg^{-1} [**p = 0.0033 (30 min), **p = 0.0023 (60 min), and **p = 0.0020 (90 min)], and 60 mg kg^{-1} [***p < 0.001 (30−90 min) doses

https://doi.org/10.1021/acsomega.3c01717
ACS Omega 2023, 8, 22809−22819

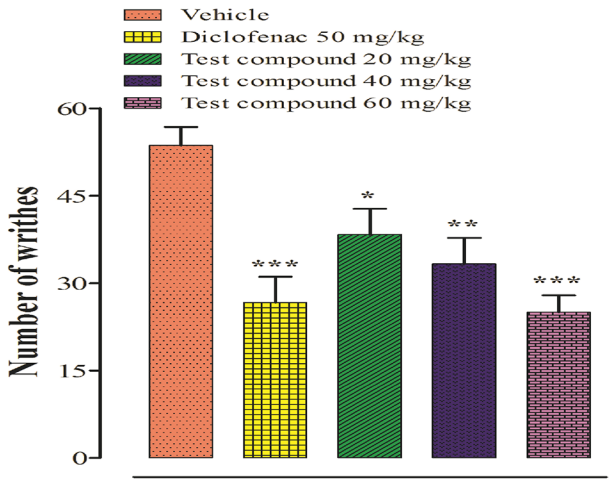


Figure 3. Antinociceptive activity of the ID. The bars represent the mean ± SEM. ANOVA accompanied by Dunnett's post hoc analysis; *p = 0.040, **p = 0.003, ***p < 0.001, (n = 6). ID administered intraperitoneally (i.p.).

of the ID in comparison to the control. Tramadol also produced an increase in the antinociceptive threshold in the hot plate analgesia for 30−90 min (***p < 0.001)] (Figure 4).

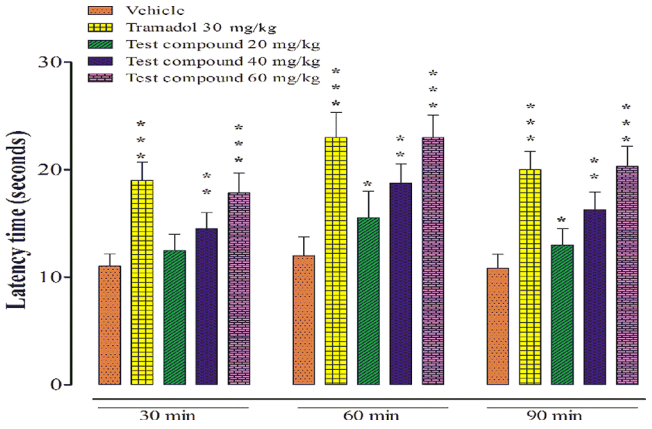


Figure 4. Hot plate analgesia of the ID at 20 mg kg^{-1} [*p = 0.020 (60 min), *p = 0.040 (90 min)], 40 mg kg^{-1} [**p = 0.0033 (30 min), **p = 0.0023 (60 min), and ** p = 0.0020 (90 min)], and 60 mg kg^{-1} [***p < 0.001 (30−90 min)] in mice. The bars depict the mean ± S.E.M. (n = 6). Dunnett's post-hoc analysis with ANOVA was utilized for determining the level of significance. ID administered intraperitoneally (i.p.).

Tail Immersion Test. In the tail immersion assay, administration of the ID to the mice exhibited a dose-dependent effect on the antinociceptive response. A statistically significant response of the ID at 20 mg kg^{-1} [*p = 0.022 (60 min), *p = 0.035 (90 min)], 40 mg kg^{-1} [**p = 0.004 (30 min), **p = 0.0034 (60 min), and **p = 0.0037 (90 min)], and 60 mg kg^{-1} (***p < 0.001) was observed as compared to the control group. Tramadol also produced a pronounced antinociceptive response (p < 0.001) through 30−90 min duration of the experiment (Figure 5).

Antagonism Studies. *Antagonism with Naloxone (NLX).* One milligram per kilogram of naloxone (NLX) was given subcutaneously 10 min before each session, and the animals' response latencies were noted at 30−90 min (Figure 6).[20] The results show that administration of NLX antagonized the

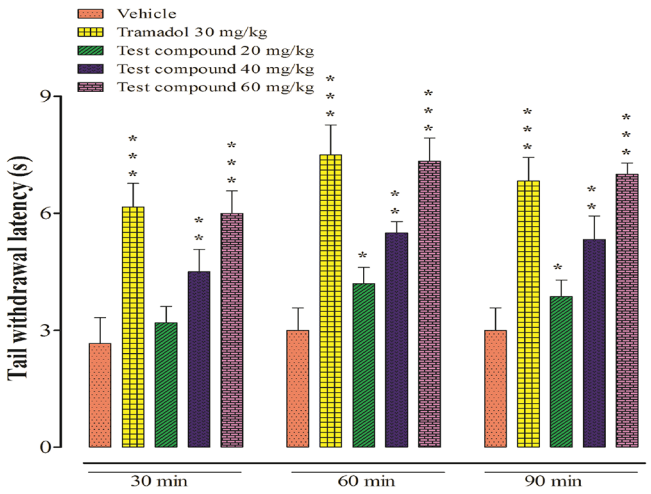


Figure 5. Tail immersion test, antinociceptive response of the ID at 20 mg kg^{-1} [*p = 0.022 (60 min), *p = 0.035 (90 min)], 40 mg kg^{-1} [**p = 0.004 (30 min), **p = 0.0034 (60 min), and ** p = 0.0037 (90 min)], and 60 mg kg^{-1} [***p < 0.001 (30−90 min)] in mice. Each bar depicts the mean ± S.E.M. (n = 6). ANOVA with Dunnett's post-hoc test was used for determining the significance level. ID administered intraperitoneally (i.p.).

antinociceptive response induced by the test compound at doses of 40 mg kg^{-1} [*p = 0.045 (30 min), *p = 0.038 (60 min), *p = 0.033 (90 min)] and 60 mg kg^{-1} [**p = 0.004 (30 min), **p = 0.005 (90 min), and ***p < 0.001 (60 min)]. Tramadol's antinociceptive reaction was shown to have substantially reversed at 30 to 90 min (***p = 0.001) in comparison to control animals (Figure 6).

Antagonism with PTZ. In the antagonism studies, PTZ did not significantly affect ID's antinociceptive response after 30 min, except with a mild effect after 60 and 90 min (*p = 0.040 and *p = 0.045). Contrarily, PTZ failed to antagonize the antinociceptive response of tramadol (30 mg kg^{-1}) at any point of time during the experimental protocol (Figure 7).

Anti-inflammatory Studies. *Carrageenan-Induced Paw Edema.* The ID showed a dose-dependent anti-inflammatory activity in the carrageenan-induced paw edema inflammatory model at dosages of 20 mg kg^{-1} [*p = 0.014 (30 min), *p = 0.026 (60 min), *p = 0.030 (90 min)], 40 mg kg^{-1} [**p = 0.004 (30 min), **p = 0.0031 (60 min), and **p = 0.0024 (90 min)], and 60 mg kg^{-1} (***p < 0.001) at 30 to 90 min. Diclofenac sodium (50 mg kg^{-1}) exhibited a more vigorous anti-edema response throughout all the testing time points (***p < 0.001) (Figure 8).

Antipyretic Activity. A substantial antipyretic effect was seen after the injection of the ID at 20 mg kg^{-1} [*p = 0.022 (30 min), *p = 0.034 (60 min), *p = 0.039 (90 min)], 40 mg kg^{-1} [**p = 0.007 (30 min), **p = 0.006 (60 min), **p = 0.007 (90 min)], and 60 mg kg^{-1} [***p < 0.001 (30−90 min)] (Figure 9). Paracetamol, on the other hand, produced a significant antipyretic response at all the time points after its administration (***p < 0.001).

Effect of the ID on Static Allodynia. The ID at doses of 40 mg kg^{-1} (*p = 0.01) and 60 mg kg^{-1} (**p < 0.001) exhibited a substantial increase in the paw withdrawal threshold in comparison to the standard control drug. Gabapentin (GP) 75 mg kg^{-1} showed a highly significant response, viz., [***p < 0.001 (30 min), ***p < 0.001 (60 min), ** p = 0.001 (90 min)], in comparison to the

https://doi.org/10.1021/acsomega.3c01717
ACS Omega 2023, 8, 22809−22819

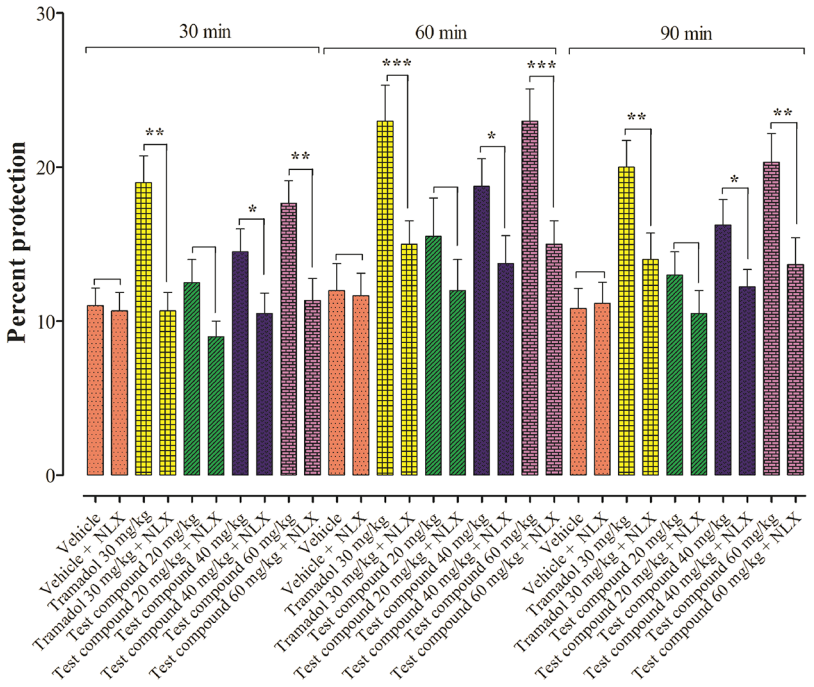


Figure 6. Effect of naloxone (NXL-1) on the antinociceptive effect of the ID in the hot plate test. The bars show the mean ± SEM. ID 40 mg kg^{-1} (*p = 0.045 (30 min), *p = 0.038 (60 min), *p = 0.033 (90 min)) and 60 mg kg^{-1} (**p = 0.004 (30 min), *p = 0.005 (90 min), and ***p < 0.001 (60 min)) in comparison to the vehicle-treated group, (ANOVA accompanied by Dunnett's post hoc analysis) (n = 6 per group). ID administered intraperitoneally (i.p.).

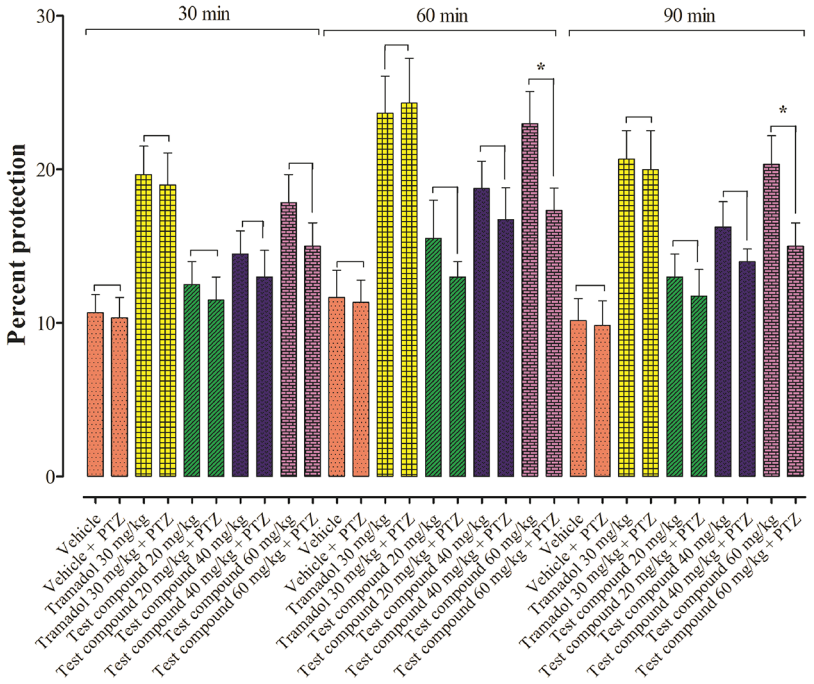


Figure 7. Effect of pentylenetetrazole (PTZ-15) on the antinociceptive effect of the ID in the hot plate test. The bars show the mean (±S.E.M.). *p = 0.040 (60 min) and *p = 0.045 (90 min) compared to the ID (alone)-treated group. ANOVA accompanied by Dunnett's post hoc analysis (n = 6 per group). ID administered intraperitoneally (i.p.).

vincristine-treated animals. The test compound ID demonstrated [**p = 0.004 (30 min), **p = 0.004 (60 min), **p = 0.009 (90 min)] at 40 mg kg^{-1} and [***p < 0.001 (30−90 min)] at 60 mg kg^{-1} compared to the vincristine-treated group (VINC). GP also showed an increasing trend in the paw withdrawal threshold of the vincristine-induced neuropathic mice to the applied force (s) as compared to the normal control animals when tested with von Frey filaments (Figure 10).

ID Binding Interactions toward COX Enzymes, Opioid Receptors, and GABA$_A$ Receptors. ID showed two hydrogen bond interactions with the active sites of amino acid. The carbonyl oxygen in indazolone ring forms bifurcated H-bonds as an acceptor with Arginine 120 and Tyrosine 355.

https://doi.org/10.1021/acsomega.3c01717
ACS Omega 2023, 8, 22809−22819

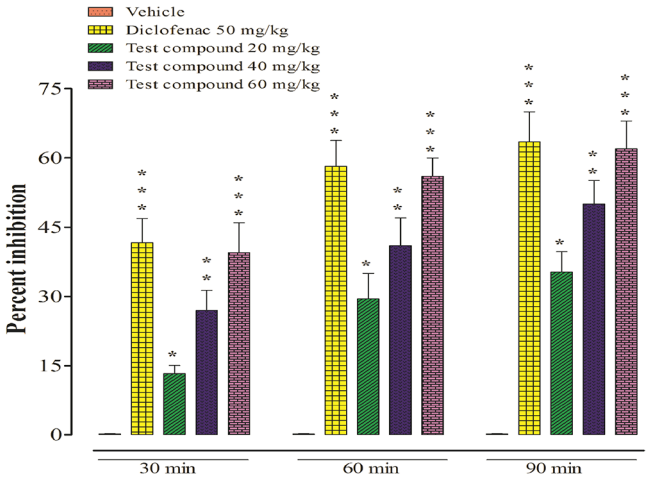


Figure 8. ID's anti-inflammatory properties in a mouse model of carrageenan-induced paw edema. Data is presented as mean ± S.E.M. (n = 6). The ID depicted a substantial response at 20 mg kg^{-1} [*p = 0.014 (30 min), *p = 0.026 (60 min), *p = 0.030 (90 min)], 40 mg kg^{-1} [**p = 0.004 (30 min), **p = 0.0031 (60 min), and **p = 0.0024 (90 min)], and 60 mg kg^{-1} [(***p < 0.001)] at 30−90 min. ANOVA accompanied by Dunnett's post-hoc analysis compared to the vehicle control (n = 6 per group). ID administered intraperitoneally (i.p.).

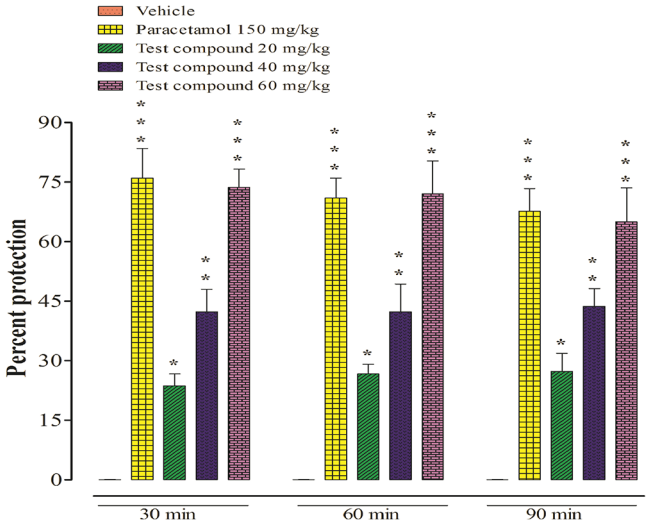


Figure 9. Antipyretic potential of the test compound ID in the standard assay of Brewer's yeast-induced pyrexia in mice. The bars show the mean ± S.E.M. The ID exhibited a significant antipyretic effect at 20 mg kg^{-1} [*p = 0.022 (30 min), *p = 0.034 (60 min), *p = 0.039 (90 min)], 40 mg kg^{-1} [**p = 0.007 (30 min), **p = 0.006 (60 min), **p = 0.007 (90 min)], and 60 mg kg^{-1} [***p < 0.001 (30−90 min)]. ANOVA accompanied by Dunnett's post-hoc analysis. ID administered intraperitoneally (i.p.).

The ID was also docked onto the active sites of COX-2, and 3D binding orientations of the compound was superimposed on the ibuprofen (ligand) in the active site of the COX-2 (Figure 11a,c), while the 2D binding show interactions (Figure 11b,d). The oxygen of the nitro group shows H-bonding interactions with Arginine 120. Meanwhile, a π-alkyl interaction was observed for COX-2 and Val 523 specific sites. The computed energy for the COX-1 and ligand complex was −4.84 kcal/mol, and that for the COX-2 and ligand complex was −6.66 kcal/mole. Meanwhile, the binding energy

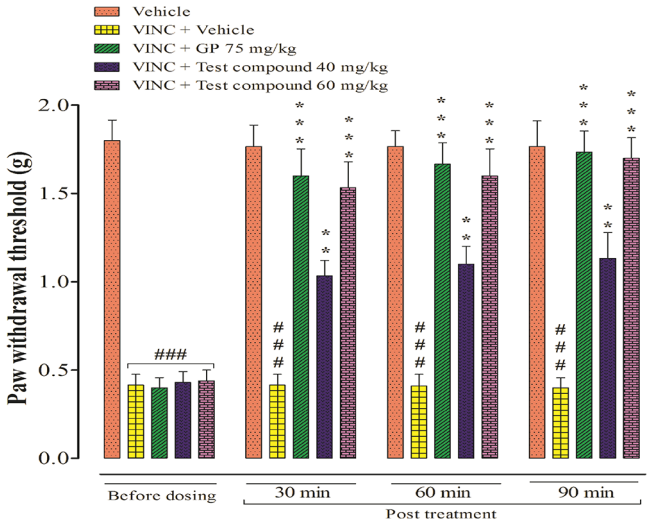


Figure 10. Effect of the ID at 20, 40, and 60 mg kg^{-1} and gabapentin (75 mg kg^{-1}) on the expression of vincristine-induced static allodynia in the hind paws of mice. GP (75 mg kg^{-1}) showed a highly significant response, viz., [***p < 0.001 (30 min), ***p < 0.001 (60 min), ***p = 0.001 (90 min)], compared to the vehicle-treated animals. The ID demonstrated [**p = 0.004 (30 min), ** p = 0.004 (60 min), **p = 0.009 (90 min)] at 40 mg kg^{-1} and [***p < 0.001 (30−90 min)] at 60 mg kg^{-1} compared to the vehicle-treated group. Data are expressed as mean ± S.E.M. ANOVA accompanied by Dunnett's post-hoc analysis compared to vincristine (VINC)-treated animals (n = 6 per group). ID administered intraperitoneally (i.p.).

values of the native ligands ibuprofen and SC-558 in the binding sites of COX-1 and COX-2 are −7.49 and −8.27 kcal/mol, respectively (Table 1).

The ID was also docked onto the active sites of the GABAergic receptor. The lowest energy 3D binding sites of the ID (pink) overlapped on the ligand (red), $GABA_A$-receptor, and methaqualone (yellow) (Figure 12a). The binding interactions of the ID with amino acid groups show that the oxygen of carbonyl establishes a H-bond with Gln-64. $\pi-\pi$ stacking was observed between the nitrophenyl ring and Tyr62, and π-alkyl interactions were detected between the ID ring and Met115 (Figure 12b). Furthermore, the ID was docked into the active sites of the opioid (μ, κ, and δ receptors) (Figure 13a−f). Binding interactions showed that it forms two H-bonds, two $\pi-\pi$ stacking interactions, and few π-alkyl interactions. The H-bond was established between the nitro group and Asn150/Glu-325. Meanwhile, Tyr-148 and Tyr-326 show $\pi-\pi$ stacking interactions with phenyl and nitrophenyl rings, respectively (Figure 13b). Two $\pi-\pi$ stacking interactions and one π-S interaction stabilize the ligand−enzyme complex. The $\pi-\pi$ stacking interaction was observed between Tyrosine (320 and 287) and the phenyl ring of the compound, while π-S interaction was observed between Met142 and another phenyl group. Figure 13e shows the best scored binding direction, which forms two H-bonds with the enzyme and ligand complex and is therefore stabilized. The nitro group and carbonyl oxygen of the ID form a H-bonding interaction with Lys108 and Lys214, respectively, as shown in Figure 13f. Meanwhile, the phenyl ring of the ID shows $\pi-\pi$ stacking interaction with Tyr308. The binding energy calculated for the ID and the native ligands in the binding site of target receptors are presented in Table 1. The Binding

https://doi.org/10.1021/acsomega.3c01717
ACS Omega 2023, 8, 22809−22819

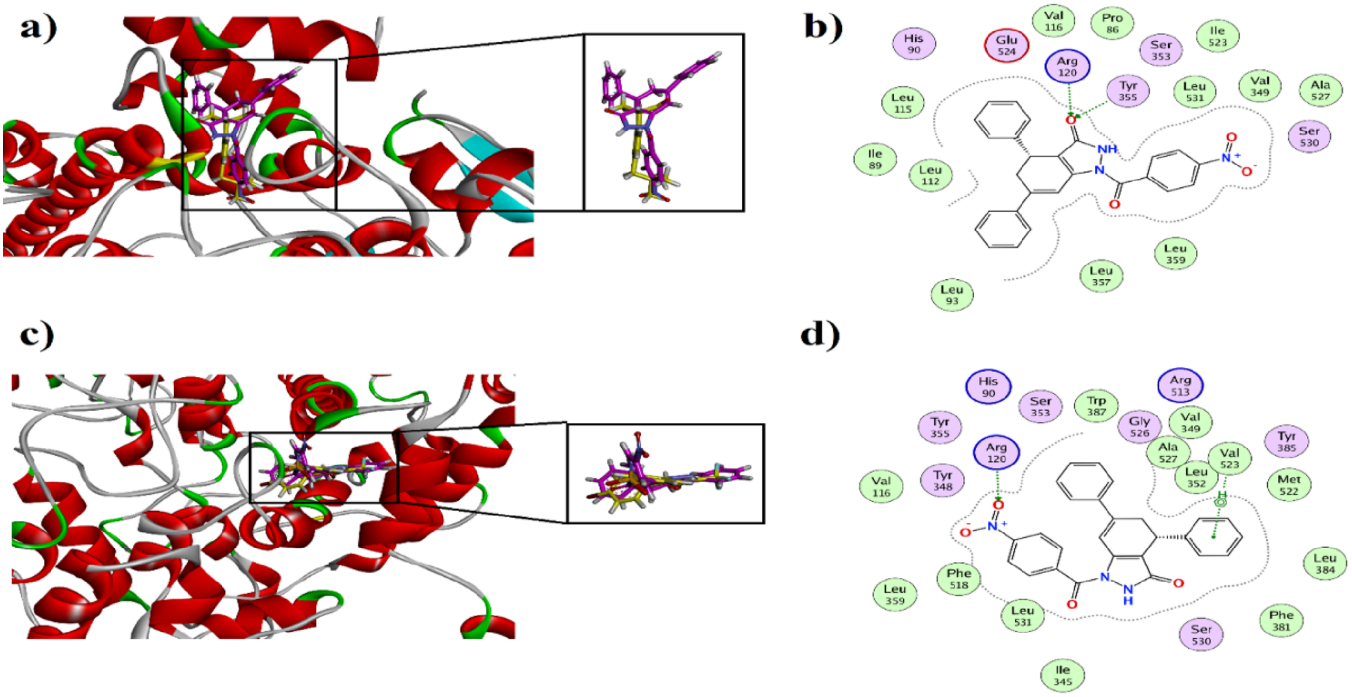


Figure 11. (a, c) 3D ribbon of the ID (pink) superimposed on the native ligands (yellow) in binding sites of COX (I and II). (b, d) 2D interaction plots of the ID against the COX (I and II) enzymes.

Table 1. Computed Binding Energy Values of Native Ligands and the Indazolone Derivative in the Binding Sites of Target Receptors

		binding energy (kcal/mol)	
target enzyme	PDB ID	native ligand	indazolone derivative
COX-1	1EQG	−7.49	−4.84
COX-2	1CX2	−8.27	−6.66
GABA receptors	4COF	−5.08	−6.06
δ receptors	4EJ4	−8.91	−7.98
μ receptors	4DKL	−8.87	−7.79
κ receptors	4DJH	−8.39	−8.21

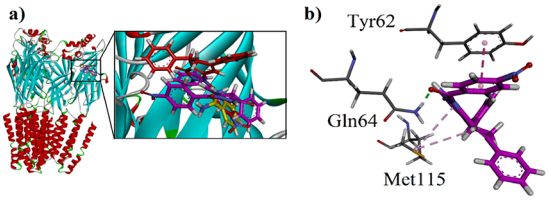


Figure 12. (a) 3D structure of bonding interactions of benzamidine (red), ID (pink), and methaqualone (yellow) in the active sites of the $GABA_A$ receptor. PDB code is 4COF. (b) 2D interaction plot for the ID.

orientations of the native ligands in the binding sites of respective enzymes are provided as Figure S-1 to Figure S-6.

Modification in the chemical structure of the existing molecules for new therapeutic uses or augmenting the existing pharmacological effects by incorporating new functional groups or derivatization has been an approach adopted by chemists to meet the challenges faced by human beings in different ages.[36,37] This study illustrated this idea and described the effects of the ID in well-known pharmacological models of pain, inflammation, pyrexia, and neuropathy in albino mice with additional and supporting evidence of *in silico* studies for the target receptors.[27] The writhing reaction produced by acetic acid is considered as a model for the assessment of peripheral pain mechanisms. Acetic acid works by activating endogenous pain mediators, which stimulate nociceptive neurons, which are responsive to a variety of medication classes, including opioids, nonsteroidal anti-inflammatory medicines, and other centrally acting analgesics.[38] Similar to diclofenac sodium, the ID increased the peripheral nociceptive threshold in a dose-dependent manner, suggesting a potential role in inhibiting the release of pain mediators. In the case of the hot plate assay, a higher latency was observed in the case of the ID as compared to the standard drug tramadol.

The opioid receptor antagonist NLX efficiently blocked the antinociceptive response of the ID in the hot plate assay, suggesting that opioidergic pathways were involved in generating its antinociceptive response. By activating the descending nerve fibers and impeding afferent nerve transmission by interacting with the opioid receptors, opioid analgesics prevent the transmission of pain.[24]

Opioid drugs, produce their pharmacological effects by binding to their receptors on the neuronal cell membranes. Opioid medications block both peripheral and centrally originating pain from a molecular perspective.[39] The literature shows that all three opioid receptors (m, δ, and κ) have a key role in numerous pharmacology actions produced by traditional opioids such as morphine.[40] These opioid receptors mediate spinal, supraspinal, and peripheral analgesia.[41]

Naloxone (NLX) is a competitive antagonist at opioid receptors. Opiates, particularly the drugs acting on *m*-opioid receptors, function as the essential analgesic agents for patients with intense pain.[42]

On the other hand, pain behavior is also mediated by GABAergic neurons and receptors.[43] Both the NLX (all doses) and PTZ (at the highest dose) blocked the antinociceptive response of the ID, indicating that its antinociceptive response

https://doi.org/10.1021/acsomega.3c01717
ACS Omega 2023, 8, 22809−22819

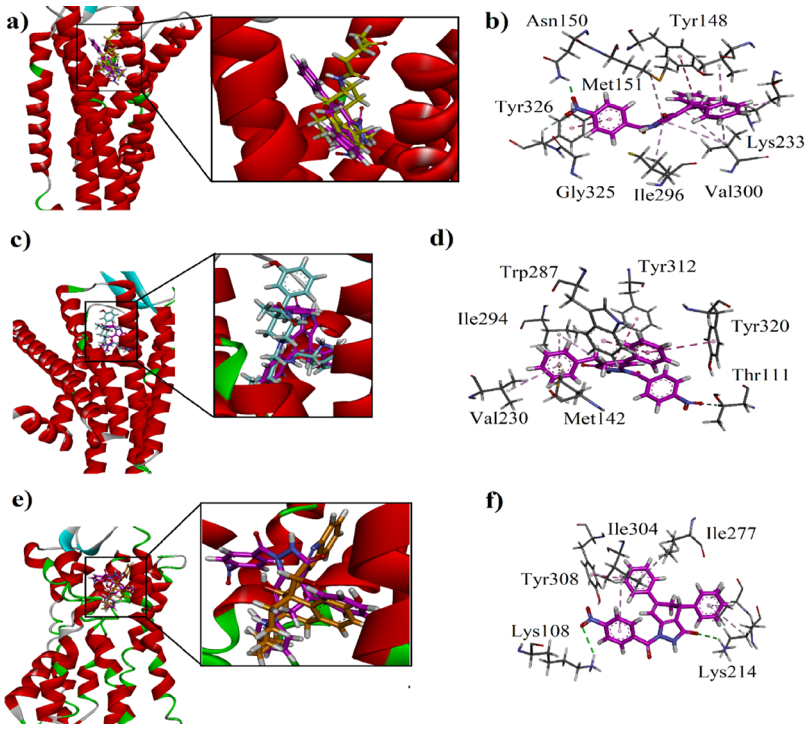


Figure 13. (a) 3D and (b) 2D structure of superimposed binding modes of benzamidine (red) in the active sites of the opoid receptors (δ) (PDB code is 4EJ4). (c) 3D and (d) 2D superimposed binding modes of the ligand in the active sites of the opioid receptor (κ) (PDB code is 4DJH). (e) 3D and (f) 2D structures of the ligand overlapping with the binding sites of opioid receptors (μ).

is being mediated by the mechanisms involving opioidergic and GABAergic pathways.[44] We have conducted standard tests regarding the mechanism of action, i.e., antagonism with naloxone (NLX) and antagonism with PTZ. These tests are considered standard because they are well-established and widely used in preclinical research to assess the involvement of these neurotransmitter systems in various physiological and behavioral responses. Naloxone (NLX) is a drug that is used as an antagonist of the opioidergic system. PTZ (pentylenetetrazol) is a drug that acts by inhibiting the activity of the GABAergic system, which is the main inhibitory system in the brain. By administering naloxone or PTZ in different experimental conditions, researchers can assess the involvement of the opioidergic and GABAergic systems in various physiological and behavioral responses. For example, by administering naloxone before administering an opioid drug, researchers can determine whether the effects of opioid are mediated by the opioidergic system. Similarly, by administering PTZ before testing, researchers can determine whether the drugs' effects are mediated by the GABAergic system.

The tail immersion test is another well-known assay that has been widely employed to evaluate the analgesic properties of opioids. It includes applying a heat stimulus to a rodent's tail and measuring how long it takes for the animal to "flip the tail" or "twitch".[45] The ID produced a significant antinociceptive response in the tail immersion test at all the tested doses. Since both the tail immersion and hot plate showed a significant decline in the nociceptive threshold, it can therefore be deduced that the ID has the potential to interfere with both the spinal and supraspinal pain pathways.

Inflammation embraces a varied range of interactions among a number of biological factors and cells that respond to injury, infections, toxic chemicals, etc. In the current study, the ID was also evaluated in a carrageenan-induced paw edema model. A group of inflammatory mediators, including histamine, serotonin, and kinins, characterizes the early phase of carrageenan-induced inflammation. The late phase, which lasts for 3 to 6 h, involves prostaglandins and lysosomal enzymes.[32] The current findings confirm that the ID successfully controlled both stages of the carrageenan-induced edema.

Since an unknown era, fever has been an essential feature or an indication of the presence of many diseases. The feverish response is controlled by the central nervous system through a multitude of mechanisms.[46,44] Fever is mostly accompanied by an ailing state and negative changes in bodily functions, including the immune system. Fever, therefore, is a major determinant of pathogenesis of many illnesses.

Compounds with an ability to constrain the prostaglandin synthesis can be used as potential antipyretic agents, like paracetamol, that inhibit the cyclooxygenase enzyme.[47] The ID decreased the febrile response compared to acetaminophen, used as a positive control. The molecular simulation findings showed that the ID possesses favorable interactions with the pain targets such as opioid receptors, COX-I and COX-II enzymes, and $GABA_A$ receptors.[48,49]

The molecular docking studies were performed for investigating the pain target site interactions and studied the COX enzyme inhibitory potential through molecular simulations for $GABA_A$, COX enzymes (I and II), and opioid (δ, κ, and μ) targets. The elongated isozymes' binding site blocks the hydrophobic route, extending from the binding area of the membrane to the catalyst. The two isoforms are dissimilar biologically from one another, and the Vl523 in the COX-2 is not retained in the COX-1 enzyme. The outcomes also showed that Tyrosine 355 and Arginine 120 make bifurcated H-bonds with the carbonyl group's oxygen atom, but Arginine 120 produces a H-bond with the nitrophenyl oxygen. Although

https://doi.org/10.1021/acsomega.3c01717
ACS Omega 2023, 8, 22809−22819

functional assays are considered the gold standard for assessing drug−receptor interactions and their pharmacological effects, there are situations where conducting such assays may not be feasible. In such cases, docking studies can still provide valuable insights into the potential mechanism of action of a drug. In this regard, we have conducted molecular docking study. Docking studies use computational methods to predict how a drug molecule binds to its target receptor and estimate the binding affinity. By analyzing the molecular interactions between the drug and the receptor, researchers can gain valuable information about the drug's potential efficacy, selectivity, and potency. This information can be used to guide the design and optimization of new drugs and to prioritize the most promising drug candidates for further experimental testing.[50,51]

The ID showed comparable activities, and its ability to interact with GABAergic and opioidergic receptors was evaluated. The 2D structure showed that the nitrophenyl ring displays a $\pi-\pi$ contact with Tyrosine 62 and alkyl interactions with Met 115.

The opioid receptor's role (μ, κ, and δ receptors) is well known in CNS. While performing docking simulation on opioid receptors, the 2D structure showed that the μ opioid receptor form two H-bonds, two $\pi-\pi$ interactions, and a small number of π-alkyl interactions. A H-bond is also formed between the nitro group and Asn150. The Tyr148 and Tyr326 shows $\pi-\pi$ interactions with the phenyl and nitrophenyl ring of indazolone. The kappa (κ) opioid receptor and ligand complex is balanced by two $\pi-\pi$ interactions and one π-s interaction. The Trp287 and Tyr320 shows $\pi-\pi$ interaction with one phenyl rings and a π-S interaction with a second phenyl ring and Met142 (PDB used was 4DJH). The molecular simulations were also performed for the ID and δ opioid receptors (PDB code was 4EJ4). The formation of two H-bonds, a π stack interaction, and only a small number of π-alkyl interactions stabilized the ligand−enzyme complex. The nitro group and carbonyl oxygen of the compound form a H-bond with Lys (108 and 214), while the phenyl group of the compound shows $\pi-\pi$ stacking interaction with Tyr308. It is important to note that docking studies have limitations and may not always accurately predict the drug's effects on the biological system.[52] For example, they may not account for the complex molecular interactions that occur *in vivo* or the impact of drug metabolism and pharmacokinetics. Therefore, it is crucial to validate the findings from docking studies through experimental testing using functional assays or other approaches. Docking studies can still provide valuable information for drug discovery and development when used appropriately and in combination with other approaches.

The ID was synthesized and characterized using NMR spectroscopy. Well-established animal models of abdominal constriction, hot plate, tail immersion, carrageenan-induced paw edema, and Brewer's yeast-induced pyrexia were employed for evaluating the potential of the ID at different doses *in vivo*.

CONCLUSIONS

A selected indazolone derivative was targetedly evaluated for antinociceptive, antineuropathic, antipyretic, and anti-inflammation potential in the standard models of rodents. The outcomes show that this indazolone compound exhibited statistically significant antinociceptive, antineuropathic, antipyretic, and anti-inflammation activities in a dose-dependent manner as compared to standard drugs like tramadol, aspirin, gabapentin, diclofenac sodium, and paracetamol, suggesting a dual mechanism as central and peripheral. Based on the results of the current study, it may therefore be concluded that the ID can be a potential drug for pain, inflammation, pyrexia, and neuropathic pain conditions, which is subject to further detailed studies before going into clinical trials.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c01717.

Binding orientations of the native ligands in the binding sites of respective enzymes (Figures S-1−S-6) (PDF)

AUTHOR INFORMATION

Corresponding Authors

Gowhar Ali − *Department of Pharmacy, University of Peshawar, Peshawar 25120, Pakistan*; Email: gowhar_ali@uop.edu.pk

Muhammad Ayaz − *Department of Pharmacy, Faculty of Biological Sciences, University of Malakand, Chakdara, KP 18000, Pakistan*; orcid.org/0000-0002-4299-2445; Email: ayazuop@gmail.com

H C Ananda Murthy − *Department of Applied Chemistry, School of Applied Natural Science, Adama Science and Technology University, Adama, Oromia 1888, Ethiopia; Department of Prosthodontics, Saveetha Dental College & Hospital, Saveetha Institute of Medical and Technical Science (SIMATS), Saveetha University, Chennai 600077 Tamil Nadu, India*; orcid.org/0000-0002-2361-086X; Email: anandkps350@gmail.com

Authors

Qarib Ullah − *Department of Chemistry, Hazara University, Mansehra 21310, Pakistan*

Zarshad Ali − *Department of Chemistry, Hazara University, Mansehra 21310, Pakistan*

Umer Rashid − *Department of Chemistry, COMSATS University Islamabad-Abbottabad Campus, 22060 Abbottabad, Pakistan*; orcid.org/0000-0002-2419-3172

Nisar Ahmad − *School of Pharmacy, Institute of Health Sciences Mardan, Mardan, KPK 23200, Pakistan*

Rasool Khan − *Institute of Chemical Sciences, University of Peshawar, Peshawar 25120, Pakistan*

Sami Ullah − *Department of Pharmacy, University of Peshawar, Peshawar 25120, Pakistan*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.3c01717

Author Contributions

Q.U.: investigation, methodology, writing - original draft; Z.A.: methodology, formal analysis; G.A.: conceptualization, project administration, supervision, writing - reviewing and editing; N.A.: methodology, analysis; R.K.: data curation, reviewing and editing; S.U.: data curation, formal analysis; M.A.: methodology, experimentation; H.C.A.M.: writing - reviewing and editing.

Notes

The authors declare no competing financial interest.

https://doi.org/10.1021/acsomega.3c01717
ACS Omega 2023, 8, 22809−22819

■ ACKNOWLEDGMENTS

The authors are thankful to their respective organizations for the support.

■ REFERENCES

(1) Khan, F. A.; Ali, G.; Rahman, K.; Khan, Y.; Ayaz, M.; Mosa, O. F.; Nawaz, A.; Hassan, S. S. u.; Bungau, S. Efficacy of 2-Hydroxyflavanone in rodent models of pain and inflammation: involvement of opioidergic and GABAergic anti-nociceptive mechanisms. *Molecules* **2022**, *27*, 5431.

(2) Bouhassira, D.; Attal, N.; Alchaar, H.; Boureau, F.; Brochet, B.; Bruxelle, J.; Cunin, G.; Fermanian, J.; Ginies, P.; Grun-Overdyking, A.; Jafari-Schluep, H.; Lantéri-Minet, M.; Laurent, B.; Mick, G.; Serrie, A.; Valade, D.; Vicaut, E. Comparison of pain syndromes associated with nervous or somatic lesions and development of a new neuropathic pain diagnostic questionnaire (DN4). *Pain* **2005**, *114*, 29−36.

(3) Merskey, H. E. Classification of chronic pain: descriptions of chronic pain syndromes and definitions of pain terms. *Pain* **1986**, 226.

(4) Ali, G.; Khan, A. New drug development process-Today: A review. *Pharmacologyonline* **2013**, *1*, 1−10.

(5) Woodcock, J.; Woosley, R. The FDA critical path initiative and its influence on new drug development. *Annu. Rev. Med.* **2008**, *59*, 1−12.

(6) Li, C.; Zhang, T.; Zeng, Z.; Liu, X.; Zhao, Y.; Zhang, B.; Feng, Y. A New Route to Indazolone via Amidation Reaction of o-Carboxyazobenzene. *Org. Lett.* **2012**, *14*, 479−481.

(7) Scala, A.; Piperno, A.; Risitano, F.; Cirmi, S.; Navarra, M.; Grassi, G. Efficient synthesis of highly substituted tetrahydroindazolone derivatives. *Mol. Diversity* **2015**, *19*, 473−480.

(8) Abouzid, K. A. M.; El-Abhar, H. S. Synthesis and antiinflammatory activity of novel indazolones. *Arch. Pharmacal Res.* **2003**, *26*, 1−8.

(9) Tse, E.; Butner, L.; Huang, Y.; Hall, I. H. The Anti-inflammatory Activity of N-Substituted Indazolones in Mice. *Arch. Pharm.* **1996**, *329*, 35−40.

(10) Muthumani, P.; Meera, R.; Venkatraman, S.; Murugan, D. P.; Devi, P. Synthesis and biological study of some novel Schiff's bases of indazolone derivatives. *J. Chem. Pharm. Res.* **2010**, *2*, 433−443.

(11) Oelschlager, H.; Matthiesen, U.; Behrendt, W. A. Analgesic compositions comprising 1H-or respectively 2H-indazolone-3 (β-morpholino-alkoxy) compounds. United States Patent No. US 3,981,997, 1976.

(12) El-Hawash, S. A. M.; Badawey, E.-S. A. M.; El-Ashmawey, I. M. Nonsteroidal antiinflammatory agents—part 2 antiinflammatory, analgesic and antipyretic activity of some substituted 3-pyrazolin-5-ones and 1, 2, 4, 5, 6, 7-3H-hexahydroindazol-3-ones. *Eur. J. Med. Chem.* **2006**, *41*, 155−165.

(13) Sarojini, B.; Mohan, B.; Narayana, B.; Sanjeev, G. Studies on in vivo radioprotection efficacy of indazolone derivative on electron beam induced oxidative stress using Drosophila Melanogaster. In *Proceedings of the international conference on radiation biology and clinical applications: a molecular approach towards innovations in applied radiobiology and a workshop on strategies in radiation research*; 2013.

(14) Rozen, D.; Grass, G. W. Perioperative and intraoperative pain and anesthetic care of the chronic pain and cancer pain patient receiving chronic opioid therapy. *Pain Pract.* **2005**, *5*, 18−32.

(15) Portenoy, R. K.; Foley, K. M. Chronic use of opioid analgesics in non-malignant pain: report of 38 cases. *Pain* **1986**, *25*, 171−186.

(16) Bovill, J. G. Mechanisms of actions of opioids and non-steroidal anti-inflammatory drugs. *Eur. J. Anaesthesiol.* **1997**, *14*, 9−15.

(17) Ong, C. K. S.; Lirk, P.; Tan, C. H.; Seymour, R. A. An evidence-based update on nonsteroidal anti-inflammatory drugs. *Clin. Med. Res.* **2007**, *5*, 19−34.

(18) Labianca, R.; Sarzi-Puttini, P.; Zuccaro, S. M.; Cherubino, P.; Vellucci, R.; Fornasari, D. Adverse effects associated with non-opioid and opioid treatment in patients with chronic pain. *Clin. Drug Invest.* **2012**, *32*, 53−63.

(19) Ahmad, N.; Subhan, F.; Islam, N. U.; Shahid, M.; Rahman, F. U.; Fawad, K. A novel pregabalin functionalized salicylaldehyde derivative afforded prospective pain, inflammation, and pyrexia alleviating propensities. *Arch. Pharm.* **2017**, *350*, No. e201600365.

(20) Aman, U.; Subhan, F.; Shahid, M.; Akbar, S.; Ahmad, N.; Ali, G.; Fawad, K.; Sewell, R. D. E. Passiflora incarnata attenuation of neuropathic allodynia and vulvodynia apropos GABA-ergic and opioidergic antinociceptive and behavioural mechanisms. *BMC Complementary Altern. Med.* **2016**, *16*, 1−17.

(21) Mahnashi, M. H.; Alshahrani, M. A.; Nahari, M. H.; Hassan, S. S. u.; Jan, M. S.; Ayaz, M.; Ullah, F.; Alshehri, O. M.; Alshehri, M. A.; Rashid, U.; Sadiq, A. In-Vitro, In-Vivo, Molecular Docking and ADMET Studies of 2-Substituted 3, 7-Dihydroxy-4H-chromen-4-one for Oxidative Stress. Inflammation and Alzheimer's Disease. *Metabolites* **2022**, *12*, 1055.

(22) Aydin, S.; Demir, T.; Öztürk, Y.; Başer, K. H. C. Analgesic activity of Nepeta italica L. *Phytother. Res.* **1999**, *13*, 20−23.

(23) Akbar, S.; Subhan, F.; Shahid, M.; Wadood, A.; Shahbaz, N.; Farooq, U.; Ayaz, M.; Raziq, N. 6-Methoxyflavanone abates cisplatin-induced neuropathic pain apropos anti-inflammatory mechanisms: a behavioral and molecular simulation study. *Eur. J. Pharmacol.* **2020**, *872*, No. 172972.

(24) Zeb, A.; Ahmad, S.; Ullah, F.; Ayaz, M.; Sadiq, A. Anti-nociceptive activity of ethnomedicinally important analgesic plant Isodon rugosus Wall. ex Benth: Mechanistic study and identifications of bioactive compounds. *Front. Pharmacol.* **2016**, *7*, 200.

(25) Khatri, N.; Garg, V. Reviewing biomedical role of Plasma Gelsolin. *Pharma Innovation* **2014**, *3*, 16.

(26) Babu, A.; Prasanth, K. G.; Balaji, B. Effect of curcumin in mice model of vincristine-induced neuropathy. *Pharm. Biol.* **2015**, *53*, 838−848.

(27) Jan, M. S.; Shahid, M.; Ahmad, S.; Hussain, F.; Ahmad, A.; Mahmood, F.; Rashid, U.; Ullah, F.; Khan, N.; Aasim, M. Synthesis of pyrrolidine-2, 5-dione based anti-inflammatory drug: in vitro COX-2, 5-LOX inhibition and in vivo anti-inflammatory studies. *Lat. Am. J. Pharm.* **2019**, *38*, 2287−2294.

(28) Ali, G.; Subhan, F.; Wadood, A.; Khan, A.; Ullah, N.; Islam, N. U.; Khan, I. Pharmacological evaluation, molecular docking and dynamics simulation studies of salicyl alcohol nitrogen containing derivatives. *Afr. J. Pharm. Pharmacol.* **2013**, *7*, 585−596.

(29) Patel, S.; Naeem, S.; Kesingland, A.; Froestl, W.; Capogna, M.; Urban, L.; Fox, A. The effects of GABAB agonists and gabapentin on mechanical hyperalgesia in models of neuropathic and inflammatory pain in the rat. *Pain* **2001**, *90*, 217−226.

(30) Shahid, M.; Subhan, F.; Ahmad, N.; Ullah, I. A bacosides containing Bacopa monnieri extract alleviates allodynia and hyperalgesia in the chronic constriction injury model of neuropathic pain in rats. *BMC Complementary Altern. Med.* **2017**, *17*, 1−12.

(31) Ullah, R.; Ali, G.; Subhan, F.; Naveed, M.; Khan, A.; Khan, J.; Halim, S. A.; Ahmad, N.; Zakiullah; Al-Harrasi, A. Attenuation of nociceptive and paclitaxel-induced neuropathic pain by targeting inflammatory, CGRP and substance P signaling using 3-Hydroxyflavone. *Neurochem. Int.* **2021**, *144*, No. 104981.

(32) Shahidul Islam, M.; Mohammed Al-Majid, A.; Nageh Sholkamy, E.; Yousuf, S.; Ayaz, M.; Nawaz, A.; Wadood, A.; Rehman, A. U.; Prakash Verma, V.; Motiur Rahman, A. F. M.; Barakat, A. Synthesis of Spiro-oxindole Analogs Engrafted Pyrazole Scaffold as Potential Alzheimer's Disease Therapeutics: Anti-oxidant, Enzyme Inhibitory and Molecular Docking Approaches. *ChemistrySelect* **2022**, *7*, No. e202203047.

(33) Khalil, A. T.; Ayaz, M.; Ovais, M.; Wadood, A.; Ali, M.; Shinwari, Z. K.; Maaza, M. In vitro cholinesterase enzymes inhibitory potential and in silico molecular docking studies of biogenic metal oxides nanoparticles. *Inorg. Nano-Met. Chem.* **2018**, *48*, 441−448.

(34) Sadiq, A.; Rashid, U.; Ahmad, S.; Zahoor, M.; AlAjmi, M. F.; Ullah, R.; Noman, O. M.; Ullah, F.; Ayaz, M.; Khan, I.; Islam, Z. U.; Ali, W. Treating hyperglycemia from Eryngium caeruleum M. Bieb: in-vitro α-glucosidase, antioxidant, in-vivo antidiabetic and molecular docking-based approaches. *Front. Chem.* **2020**, *8*, No. 558641.

https://doi.org/10.1021/acsomega.3c01717
ACS Omega 2023, 8, 22809−22819

(35) Ahmad, A.; Ullah, F.; Sadiq, A.; Ayaz, M.; Saeed Jan, M.; Shahid, M.; Wadood, A.; Mahmood, F.; Rashid, U.; Ullah, R.; Sahibzada, M. U. K.; Alqahtani, A. S.; Mahmood, H. M. Comparative cholinesterase, α-glucosidase inhibitory, antioxidant, molecular docking, and kinetic studies on potent succinimide derivatives. *Drug Des., Dev. Ther.* **2020**, 2165−2178.
(36) Islam, M. S.; Al-Majid, A. M.; Sholkamy, E. N.; Yousuf, S.; Ayaz, M.; Nawaz, A.; Wadood, A.; Rehman, A. U.; Verma, V. P.; Bari, A.; Haukka, M.; Soliman, S. M. Synthesis, molecular docking and enzyme inhibitory approaches of some new chalcones engrafted pyrazole as potential antialzheimer, antidiabetic and antioxidant agents. *J. Mol. Struct.* **2022**, *1269*, No. 133843.
(37) Mahmood, F.; Ali, R.; Jan, M. S.; Chishti, K. A.; Ahmad, S.; Zeb, A.; Ayaz, M.; Ullah, F.; Aasim, M.; Khan, N. Z.; Rahim, H. Chemical characterization and analgesic potential of Notholirion thomsonianum extract. *Lat. Am. J. Pharm.* **2019**, *38*, 807−812.
(38) Ahmad, N.; Subhan, F.; Islam, N. U.; Shahid, M.; Ullah, N.; Ullah, R.; Khurram, M.; Amin, M. U.; Akbar, S.; Ullah, I.; Sewell, R. D. E. Pharmacological evaluation of the gabapentin salicylaldehyde derivative, gabapentsal, against tonic and phasic pain models, inflammation, and pyrexia. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **2021**, *394*, 2033−2047.
(39) Wang, Y.; Lei, J.; Gupta, M.; Peng, F.; Lam, S.; Jha, R.; Raduenz, E.; Beitz, A. J.; Gupta, K. Electroacupuncture in conscious free-moving mice reduces pain by ameliorating peripheral and central nociceptive mechanisms. *Sci. Rep.* **2016**, *6*, 34493.
(40) Günther, T.; Dasgupta, P.; Mann, A.; Miess, E.; Kliewer, A.; Fritzwanker, S.; Steinborn, R.; Schulz, S. Targeting multiple opioid receptors−improved analgesics with reduced side effects? *Br. J. Pharmacol.* **2018**, *175*, 2857−2868.
(41) Stein, C.; Millan, M. J.; Shippenberg, T. S.; Peter, K.; Herz, A. Peripheral opioid receptors mediating antinociception in inflammation. Evidence for involvement of mu, delta and kappa receptors. *J. Pharmacol. Exp. Ther.* **1989**, *248*, 1269−1275.
(42) Ma, H.; Obeng, S.; Wang, H.; Zheng, Y.; Li, M.; Jali, A. M.; Stevens, D. L.; Dewey, W. L.; Selley, D. E.; Zhang, Y. Application of Bivalent Bioisostere Concept on Design and Discovery of Potent Opioid Receptor Modulators. *J. Med. Chem.* **2019**, *62*, 11399−11415.
(43) Pedersen, L. H.; Scheel-Krüger, J.; Blackburn-Munro, G. Amygdala GABA-A receptor involvement in mediating sensory-discriminative and affective-motivational pain responses in a rat model of peripheral nerve injury. *Pain* **2007**, *127*, 17−26.
(44) Karim, N.; Khan, I.; Abdelhalim, A.; Halim, S. A.; Khan, A.; Altaf, N.; Ahmad, W.; Ghaffar, R.; Al-Harrasi, A. Involvement of selective GABA-A receptor subtypes in amelioration of cisplatin-induced neuropathic pain by 2′-chloro-6-methyl flavone (2′-Cl-6MF). *Naunyn-Schmiedeberg's Arch. Pharmacol.* **2021**, *394*, 929−940.
(45) Vidal-Torres, A.; De La Puente, B.; Rocasalbas, M.; Touriño, C.; Bura, S. A.; Fernández-Pastor, B.; Romero, L.; Codony, X.; Zamanillo, D.; Buschmann, H.; Merlos, M.; Baeyens, J. M.; Maldonado, R.; Vela, J. M. Sigma-1 receptor antagonism as opioid adjuvant strategy: enhancement of opioid antinociception without increasing adverse effects. *Eur. J. Pharmacol.* **2013**, *711*, 63−72.
(46) Shaskan, E. G. *Hypothalamic and wholebrain monoamine levels in bats: Some aspects of central control of thermoregulation*; The University of Arizona, 1969.
(47) Aronoff, D. M.; Neilson, E. G. Antipyretics: mechanisms of action and clinical use in fever suppression. *Am. J. Med.* **2001**, *111*, 304−315.
(48) Pasternak, G. W.; Pan, Y.-X. Mu opioids and their receptors: evolution of a concept. *Pharmacol. Rev.* **2013**, *65*, 1257−1317.
(49) Varga, B. R.; Streicher, J. M.; Majumdar, S. Strategies towards safer opioid analgesics—A review of old and upcoming targets. *Br. J. Pharmacol.* **2023**, *180*, 975−993.
(50) Ghufran, M.; Rehman, A. U.; Shah, M.; Ayaz, M.; Ng, H. L.; Wadood, A. In-silico design of peptide inhibitors of K-Ras target in cancer disease. *J. Biomol. Struct. Dyn.* **2020**, *38*, 5488−5499.
(51) Ghufran, M.; Ullah, M.; Khan, H. A.; Ghufran, S.; Ayaz, M.; Siddiq, M.; Abbas, S. Q.; Hassan, S. S. U.; Bungau, S. In-silico lead druggable compounds identification against SARS COVID-19 main protease target from in-house, chembridge and zinc databases by structure-based virtual screening, molecular docking and molecular dynamics simulations. *Bioengineering* **2023**, *10*, 100.
(52) Ghufran, M.; Khan, H. A.; Ullah, M.; Ghufran, S.; Ayaz, M.; Siddiq, M.; Hassan, S. S. u.; Bungau, S. In Silico Strategies for Designing of Peptide Inhibitors of Oncogenic K-Ras G12V Mutant: Inhibiting Cancer Growth and Proliferation. *Cancers* **2022**, *14*, 4884.

https://doi.org/10.1021/acsomega.3c01717
ACS Omega 2023, 8, 22809−22819