Anti-Inflammatory and Analgesic Activities of 7-Chloro-4-(Piperazin-1-yl) Quinoline Derivative Mediated by Suppression of Inflammatory Mediators Expression in Both RAW 264.7 and Mouse Models

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Introduction

Inflammation is a complex biological defense response that can be triggered by various factors such as infections, tissue injury, and toxic compounds. This defense mechanism acts on the removal of harmful stimuli and initiates the healing process. Chronic inflammation and sepsis could be developed due to the excessive release of cytokines with concurrent migration of inflammatory cells to the affected regions.

Abstract

Background: 4-Aminoquinoline derivatives possess various potential biological properties. The introduction of additional piperazine heterocyclic pharmacophoric moiety tends to have profound impact in increasing the activity. The present work was undertaken to investigate the in-vitro and in-vivo anti-inflammatory activity as well as the peripheral and central analgesic activities of compound 1-(4-(7-chloroquinoline-4-yl)piperazin-1-yl)-2-(4-phenylpiperazin-1-yl)ethanone (5) in experimental models.

Methods: The percentage inhibition of the lipopolysaccharide induced NO release of 7-chloro-4-(piperazin-1-yl)quinoline derivatives 1-9 was determined in RAW 264.7 murine macrophage model. Western blot analysis was performed to evaluate the effect of compound 5 on protein expression of inducible nitric oxide synthase (iNOS). Gene expression of inflammatory markers was evaluated using real-time polymerase chain reaction. The peripheral and central analgesic activities of compound 5 were evaluated in mice using writhing and hot-plate tests, respectively. Anti-inflammatory activity was assessed using carrageenan-induced paw edema assay in mice and serum NO and COX-2 levels were measured.

Results: Compound 5 demonstrated the highest NO inhibitory activity that was accompanied by inhibition of iNOS protein expression and decreased gene expression levels of inflammatory markers. It revealed a potential peripheral analgesic effect through inhibition of abdominal writhing in mice treated with doses of 15 and 30 mg/kg and its effect was comparable to diclofenac sodium. Compound 5 possessed an analgesic activity starting from 15 min post administration and reached its peak at 45 min which was significantly higher than that of tramadol hydrochloride suggesting its potential as central analgesic agent. It also showed percentage of inhibition of edema of 34, 50 and 64% at 1, 2, and 3 h respectively, post carrageenan challenge together with a significant decrease in serum NO and COX-2 levels.

Conclusion: The remarkable anti-inflammatory and analgesic activities of compound 5 could be attributed to the advantageous introduction of the heterocyclic 7-chloro-4-(piperazin1-yl) quinoline scaffold incorporated with N-phenylpiperazine functional groups linked together with the ethanone pharmacophoric chain.
Upon inflammatory stimuli, macrophages secrete pro-inflammatory mediators such as tumor necrosis factor-alpha (TNF-α), interleukin-1beta (IL-1β), and IL-6 and promote prostaglandin synthesis which leads to up-regulation of inflammatory reactions. In addition, stimulation of the immune cells increases the production of pro-inflammatory proteins/enzymes such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) which induces nitric oxide (NO) and prostaglandin release, respectively. NO and COX subsequently induces inflammatory responses that are involved in the progression of inflammation-related diseases, such as cardiovascular disease and cancer.

Quinoline moiety derivatives plays vital role in the development of numerous classes of newer drugs having various biological properties such as antimalarial, anticancer, antibacterial, anti-tubercular, and anti-inflammatory and analgesic activities. Moreover, glafenine, floctafenine and antrafenine (Figure 1) are analgesic and non-steroidal anti-inflammatory drugs (NSAIDs) related to the fenamate class and can be considered as 4-aminoquinolines. Introducing another heterocyclic moiety to the quinolone ring initiates the activity, as noticed by the piperazine heterocyclic pharmacophoric moiety. Meanwhile, the 7-chloro-4-(piperazin-1-yl)quinoline structural skeleton constitutes the principle component of the antimalarial piperaquine, which support this scaffold as an effective candidate with multiple pharmacological potentials.

Non-steroidal anti-inflammatory and analgesic drugs (NSAIDs) are prescribed for pain relief and improvement of the patient’s life style, but they are often associated with adverse effects, therefore studies are still needed for safer candidates. Aboul-Enein et al., disclosed the synthesis and the vascular endothelial growth factor receptor-II (VEGFR-II) inhibitory effect of several 1-(4-(7-chloroquinoline-4-yl)piperazin-1-yl) derivatives (1-9, Figure 2). Their structures demonstrated 4-aminoquinoline and aminocetamide “1,2-diamoethanone” moieties which are noticed features in many marketed NSAIDs and analgesics (Figure 1). Therefore, in the current work, it was deemed of interest to start with the in vitro anti-inflammatory screening of compounds 1-9. These derivatives demonstrated significant inhibition of NO level in RAW 264.7 cells. It is worth mentioning that, compound 5 namely; 1-(4-(7-chloroquinoline-4-yl)piperazin-1-yl)-2-(4-phenylpiperazin-1-yl)ethanone showed the highest NO inhibitory activity. The structural feature of compound 5 shows a quinoline moiety incorporated with two piperazinyl functional groups linked together with the ethanone pharmacophoric chain (Figure 1). Therefore, as an extension of the previous study and encouraged by the current in vitro NO inhibition anti-inflammatory findings, it was of significance to evaluate the analgesic and anti-inflammatory effect of compound 5 both in vitro and in vivo.

Materials and Methods

In vitro assays

Cell culture and treatment

The murine RAW 264.7 macrophage cells (ATCC), cultured and maintained in Dulbecco’s Modified Eagle’s Medium (DMEM from Lonza, Verviers, Belgium) in a humidified 5% CO₂ atmosphere (Certomat' CS 20
Inhibition of LPS-induced NO release

RAW 264.7 model was employed to assess the in vitro anti-inflammatory potential as previously described. 20 Briefly, cells (5 × 10^5 cells/ml) were plated overnight in 96-well microplates. Overnight grown cultures were treated with either vehicle (0.1% v/v dimethyl sulphoxide vehicle (DMSO, Seva electrophoresis, Catalogue number 39757.02, negative control LPS), 100 ng/ml lipopolysaccharide (LPS; Sigma-Aldrich, from E. coli serotype O111:B4) or with LPS containing 30 µM as a cut off concentration 21 from the studied 7-chloro-4-(piperazin-1-yl)quinoline derivatives 1-9, dissolved in 0.1% DMSO (v/v). Following 24 h treatment, Griess assay 22 was used to measure NO in triplicate aliquots of culture medium from each treatment group. The assay was performed by mixing 100 µl of culture medium from each well with 100 µl of Griess reagent [equal volumes of solution A (0.1% wt/v N-(1-naphthyl)ethylenediamine hydrochloride) and solution B (1 % wt/v of sulphanilamide in 5% (v/v) phosphoric acid )]. Absorbance was monitored at 540 nm using a Tristar lb 942 microplate reader (Berthold, Germany). NO Inhibition (%) was estimated for each group relative to the LPS only control.

Western blot analysis

The relative protein expression of the proinflammatory marker inducible nitric oxide synthase (iNOS) was performed using Western blotting. RAW 264.7 cells were plated and cultured overnight in 6-well plates as 1.5 × 10^5 cells/well. Cultures were treated with compound 5 (0-30 µM). After 24 h treatment time, cells were washed using ice-cold phosphate-buffered saline (PBS) and scarpred in RIPA lysis buffer (Catalogue number 89900, Thermoscientific, USA). After incubation for 20 min on ice, cell lysates were centrifuged at 15000 × g for 20 min at 4°C and protein concentration was measured on a spectrophotometer (Thermo nano-drop). Samples (80 µg total protein) of cell lysates were resolved on 10% polyacrylamide gel electrophoresis (Bio-Rad Tetra Cell) and electro-plotted onto nitrocellulose membrane using a Trans-blot mini module (Bio-rad, USA). The membrane was blocked using 5% skim milk (catalogue number 42590.01, Serva Electrophoresis, Germany) in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1 h at room temperature, followed by an overnight incubation at 4°C with 1:1000 dilution of iNOS primary antibody (Merck Millipore, Massachusetts, USA) and β-actin was used as loading control. Following 4 x 5 min washes with TBST, the membranes were incubated with 1:10,000 dilution of the horseradish peroxidase-conjugated secondary antibody (catalogue number AB97240, Abcam, UK) for 1 h at room temperature, followed by another 4 x 5 min washes with TBST. Membrane proteins were detected using the ECL western blotting detection substrate (Novex™, Catalogue number WP20005, Thermoscientific, USA) and imaged using a UVP Biospectrum Image (analytik Jena, Germany). Densitometric analysis of proteins bands were performed using the image’s built-in software (VisionWorks LS, analytik Jena, Germany) according to manufacturer instructions. Protein Expression of iNOS was normalized to that of β-actin and expressed as fold of normalized expression to the LPS+ control.
primers used for TNF-α, IL-6, IL-1β, β-actin, iNOS, COX-2 are represented in Table 1. Reactions were initiated through incubation at 50 °C for 2 min followed by 95 °C for 10 min and then 40 cycles consisted of denaturation (94 °C) for 15 s, different annealing temperatures (56-64 °C) for each pair of primer for 12 s and extension (72 °C) for 25 s. Data from RT-qPCR were analyzed using a specific software (Applied Biosystems) for the detection and analysis of sequences (v1.7). Relative levels of iNOS, COX2, IL-6, IL-1β and TNF-α gene mRNAs were normalized to that of β-actin (internal reference gene) using the comparative Ct method and were reported as fold changes.

**Pharmacological studies**

**Animals**

Adult male albino mice weighing 20–25 g were used for the assessment of the analgesic and anti-inflammatory activities. Mice were purchased from the Animal-Breeding Unit of the National Research Centre (Giza, Egypt). Animals were housed in ventilated cages with *ad libitum* access to tap water and standard pelleted diet under controlled conditions of temperature (23 ± 2°C), light cycle (12 h light/dark), and relative humidity (55 ± 5%). All animal procedures were carried out in compliance with the regulations of the ethical committee of the National Research Centre for use of laboratory animals (Number: 16155).

**Analgesic activity assays**

The analgesic profile of compound 5 was evaluated in mice (*n = 6*) by adopting acetic acid-induced writhing test to determine the peripheral analgesic effect at doses of 15 and 30 mg/kg body weight, in addition to the hot-plate technique to determine the central analgesic effect at a dose level of 30 mg/kg body weight.

**Writhing test**

Peripheral analgesic effect of compound 5 was determined *in vivo* using the writhing test. Mice were classified as: group I served as the control group and received the vehicle (1% Tween-80 aqueous solution); group II received tramadol hydrochloride (25 mg/kg, *i.p.*); and group III received compound 5 (30 mg/kg, *i.p.*). The mice were placed gently into a 1-l dry glass beaker, and the temperature was adjusted to 55–56°C. The mice were pretested, and those having a latency time greater than 15 s were excluded from the testing. The reaction time was measured and it was considered as the time interval (s) starting when the mouse reached the hot beaker until paw licking or jumping occurred. The normal reaction time was determined three times at 5-min intervals and the average was calculated for all animals before injection of the vehicle, reference drug, or test compounds. The reaction time was measured from 0.05 ml of 0.5% suspension of carrageenan in saline i.p. into the plantar tissue of one hind paw. Meanwhile, equal volume of saline was injected into the other hind paw for control measurements. Results were calculated using the following equation:

\[
\% \text{ Inhibition of abdominal writhings} = \frac{N_C - N_I}{N_C} \times 100
\]

where *N*I is the number of writhes of the control group and *N*C is the number of writhes of the treated group.

**Hot-plate test**

The hot-plate method involves comparing the response of mice to pain stimulus in treated and untreated mice at definite time intervals. Mice were classified as: group I served as the control group and received the vehicle (1% Tween-80 aqueous solution); group II received tramadol (25 mg/kg, *i.p.*), with diclofenac sodium (10 mg/kg, *i.p.*) as reference standard; and group III received compound 5 (30 mg/kg, *i.p.*). The mice were placed gently into a 1-l dry glass beaker, and the temperature was adjusted to 55–56°C. The mice were pretested, and those having a latency time greater than 15 s were excluded from the testing. The reaction time was measured and it was considered as the time interval (s) starting when the mouse reached the hot beaker until paw licking or jumping occurred. The normal reaction time was determined three times at 5-min intervals and the average was calculated for all animals before injection of the vehicle, reference drug, or test compounds. The reaction time was determined at 15-, 30-, 45-, 60-, 90-, and 120-min intervals after vehicle, reference drug, or compound 5 injection.

**Anti-inflammatory activity**

**Carrageenan-induced paw edema assay**

Anti-inflammatory activity was assessed in an acute model via the carrageenan-induced paw edema assay. Adult male albino mice (*n = 6*) were classified into III groups. Group I (control group) received the vehicle 1% Tween-80 aqueous solution. Group II received indomethacin (5 mg/kg, *i.p.*), as reference standard, whereas group III was *i.p.* dosed with 30 mg/kg of compound 5 dissolved in Tween-80 (1% aqueous solution) one hour before carrageenan challenge. The paw edema was induced with subplantar injection of 0.05 ml of 0.5% suspension of carrageenan in saline into the plantar tissue of one hind paw in all groups. Meanwhile, equal volume of saline was injected into the other hind paw for control measurements. Results were
recorded by measuring the thickness of both hind paws using a Vernier Caliper (SMEC, Shanghai, China) 1, 2, and 3 h after carrageenan challenge. The percentage swelling as well as the percent inhibition of edema of the mouse paw was calculated using the following equations:

\[
\% \text{ Swelling} = \frac{V_c - V_s}{V_s} \times 100
\]

where \(V_c\) is the thickness of the carrageenan paw and \(V_s\) is the thickness of the saline paw at each time interval. The average paw swelling in both compound 5 and indomethacin-treated mice were compared with that of the vehicle treated mice.

\[
\% \text{ Inhibition of edema} = \left[1 - \frac{\% \text{ Swelling of treated group}}{\% \text{ Swelling of carrageenan group}}\right] \times 100
\]

**Blood collection**

Three hours post-carrageenan injection, blood samples were collected from different groups via intracardiac puncture, allowed to clot then serum was separated through centrifugation at 3000 rpm at 4°C for 15 min. Collected sera were then aliquoted to be stored at -80°C till next use.

**Determination of serum nitric oxide and cyclooxygenase-2 level**

Levels of nitric oxide (NO) and cyclooxygenase-2 (COX-2) level were evaluated in the serum of normal mice administered with the vehicle as well as mice receiving the vehicle (control), compound 5 (30 mg/kg, i.p) and indomethacin (5 mg/kg, i.p.) then injected with carrageenan, using mouse NO and COX-2 enzyme-linked immunosorbent assay (ELISA), respectively (MyBioSource, Inc., San Diego, CA) according to the manufacturer’s instructions.

**Acute toxicity test**

Compound 5 was administered in different doses (10-500 mg/kg) given via i.p. route to respective adult healthy groups of mice followed by continuous observation for any deaths or gross behavioral changes during the first 2 h. The mice were occasionally observed starting from the next 4 to 24 h and for the occurrence of any delayed effects. Examination of mice was further prolonged for 72 hours for any behavioral changes or toxic signs. Mice were kept on observation for a total period of 14 days.

**Statistical analysis**

All results were represented as mean±SEM. Comparison between different groups was performed using one-way analysis of variance (ANOVA). Student–Newman–Keuls method was used as a post-hoc analysis to compare means.

Results were considered statistically significant when \(P<0.05\).

**Results**

**In vitro anti-inflammatory activity**

In the present study, 1-(4-(7-chloroquinoline-4-yl)piperazin-1-yl) derivatives 1-9 were initially tested in vitro for their anti-inflammatory potential. We employed RAW 264.7 murine macrophage model as a sensitive and well recognized cells for inflammation induction by LPS, a pattern recognition molecule that is a constituent of the cell wall of Gram negative bacteria. As displayed in Figure 3, the screening revealed differential inhibition of the LPS-induced NO release by the tested compounds at 30 µM. Among the tested compounds, compounds 5 was the most potent to inhibit NO release, recording 74.1% ± 2.2 inhibition of LPS-induced NO, as assessed with Greiss assay.

To evaluate the impact of compound 5 on the inhibition of LPS-induced iNOS protein expression intracellularly, RAW 264.7 cells were co-treated with LPS and increasing concentrations of compound 5. Western blotting of cell lysate proteins revealed a concentration-dependent inhibition of the iNOS protein expression induced by LPS (LPS+). Densitometric analysis of iNOS bands normalized to β-actin revealed a gradual inhibition by increasing doses of compound 5, recording only 0.2 fold of LPS+ group (i.e. 80% inhibition of LPS-induced iNOS expression at concentration of 30 µM), as displayed in Figure 4.

![Figure 3. Inhibition of LPS-induced NO release by the indicated compounds in RAW 264.7 cells. Cells were co-treated for 24 h with either LPS (100 ng/ml) alone (LPS+) or with 30 µM of compounds. NO was determined as described in the Methods section. Data are means ± SEM (n=3), *P<0.05 compared to LPS+ control.](image-url)
Effect of compound 5 on the gene expression of inflammatory markers in LPS-stimulated RAW 264.7 macrophages

To further explore the anti-inflammatory effect of compound 5, the influence of compound 5 on the proinflammatory markers iNOS, COX2, IL-6, IL-1β and TNF-α was studied in LPS-induced RAW 264.7 macrophages. As shown in figure 5, LPS (100 ng/ml) markedly increased the transcriptional expression of iNOS (5.6 fold), TNF-α (3.9 fold), COX2 (4 fold), IL-1β (5.3 fold), and IL-6 (3.5 fold), compared with the vehicle-treated macrophages (p<0.05). Co-treatment of RAW 264.7 macrophages with compound 5 (30 µM) and LPS for 5h was able to significantly decrease the expression levels of the aforementioned inflammatory markers compared to LPS group (p<0.05). It can also be observed that compound 5 has nearly the same effect as indomethacin control on the induction of TNF-α, COX2, and IL-1β expression.

Pharmacological studies

Analgesic activity

Effect of compound 5 on peripheral analgesic activity (writhing test)

Treatment with compound 5 at doses of 15 mg/kg and 30 mg/kg has resulted in percentage inhibition of abdominal writhing by 33 and 62%, respectively. The decreased the number of writhings in the treated groups at both dose levels was significant compared with acetic acid-induced group that received the vehicle while not significant compared to the diclofenac sodium (10 mg/kg). Furthermore, the maximum effect of compound 5 was observed at 30 mg/kg where it reported a significant decrease in the number of abdominal writhing compared with compound 5 at 15 mg/kg (Figure 6).

Effect of compound 5 on central analgesic activity (hot-plate test)

The central analgesic activity of compound 5 was evaluated via the hot-plate test and results were summarized in Table 2 and Figure 7. In the current study, the analgesic effect was measured at 0, 15, 30, 45, 60, 90, and 120 min post i.p. administration of either the vehicle control, tramadol hydrochloride reference drug (25 mg/kg), or compound 5 (30 mg/kg). The analgesic activity started to increase at the 15 min time point where both tramadol and compound 5 possessed significant analgesic activities compared to control. The highest analgesic activity of tramadol hydrochloride was observed at 30 min post administration which was similar to that achieved by compound 5 at the same time point. Interestingly, compound 5 maintained its analgesic activity and achieved the highest analgesic peak at 45 min post administration which was much higher than tramadol hydrochloride. Although the analgesic activity of compound 5 started to decrease at the 60 min time interval, but it was still significantly higher than that of tramadol. Furthermore, the analgesic activity of
Pharmacological Effect of Quinoline Derivative

Sub-plantar injection of carrageenan in mice has resulted in an increase in the percentage swelling of the mouse paw by 50, 55.6 and 62 % at 1, 2, and 3 h post injection (Figure 8). Compound 5 at dose 30 mg/kg obviously inhibited the carrageenan-induced swelling in a time dependent manner. The percentage of inhibition of edema by compound 5 was 34, 50 and 64% at 1, 2, and 3 h, respectively, post carrageenan challenge while treatment with the reference standard indomethacin exhibited percentage inhibition of edema of 12, 41 and 58% at the same time intervals (Figure 9). Both compound 5 and indomethacin demonstrated significant decrease in the percentage swelling compared to the carrageenan group at measurements taken 1, 2 and 3 h post carrageenan. In addition, compound 5 showed a significant decrease in percentage swelling compared to the reference standard at the first hour post carrageenan challenge. Interestingly, this significant inhibitory effect persisted in the 2nd and the 3rd hour demonstrating the potential anti-inflammatory response of compound 5.

Effect of compound 5 on serum NO and COX-2 levels

In order to evaluate the anti-inflammatory mechanisms of compound 5 in vivo, serum NO and COX-2 levels were examined by ELISA. Data represented in Figure 10 demonstrated significant increase in NO level in serum samples of mice collected 3 hours post carrageenan-injection (control group) compared to the normal (negative control) group. In addition, compound 5 (30 mg/kg) and indomethacin (5 mg/kg) significantly decreased the NO levels in serum compared with the control (carrageenan) group by 57 and 64%, respectively. Furthermore, treatment with compound 5 and indomethacin significantly decreased COX-2 serum level in mice challenged with carrageenan by 55 and 65%, respectively, relative to the control group (Figure 11). No significant differences in serum NO and COX-2 levels were observed between indomethacin and compound 5 treated groups.

Table 2. Effect of compound 5 in the hot-plate test in adult male albino mice.

| Treatments           | Time (s) |
|----------------------|----------|
|                      | 0        |
|                      | 15       |
|                      | 30       |
|                      | 45       |
|                      | 60       |
|                      | 90       |
|                      | 120      |
| Control (Vehicle)    | 10.0±0.60| 10.40±0.54*| 10.67±0.42*| 10.50±0.43*| 10.80±0.37*| 10.43±0.57*| 10.40±0.51*|
| Tramadol (25 mg/kg)  | 10.67±0.49| 16.13±0.71*| 22.21±0.51*| 17.65±0.55*| 16.47±0.61*| 15.29±0.42*| 14.71±0.34*|
| Compound 5 (30 mg/kg)| 10.78±0.40| 14.14±0.35**| 22.00±0.53*| 25.29±0.42**| 18.57±0.51**| 14.45±0.57*| 13.82±0.33*|

The mice were observed for their response toward pain stimulus at definite time intervals. Each value represents the mean reaction time in second ± SEM. *P < 0.05 compared with control; **P < 0. 05 compared with tramadol hydrochloride.

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Acute toxicity studies in albino mice with compound 5 at dose up to 500 mg/kg did not cause any signs of toxicity or behavioral changes or mortality during the observation period. In addition, no changes in fur and skin, mucous membranes, and eyes or behavioral pattern. Convulsions, tremors, coma sleep, lethargy, diarrhoea, and salivation were not observed.

Discussion
Nitric oxide (NO) has been previously demonstrated as one of the most important inflammatory mediators. Sensitization with inflammatory inducers such as LPS causes the release of NO from immune cells, a process that is catalyzed by iNOS. Several studies have pointed out the role of NO as a critical regulatory molecule of various physiological processes including host defense and neural coordination. However, overproduction of NO was associated with oxidative damage and inflammatory
Pharmacological Effect of Quinoline Derivative

The progression of inflammatory diseases involve pro-inflammatory mediators such as COX2, iNOS, TNF-α, IL-6, and IL-1β which are also known to play a substantial role in different autoimmune diseases. Therefore, the levels of NO and the pro-inflammatory markers are regarded as indicators of the degree of inflammation.

In the present study, nine quinoline derivatives showed significant inhibition of NO level in RAW 264.7 cells. Remarkably, compound 5 demonstrated the highest NO inhibitory activity which could be attributed to the presence of two privileged piperazinyl heterocyclic moieties connected by ethanone spacer, and linked to the versatile quinoline scaffold in one molecule. The NO inhibitory activity of compound 5 in LPS-induced RAW 264.7 macrophages was explained to be as result of the inhibition of iNOS protein expression in a concentration-dependent manner as assessed with Western technique. Furthermore, co-treatment of RAW 264.7 macrophages with compound 5 decreased significantly the gene expression levels of iNOS, COX2, TNF-α, IL-1β and IL-6 in LPS-induced macrophages cells suggesting that compound 5 may exert its anti-inflammatory effect through the suppression of the expression of these proinflammatory mediators. The inhibitory action of compound 5 was much higher for COX2 and IL-6 relative expression levels.

The promising in vitro NO inhibitory activity, iNOS protein expression and inflammatory mediators gene expression results encouraged to further explore the in vivo anti-inflammatory and analgesic potentials of compound 5. Acetic acid-induced abdominal writhing test is a well-established, reliable and sensitive in vivo test model that is widely used for the assessment of peripheral analgesic activity. The mechanism of acetic acid-induced pain and abdominal writhing involves the release of endogenous mediators such as arachidonic acid, prostaglandins E2 and F2, COX, bradykinin, serotonin, histamine and cytokines in the peritoneal cavity. These mediators prompt local peritoneal nociceptive receptors and cause peripheral nociceptive sensitization as well as inflammatory pain in the peritoneal area. In the present study, compound 5 revealed a potential peripheral analgesic effect through possessing a significant decrease in the number of abdominal writhings compared to the control group injected with acetic acid only. The produced analgesic effect of compound 5 was comparable to that produced by diclofenac sodium (10 mg/kg). These results strongly suggest that compound 5 retains peripheral analgesic activity through inhibition of local endogenous mediators involved in pain and inflammation. In agreement with our study, Hasan et al. reported that the mechanism of peripheral pain inhibition which involves inhibition of prostaglandin biosynthesis is possibly caused by any substance that inhibits acetic acid-induced writhing. In order to investigate the central analgesic activity of compound 5, the hot-plate test was used. It is a well-established behavioral model of nociception and determination of central analgesic activity of compounds as it includes higher brain function. The mechanism involved in the elevation of the reaction time of the two behavioral responses integrated in the hot-plate test including; paw licking and jumping reactions, are supraspinal mechanisms. The mechanism of supraspinal/spinal signal integration in neurologic pain includes the release of endogenous opioids, serotonin, noradrenaline, and acetylcholine. In the hot-plate test, centrally acting analgesics exert their action on the spinal cord by acting on the opioid receptors. In general, µ opioid receptors mediate spinal analgesia while in particular, µ opioid receptor subtype 1, mediates supraspinal analgesia. The reference standard “Tramadol” is a µ opioid receptor agonist that also inhibits the neuronal re-uptake of serotonin and norepinephrine resulting in analgesic effect. In the current study, compound 5 possessed an increase in analgesic activity starting from 15 min post administration where it reached its maximum at 45 min time point and was significantly higher than that of tramadol hydrochloride suggesting its potential as central analgesic agent.

The anti-inflammatory activity of compound 5 was evaluated in in vivo using carrageenan-induced paw edema model in mice. This model is commonly used to test the anti-inflammatory activity of compounds acting on mediators of acute inflammation. In carrageenan challenge, inflammation is mediated by the in situ liberation of proinflammatory mediators such as histamine, bradykinin, tachykinins, complement and reactive oxygen and nitrogen species. In addition, carrageenan-induced inflammation results in neutrophils infiltration and migration to sites of inflammation as well as the production of neutrophils-derived free radicals. In general, carrageenan injection causes dilation of the postcapillary venules which results in exudation of inflammatory fluid and cells and release of proinflammatory mediators. Thus, inhibition of such events which represent the early exudative inflammatory phase would lead to the inhibition or termination of the inflammatory process. Injection of carrageenan results in edema formation which is considered a biphasic event. The initial phase is a nonphagocytic edema that is associated with trauma and the liberation of acute mediators such as serotonin, histamine and bradykinin, and their effect on vascular permeability occurring at 1 or 1.5 h and is primarily. The second phase is mainly ascribed to the overproduction of prostaglandins and release of inducible cyclooxygenase and lysosome enzymes for 2–3 h. Thus, carrageenan challenge is usually linked with the activation of the cyclooxygenase pathway. In the current study, the ability of compound 5 to inhibit the carrageenan induced swelling in time dependent manner was demonstrated. The percentage inhibition obtained after 2 and 3 h following carrageenan challenge was 50 and 64%, respectively, indicating that compound 5 possibly exerts its anti-inflammatory effect via inhibition of
the cyclooxygenase pathway. This postulation was further confirmed through the assessment of the serum levels of NO and COX-2 in mice treated with compound 5 at 3 h post-carrageenan challenge. The results revealed a significant inhibition of serum NO and COX-2 levels demonstrating the potential mechanism of action of compound 5 is via inhibition of NO and COX-2 in vivo. This finding is in agreement with Mazzoni et al. where maximum activity of the quinolone derivative namely; methyl 1-(4'-methylbenzoyl)-6-iodo-4-oxo-1,4-dihydroquinoline-2-carboxylate, demonstrated 71% inhibition of edema at the third hour post carrageenan challenge and is also the maximum of the whole experiment. In addition, the most of 4-oxoquinoline-2-carboxylic acid derivatives exhibited good analgesic and anti-inflammatory activities in carrageenan-induced rat paw edema and acetic acid writhing test in mice, respectively. Although compound 5 previously demonstrated no pharmacological impact in terms of in-vitro antitumor activity, but the current findings suggest that the structure features of compound 5 consists of 7-chloro-4-(piperazin-1-yl)quinoline heterocyclic scaffold incorporated with N-phenylpiperazine functional groups linked together with the ethanone pharmacophoric chain potentiated the analgesic and anti-inflammatory activities.

Conclusion
The current study demonstrated the promising anti-inflammatory therapeutic effect of compound 1-(4-(7-chloro quinoline-4-yl)piperazin-1-yl)-2-(4-phenylpiperazin-1-yl)ethanone (5) in cellular RAW 264.7 macrophages and animal models. It was capable of inhibiting NO, iNOS protein and gene expression together with the inflammatory mediators: COX-2, IL-6, IL-1β and TNF-α gene expression. In addition, the in vivo carrageenan-induced paw edema assay showed a significant decrease in percentage swelling in mice treated with compound 5 compared to indomethacin that was accompanied by a significant inhibition of serum NO and COX-2 levels. Compound 5 revealed a potential peripheral analgesic effect through the significant inhibition of abdominal writhing in mice. Moreover, it possessed a central analgesic activity through raising the pain threshold. Conclusively, compound 5 could be considered as a promising bioactive anti-inflammatory and analgesic candidate.

Acknowledgments
We are deeply greatful to Prof. Dr. Aboul-Enein, M. N. and Prof. Dr. El-Azzouny A. M. who were responsible for the study entitled: "Design, Synthesis, and Cytotoxic Evaluation of Certain 7-Chloro-4-(piperazin-1-yl) quinoline Derivatives as VEGFR-II Inhibitors" and for providing us with compounds 1-9.

Ethical Issues
All animal procedures were carried out in compliance with the regulations of the ethical committee of the National Research Centre for use of laboratory animals (Number: 16155).

Author Contributions
MEA, ARH, EKA: Contributed to the idea and design of the study and revision of the manuscript. MEA: Wrote the paper and conducted the in vivo analgesic and anti-inflammatory activities, determination of serum NO and COX-2 levels and acute toxicity test and wrote the relevant sections. ARH and EKA: Conducted the in vitro anti-inflammatory activity, including maintaining macrophage cell culture and treatment, inhibition of LPS-induced NO release, Western blot analysis and wrote the relevant sections. EKA: Conducted the RNA extraction and quantitative real-time polymerase chain reaction and wrote the relevant sections. MFH: prepared compounds 1-9. All authors have read and agreed to the published version of the manuscript.

Conflict of Interest
The author declare there is no conflict of interest in this study.

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