RESEARCH ARTICLE

Pharmacological Evaluation of New 4, 5-dihydro-1H- Pyrazole-1-yl acetate Derivatives as Anti-inflammatory Agents

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Received: 10th September, 2022; Revised: 25th October, 2022; Accepted: 27th November, 2022; Available Online: 25th December, 2022

ABSTRACT

A series of nine novel 4, 5-dihydro-1H- pyrazole-1-yl acetate derivatives (IV_{a-i}) by Shahla *et al.* were investigated *in-vitro* for their ability to prohibit arachidonic acid (AA) from becoming prostaglandin H2 (PGH2), the inhibitory effects were found at 0.016 and 0.02 nM for compounds IV_e and IV_f, respectively, this was due to high affinity for binding with cyclooxygenase (COX) enzymes. kinetics study and binding affinity results of these compounds showed good K_d constant of IV_e and IV_f with COX at (0.008 and 0.003 nM), respectively. Cytotoxicity of these two compounds in RAW 264.7 macrophages cell lines were performed for anti-inflammatory testing, determine their non-cytotoxic concentration to make sure that their anti-inflammatory activity not caused by cytotoxicity, results showed that IV_e (IC_{50} = 4.2 µM) and IV_f (IC_{50} = 2.7 µM), respectively. Compound IV_e decreased the COX fold activity followed by compound IV_f more than the positive control at the same concentration. During the evaluation of pro-inflammatory marker nitric oxide (NO) level with these compounds, results showed that the level significantly reduced in the presence of IV_e & IV_f at (5 and 10 µM) and (2.5 and 5 µM) respectively. Determination the level of cytokines with these compounds also performed, compounds IV_e at concentration of (10 µM) and IV_f (5 µM) significantly reduced the mean level of TNF-α. Significant reduction of proinflammatory IL-1β and IL-6 mean level production was observed at 10 µM of compound IV_e and 2.5, 5 µM of compound IV_f. The results showed good indication for the anti-inflammatory activity of these compounds and to optimize activity, further structural optimization is required. in the future study.

Keywords: 4, 5-dihydro-1H- Pyrazole-1-yl acetate, Anti-inflammatory, COX enzyme, Cytokines, Pro-inflammatory marker, Pyrazole.

International Journal of Drug Delivery Technology (2022); DOI: 10.25258/ijddt.12.4.40

How to cite this article: Abdul-Majeed SZ, Mahdi MF, Al-Mugdadi SFH, Pharmacological Evaluation of New 4, 5-dihydro-1H-Pyrazole-1-yl acetate Derivatives as Anti-inflammatory Agents. International Journal of Drug Delivery Technology. 2022;12(4):1733-1741.

Source of support: Nil.

Conflict of interest: None

INTRODUCTION

Considered one of our bodies' defense strategies against invasive pathogens is inflammation.[1] Additionally, it speeds up the mending of damaged tissue and helps the cells go back to normal. Despite these positive effects, it may also cause cardiovascular diseases,[2] tumors,[3] inflammatory bowel syndrome,[4] arthritis,[5] pulmonary disorders,[6] Alzheimers,[7] etc., as many negative effects. Understanding the function of inflammatory mediators, which directly cause inflammatory reactions, is essential for treating inflammation. Plasma proteins or certain cell types, such as platelets, neutrophils, monocytes, mast cells and macrophages, can produce inflammatory mediators. Host cell proteins or bacterial toxins set them off. On specific receptors on the target cells the inflammatory mediators bind and increase neutrophil chemotaxis and vascular permeability, cause contraction of smooth muscle, have an immediate impact on enzymatic activity, cause pain, or cause oxidative damage. They can also cause smooth muscle contraction. Despite having brief lives on average, these chemical mediators have negative impacts.[1] Vasoactive amines (such as 5-HT and histamine), eicosanoids (such as prostaglandins and leukotrienes), and cytokines (such as tumor necrosis factor (TNF) and interleukin-1 (IL-1)) are examples of the inflammatory chemical mediators. Arachidonic acid is transformed by cyclooxygenase-2 (COX-2) into prostaglandin H2 (PGH2), a mediator of inflammation.[8] Another effective strategy for the treatment of inflammation is to reduce PGE2 synthesis by inhibiting enzymatic activity and/or COX-2 protein expression. Nitric oxide (NO) also makes a significant role to the development of inflammation (under other normal physiological conditions it could produce anti-inflammatory effect).[9-11] However, in abnormal circumstances, it functions as

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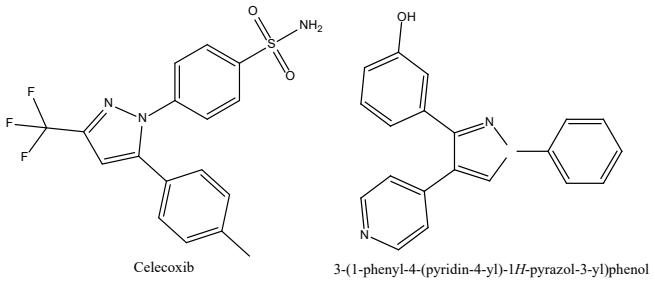

Figure 1: structural of celecoxib and 3-(1-phenyl-4-(pyridin-4-yl)-1H-pyrazol-3-yl) phenol as inhibitor of PGE2 and NO release

a proinflammatory mediator to cause a localized inflammatory response due to increased secretion. During an inflammatory response, the enzyme inducible nitric oxide synthase (iNOS) produces NO. At the location of inflammation, NO causes localized vasodilation, which causes edema.[12] Therefore, lowering NO synthesis through iNOS enzymatic activity suppression and/or iNOS protein expression inhibition may be advantageous for controlling inflammation, comparable to the inhibition of PGE2 production. Recently, it has been discovered that numerous substituted pyrazole compounds exhibit anti-inflammatory properties.[13-16] In our study, we evaluated a series of 4, 5-dihydro-1H- Pyrazole-1-yl acetate as a pyrazole-based anti-inflammatory agent derivatives with a structural likeness to celecoxib (Figure 1) as inhibitors of LPS-induced NO and PGE2 productions. Vicinal diaryl heterocycles as COX-2-inhibiting anti-inflammatory agents such as celecoxib have been reported. The presence of vicinal diaryl pyrazole scaffold in the structures of our target compounds encouraged us to investigate their anti-inflammatory activity.[17] Moreover, 3-(1-phenyl-4-(pyridin-4-yl)-1H-pyrazol-3-yl)phenol (Figure 1) possessing tri aryl pyrazole nucleus as inhibitor of PGE2 and NO release has been reported.[18]

MATERIALS AND METHODS

In-vitro Characterization Study of Synthesized Compounds Against COX Enzyme

The ability of the previously 9 synthesized compounds (IV_{a-i}) by Shahla *et al.* (2022) to prohibit the formation of PGH2 from arachidonic acid (AA) was assessed using a COX inhibitor screening assay kit according to the Abcam (No. ab139432) manufacturer's guidelines (UK). The assay was run in duplicate with 9 solutions of compounds that were prepared in dimethyl sulfoxide (DMSO) at four concentrations (0.008, 0.012, 0.016 and 0.02 nM). According to the instructions in the kit handbook, a standard curve of six prostaglandin concentrations, a non-specific binding sample, and a maximal binding sample were utilized to calculate the inhibition of the test chemical by using the multiple regression generated best-fit line. The 100% initial activity plate for the two enzymes cyclooxygenase-1 and cyclooxygenase-2 contained 10 mL hematin and 10 mL of the enzyme in 160 mL reaction buffer. Then 10 mL from (IV_{a-i}) compounds of concentrations mentioned above were added to the wells of inhibitor for the two enzymes and 10 mL of vehicle (DMSO) was added to 100% initial activity and background wells. The plate was incubated for 10 minutes at 37ºC and the reaction was started by adding 10 mL of cold COX chemiluminescent substrate, then immediately inject ten microliter of a cold, diluted solution of arachidonic acid wells. After that read in a luminometer for 5 seconds. The following equation used to estimate the percentage inhibition d by comparing the test compounds with the blank:

$$[PGE2]_{vehicle} - [PGE2]_{drug} \times 100/[PGE2]_{vehicle}$$

In-vitro Kinetics Study of Synthesized Compounds as a Function of the Concentrations of COX Enzyme

Synthesized compounds (IV_{a-i}) kinetics were validated using a COX inhibitor screening assay kit (cat.no. ab204699) 10 µL from 0.008, 0.012, 0.016 and 0.02 nm from IV_e and IV_f compounds, respectively were added to COX enzyme 5 U/well and kept 37°C with concentrations of 50 µL COX Chemiluminescent substrate and 50 µL at 5 mM arachidonic acid. Reactions were stopped by added of 50 µL of 1 M HCl at times (5, 15, 30 and 45 minutes) then measured fluorescence at a wavelength between Ex. 360 nm and Em. 460 nm.

Studying the Therapeutic Effect of Compounds as Anti-inflammatory

To evaluate the pharmacological interventions for synthesized compounds to inhibit COX isoenzyme as anti-inflammatory, IV_e and IV_f compounds were used to study the anti-inflammatory in RAW 264.7 cell line.

The RAW 264.7 cells line from BALB/c mice were treated with different concentrations from (100–0.15 µM) of IV_e and IV_f compounds. After 72 hours, to analyze the degree of cytotoxicity of these compounds MTT assay test was run on RAW 264.7 cells. IV_e and IV_f inhibition ability to lipopolysaccharide-induced pro-inflammation in RAW 264.7 cells, pro-inflammatory markers level like nitric oxide (NO), tumor necrosis factor (TNF-α), interleukin (IL-1β and IL-6) were assessed.

In-vitro Cytotoxicity

Cell Culture Mouse Cell Lines

RAW 264.7 cell line was purchased from American Type Culture Collection ATCC and in the Cell Bank of the Biomedical Research Centre at the University of Mustansiriyah was stored. RAW 264.7 cell line was used as model cancer cells for this study.

Maintenance, Storage and Resuscitation of Cell Line

RAW 264.7 cells in Dulbecco's Modified Eagle's Medium (DMEM) liquid medium were cultured, with fetal bovine serum (FBS) 10% and penicillin–streptomycin 1% as antiseptic. The -80ºC was used to store obtained cells pellet for 24 hours and for long time were stored under liquid nitrogen. All steps for maintenance, storage and resuscitation done according to Marin V *et al.* and Qusay A *et al.*[19,20]

Cell Viability and Inhibitory Concentration (IC_{50}) by MTT Assay Colorimetric Assay

The MTT assay was used to assess the effects of compounds IV_{a-i} on RAW 264.7 cells viability. A 100 µL from all cells suspensions were dispensed into 96 well flat-bottom tissue culture plates at concentrations of 5 x 10^3 cells/well and incubated for 24 hours under standard conditions, 4 x 10^3 cells/well for 48 hours incubation and 3 x 10^3 cells/well for 72 hours incubation. After 24 hours, the cells were treated with (0.15, 0.32, 0.75, 1.5, 3.12, 6.25, 12.5, 25, 50 and 100 µM) of the compounds IV_{a-i} in the presence of 100 ng/mL lipopolysaccharide (LPS) for 24 hours. Compared to untreated control cells, the vitality of RAW 264.7 cells in each well was displayed as a percent. The inhibitory concentration 50% (IC_{50}) was evaluated after 72 hours.[21]

Effect of IV_e and IV_f Compounds on Nitric Oxide Production in RAW 264.7 Cell Line

By detecting the nitrite levels in the culture media using the Griess reagent test, NO generation was indirectly evaluated. In a 96 well plate, RAW 264.7 cells were planted for 24 hours at a density of 5 104 cells per well. The cells were handled following incubation. with 5 and 10 µM of IV_e and 2.5 and 5 µM of IV_f compounds in the presence of LPS 100 nm/mL for 24 hours. Each of 100 mL of DEMEM culture media was mixed with an equal volume of Griess reagent I (1% sulfanilamide, 0.1% naphthyl ethylenediamine dihydrochloride and 5% phosphoric acid) then incubated at room temperature for 10 minutes. The absorbance was measured at 540 nm with a microplate reader. A standard curve made from sodium nitrite was used to calculate the nitrite levels.

Determination the TNF- α

1×10^5/100 µL RAW 264.7 cells were seeded per well in 96 well plate. Cells were incubated with 5 and 10 µM of IV_e and 2.5 and 5 µM of IV_f compounds in the presence of LPS (100 ng /mL) for 24 hours. Cell culture supernatants were centrifuged at 5000 x g for 3 minutes at 4°C to remove insoluble material. Secreted TNF-α was measured in cell culture supernatants using commercially available TNF-α ELISA kit (Abcam, no. ab208348, UK) following the instructions provided by the manufacturers. Briefly, 50 µL of IV_e and IV_f were added to appropriate wells, then 50 µL of the antibody cocktail was added to each well. Covered the plate and incubated for 1-hour at room temperature with shaking, after wash three times, 100 µL of TMB development solution was added and incubated for 10 minutes in the dark on a plate shaker at 400 rpm. Stopped the reaction by adding 100 µL of stop solution, plate shaken for 1-minutes. The absorbance at 450 nm for each compound was read. For statistical analysis of three independent experiments results were used.

Determination the Interleukin IL-1β

The 5× 10^4 RAW 264.7 cells per well were seeded in 96 well plates and incubated with 5 and 10 µM of IV_e and 2.5 and 5 µM of IV_f compounds for 24 hours while exposed to LPS (100 ng/mL). To remove insoluble substances, cell culture upper layer was centrifuged at 5000 x g for three minutes at 4°C. Secreted IL-1β was measured in cell culture upper layer using commercially available Interleukin IL-1β ELISA kits (Abcam, no. ab100704, UK) following manufacturer's instructions briefly, incubated the cells with 100 µL of IV_e and IV_f in well for 2.5 hours at room temperature with shaking. After washing four times. The 100 µL of 1X Biotinylated IL-1β detection antibody was added and incubated for 1-hour at room temperature with shaking and wash. The 100 µL of 1X HRP-Streptavidin solution was incubated for 45 minutes at room temperature with shaking then wash. TMB substrate reagent of 100 µL was added and incubated for 30 minutes at room temperature in the dark room with shaking. Finally, stop the reaction by added 50 µL from stop solution to each well and read at 450 nm immediately. For statistical analysis results of three independent experiments were used.

Determination the Interleukin IL-6

The supernatant of RAW 264.7 cells cultured was used for measuring IL-6 cytokines using commercially available ELISA kit (Abcam, no. ab100713, UK). The 100 µL of 5 and 10 µM of IV_e as well as 2.5 and 5 µM of IV_f compounds were added into appropriate wells in the presence of LPS (100 ng/mL) and incubated for 2.5 hours at room temperature with shaking. After washing four times, hundred microliters of 1X biotinylated IL-6 detection antibody was added and incubated for one hours with shaking at room temperature, then washed four times. The 100 mL of 1X HRP-Streptavidin solution was added to each well and incubated for 45 minutes with shaking at room temperature, then washed four times with washing solution. Finally, 100 mL of TMB substrate reagent was added and incubated for 30 minutes at room temperature with shaking in the dark. The reaction stopped by adding 50 mL from stop solution to each well and read at 450 nmb immediately. Results of three independents experiments were used for statistical analysis.

In-vitro COX-1/COX-2 Inhibition Assay in RAW 264.7 Cell Line

Using a cyclooxygenase activity assay and a fluorometric kit(Abcam, no. ab204699, UK), the capacity of the test IV_e and IV_f drugs to inhibit COX-1 and COX-2 isoenzymes was assessed *in-vitro.* The 2×10^6 RAW 264.7 cells were harvested. Firstly in 75 cm^3 flask at 24 hours. Cells was washed with 10 mL cold PBS (1X) and transferred to a one-half mL tube. Centrifuged at 500 x g for 3 minutes. Resuspend cell pellet after discard supernatant in 0.2–0.5 mL of lysis buffer with protease inhibitor cocktail. Then, incubated on ice for 5 minutes and centrifuged the cell lysate at 12,000 x g, 4°C for 3 minutes 200 mL of cell lysate was added in each 12 well plate then add 100 mL from five and 10 mL of IV_e as well as 2.5 and 5 mL of IV_f compounds and 0.02 nm SC560 (cyclooxygenase-1 inhibitor as a positive control) and 0.02 nano micromolar Celecoxib (cyclooxygenase-2 inhibitor as a positive control) 20 mL of DMSO was applied to the 100% initial activity and background wells and the enzyme inhibitor wells. The reaction was begun

by pouring 580 µL of the reaction mixture (20 µL COX Probe + 40 µL COX Cofactor + 520 µL COX assay buffer) into each well of the plate, which had been incubated at 37ºC for 10 minutes. After injecting 100 µL of diluted cold arachidonic acid solution to all the reaction wells, the fluorescence at Ex/Em = 535/587 nm was measured.

Data Analysis

All statistical analysis of compounds characterization using the nonlinear curve fitting software Origin 9.1 software were used to performed kinetic. IC_{50} was done by using the nonlinear curve fitting software prism pad software. NO, TNF-α, IL-1β and IL-6 levels were evaluated by using data comparison between all groups within the same plate by one-way ANOVA with Tukey (prism pad software). Values of $p < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

In-vitro Characterization Study of Inhibitors for the Cyclooxygenase (COX) Activity Enzyme by nine Synthesized Compounds (IV_a-IV_f) as Compared with Anti-Inflammatory Controls

9 compounds (IV_a-IV_i) were selected to be further studied. *In-vitro* cyclooxygenase inhibition assay kit was used to identify the most potent and the most selective group. The results fluorometric COX activity assay is summarized in Figure 2. In this figure, axis Y representing fluorescence data of COX activity for all compounds divided by fluorescence data of COX activity at zero time while axis X representing logarithm concentrations of 0.008, 0.012, 0.016 and 0.02 nm from (IV_a- IV_i) compounds compared with the activity of 0.02 nM SC560 and celecoxib as a positive control. The effects of compounds IVe and IVf were further observed to reduce COX enzyme fold activity, as seen in Figure 3, compared to positive control SC560 and celecoxib at 0.02 nM. The inhibitory effects were found at 0.016 and 0.02 nM for compounds IV_e and IV_f, respectively. Such inhibition in fold COX activity is due to high affinity of these two compounds among the 9 tested compounds to bind with COX enzymes. Compounds IV_{a-d} and

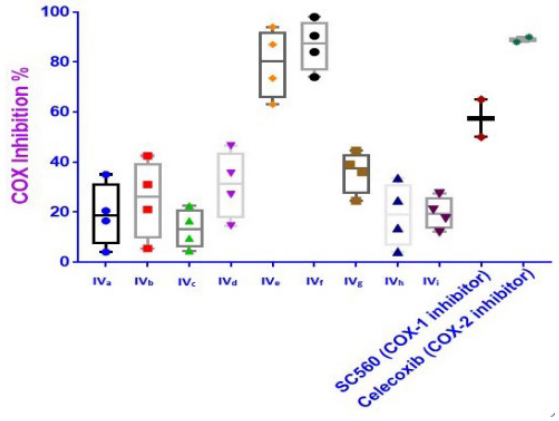

Figure 2: Measurement of inhibitors COX activity in all compounds with Cyclooxygenase inhibitor screening activity assay Kit.

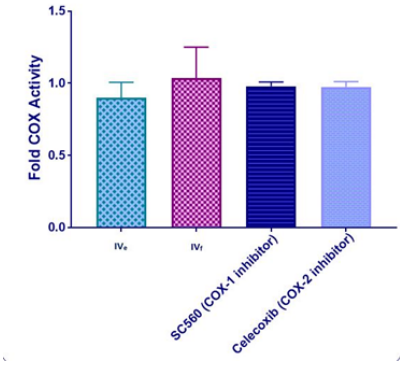

Figure 3: Measurement of COX activity in IVe and IVf compounds with Cyclooxygenase activity assay Kit.

IV_{g-i} have activity against COX isoenzymes but the inhibition is less than 50%.

Subsequently, the data of COX inhibitors characterization were fitted on mathematical equation:

$$\frac{1}{1+\frac{K}{X-\sqrt{A+X+K-(A+X+k)\text{^}2-4*A*X}}}$$

K= Dissociation constant K_d

A= Constant (log Concentrations of nine compound used in this study)

X= Independent variable (inhibitors activity for each compound)

Using Origin 9.1 software to determine the dissociation constant k_d and association constant k_a to choose the best compound to complete the study of kinetics and binding affinity of these compounds using nonlinear regression analysis. Data showed good K_d constant of IV_e with COX (0.008 nM) and IV_f with COX (0.003 nM) compared to all nine compounds in this study, as illustrated in Table 1 and Figures 4 and 5. Thus, IV_e and IV_f used for further COX kinetics study.

COX kinetics as a function of the concentrations of tested compounds

A velocity vs substrate concentration [S] graph is needed to determine the Michaelis constant (K_m) and velocity maximum (V_{max}) values. After measuring COX for 5, 15, 30, and 45 minutes, the slope of these four locations was used to calculate an initial velocity. Due to the difficulty in accurately

Table 1: K_d and K_a constants ± SEM for all synthesized compounds

Compounds	$K_{d\ (nM)}$	$K_{a\ (nM)}$	*R-square*
IV_a	-13.3 ± 3.5	-	Not good
IV_b	-15.65 ± 1.3	-	Not good
IV_c	-20.65 ± 4.9	-	Not good
IV_d	-2.44 ± 1.9	-	Not good
IV_e	0.008 ± 3.5	125 ± 0.01	0.99999
IV_f	0.003 ± 0.46	333 ± 0.045	0.99989
IV_g	-0.02 ± 7.01	-	Not good
IV_h	-0.0105 ± 6.8	-	Not good
IV_i	-10.01 ± 3.7	-	Not good

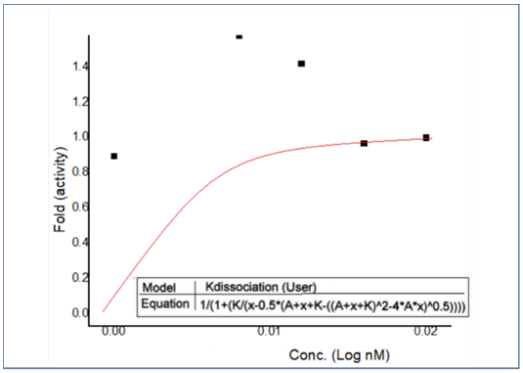

Figure 4: The best fit of the data yielded a K dissociation constant of IV_e with COX= 0.008nM.

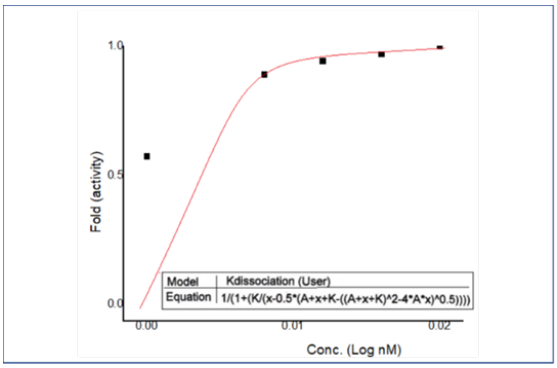

Figure 5: The best fit of the data yielded a K dissociation constant of IV_f with COX= 0.003nM.

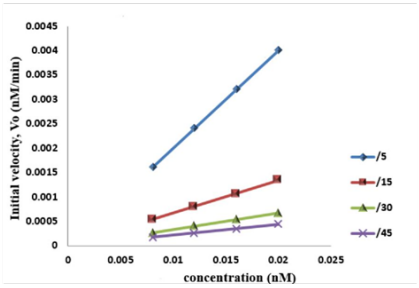

Figure 6: A plot of the reaction velocity (V_0) versus compound IV_e concentration (nM) for COX enzyme that obeys Michaelis-Menten kinetics shows that the maximal velocity (V_{max}) is approached asymptotically.

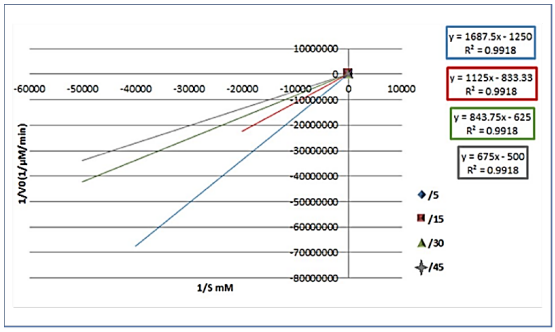

Figure 7: A double-reciprocal plot of COX kinetics for compound IV_e is generated by plotting $1/V_0$ as a function 1/[S].

determining V_{max} on the graph, velocity versus substrate [S], double reciprocal plots were utilized to calculate both V_{max} and K_m. Regarding the results, further kinetics study can be done on compounds IV_e and IV_f as they showed the inhibitors COX enzyme activities as well as the good value of K_d, Figure 6 illustrates compound IV_e progress curve analysis at different concentrations (0.008, 0.012, 0.016 and 0.02 nM) and demonstrated that at 37°C, V_{max} for the COX reaction is reached in 45 minutes. As the concentration of compound IV_e grows, the rate of catalysis rises linearly until beginning to level off and approach a maximum at 0.02nM concentrations.

Figure 7 and Table 2 summarized information about compound IV_e, K_m value and V_{max}. At each time under investigation, the relationship between initial velocity and compound IV_e concentration was plotted as a hyperbolic curve, and all Lineweaver-Burk plots were linear. The kinetic mechanism of compound IV_e suppression through improved binding of compound IV_e with the COX enzyme was confirmed by a lower K_m value, as shown in Table 5. The maximum measured speed is V_{max}. of the reaction catalyzed by compound IV_e at concentrations (0.008, 0.012, 0.016 and 0.02 nM) were less than the K_m and it is constant at 1.3 nM, that is mean the reaction is noncompetitive inhibitor which can binds to a different site on the COX enzyme; compound IV_e doesn't block substrate binding, but it causes other changes in the COX enzyme so that it can no longer catalyze the reaction efficiently.

As a function of time for a series of compound IV_e concentrations (Figure 8) the extent of COX enzyme inhibitor formation is determined. In each case, COX-formed activity increases with time at maximum product concentration (62 nM) with compound IV_e concentration (0.02 nM) at 45 minutes. However, there comes the point when there is no longer any net change in the concentration of component IV_e. The most crucial aspect of this graph is that all concentrations of compound IV_e significantly increased the velocity of COX catalyze reaction thereby decreasing the activity of COX enzyme as compared with positive control 0.02 nM SC560 and Celecoxib, $p < 0.001$.

In the same context, K_m and V_{max} of COX enzyme with compound IV_f at series time 5, 15, 30 and 45 minutes demonstrated in Table 3. In Figures 9 and 10, kinetic examination of COX enzyme activity with compound IV_f showed. At a fixed concentration of COX enzyme, V_0 is almost constant to compound IV_f concentration. K_m value is low and increased that means the reaction is competitive

Table 2: V_{max} and K_m for compound IV_e ± standard error

	45 minutes		30 minutes		15 minutes		5 minutes	
IV_e	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m
	0.002 ± 0.001	1.35 ± 0.087	0.0016 ± 0.0023	1.35 ± 0.087	0.001200 ± 0.0102	1.35 ± 0.087	0.0008 ± 0.0001	1.35 ± 0.087

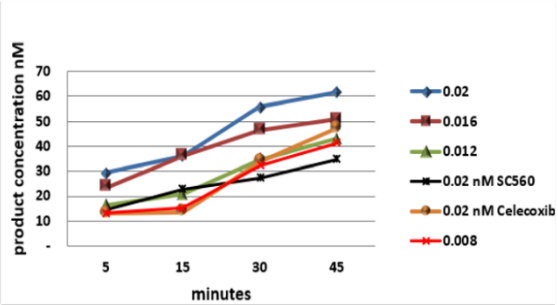

Figure 8: The amount of product formed at different concentrations of IV_e with 0.02nM of SC560 and Celecoxib are plotted as a function of time, $p < 0.001$.

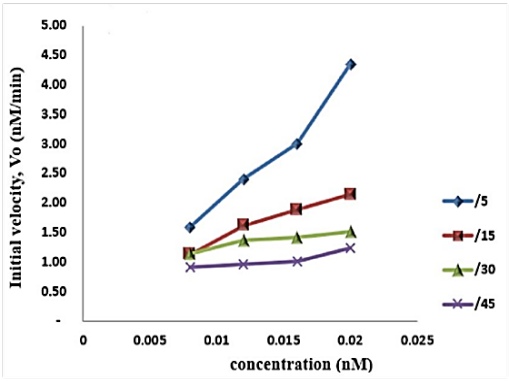

Figure 9: A plot of the reaction velocity (V_0) versus compound IV_f concentration (nM) for COX enzyme that obeys Michaelis-Menten kinetics shows that the maximal velocity (V_{max}) is approached asymptotically.

inhibition, binds to the active site and prevents the substrate from binding there.

To examine the inhibition of compound IV_f, the dependence of compound IV_f concentration on COX activity at different time 5, 15, 30, 45 minutes was determined. In Figure 11, compound IV_f is seen to activate the COX in time course at different concentration as well as SC560 and celecoxib. These results demonstrate, for the first time, that compound IV_f can accelerate the reaction at 0.02 nM more than the other concentrations with maximum product concentration (55.37 nM at 45 minutes). Likewise, all the concentrations of compound IV_f were significantly increased the velocity of COX catalyze reaction thereby decreasing the activity of COX enzyme as compared with positive control with 0.02 nM SC560 and celecoxib, $p < 0.05$.

Cytotoxicity and Anti-inflammatory Activity of Compounds (IV_e and IV_f) in RAW 264.7 Cell Lines

The RAW 264.7 cells were treated with different concentrations from (0.15–100 µM) of compounds (IV_e and IV_f). After 72 hours, to analyze the degree of cytotoxicity of these compounds

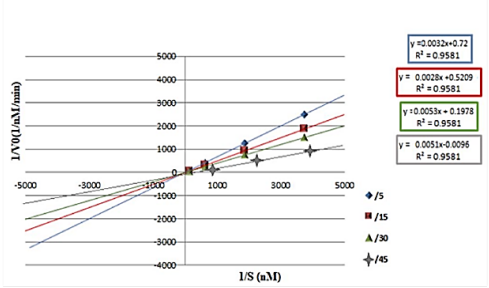

Figure 10: A double-reciprocal plot of COX kinetics for compound IV_f is generated by plotting $1/V_0$ as a function 1/[S].

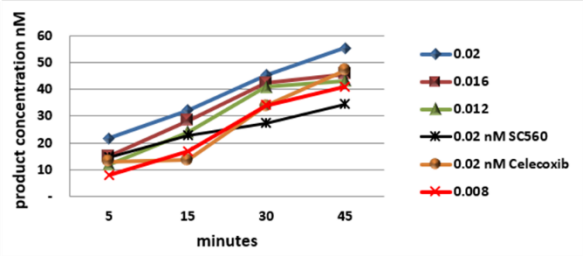

Figure 11: The amount of product formed at different concentrations of compound IV_f (0.008nM, 0.012nM, 0.016nM, 0.02nM with 0.02nM for SC560 and Celecoxib) are plotted as a function of time, $p < 0.05$.

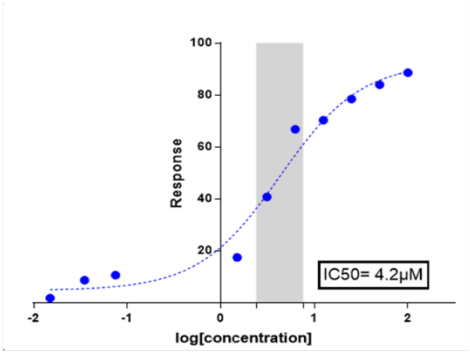

Figure 12: Dose-response curve for compound IV_e. RAW 264.7 cells were treated for 72h with 0.15, 0.35, 0.75, 1.56, 3.12, 6.25, 12.5, 25, 50 and 100µM dose ranges of compound IV_e.

on RAW 264.7 cells MTT assay was performed as seen in Figures 12 and 13.

Inhibitor COX Activity by Compounds (IV_e and IV_f) as Compared with SC560 (COX-1 inhibitor) and Celecoxib (COX-2 inhibitor) in RAW 264.7 Cell Line

Fold activity of COX was inhibited by increasing the concentration of Compounds (IV_e and IV_f) from 1–5 µM in comparison with positive control (SC560 and celecoxib). The

Table 3: V_{max} and K_m for compound IV_f ± standard error.

	45 minutes		*30 minutes*		*15 minutes*		*5 minutes*	
IV_f	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m
	10 ± 1.31	0.059 ± 0.001	10 ± 1.31	0.053 ± 0.003	10 ± 1.312	0.028 ± 0.002	10 ± 1.31	0.021 ± 0.005

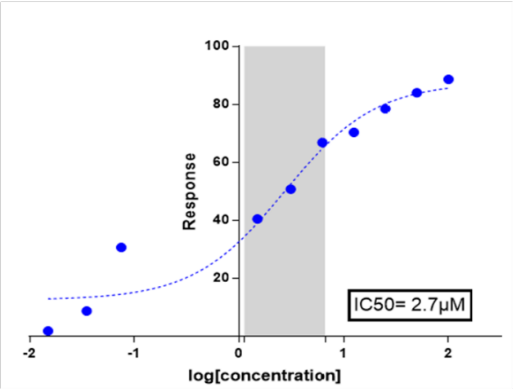

Figure 13: Dose-response curve for compound IV_f. RAW 264.7 cells were treated for 72h with 0.15, 0.35, 0.75, 1.56, 3.12, 6.25, 12.5, 25, 50 and 100μM dose ranges of compound IV_f.

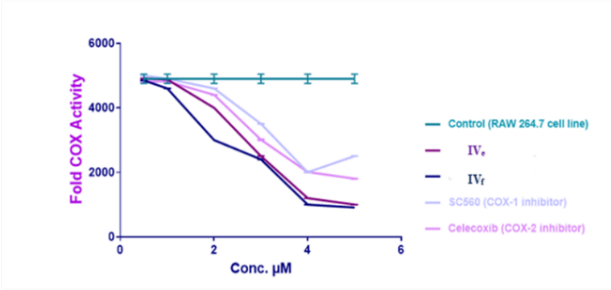

Figure 14: Measurement the inhibitory effect of COX activity by Compounds (IV_e and IV_f) with Cyclooxygenase activity assay Kit in RAW 264.7 cell line.

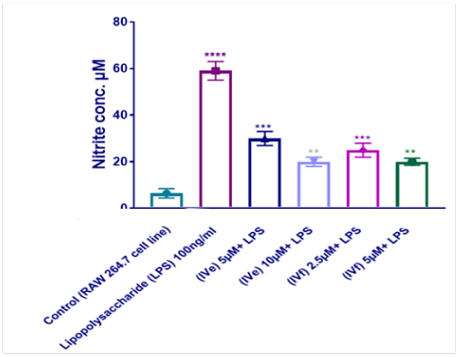

Figure 15: NO production in LPS-stimulated RAW 264.7 cells. Compounds (IV_e and IV_f) inhibited LPS-induced cell inflammation in RAW 264.7 cells.

result showed that compound IV_e decreased the COX fold activity followed by compound IV_f more than the positive control at the same concentration, as demonstrated in Figure 14.

Evaluation the Level of Pro-inflammatory Marker Nitric Oxide (NO) with Compounds (IV_e and IV_f) in RAW 264.7 Cell Line

LPS is endotoxin of gram-negative bacteria which induce proinflammatory of many cytokines, thus LPS induce release

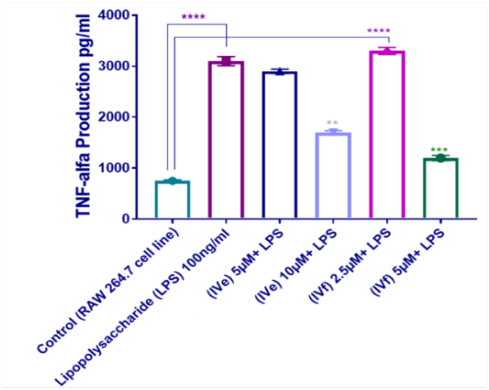

Figure 16: TNF-α production in LPS-stimulated RAW 264.7 cells. Compounds (IV_e and IV_f) inhibited LPS-induced cell inflammation in RAW 264.7 cells.

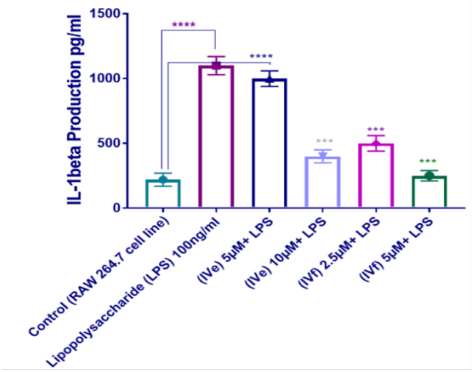

Figure 17: IL-1β production in LPS-stimulated RAW 264.7 cells. Compounds (IV_e and IV_f) inhibited LPS-induced cell inflammation in RAW 264.7 cells.

of these cytokines considered good method for novel synthesis compounds study *in-vitro*. To elucidate whether compounds (IV_e and IV_f) might reduce the production of NO, a sign of pro-inflammatory activity, in RAW 264.7 cells when lipopolysaccharide (LPS) induces inflammation (NO) was assessed. Regarding to best concentration of compounds (IV_e and IV_f), IV_e at (5 μM and 10 μM) with LPS was more potent to reduce the level NO significantly (30 ± 3.1 μM) and (20 ±

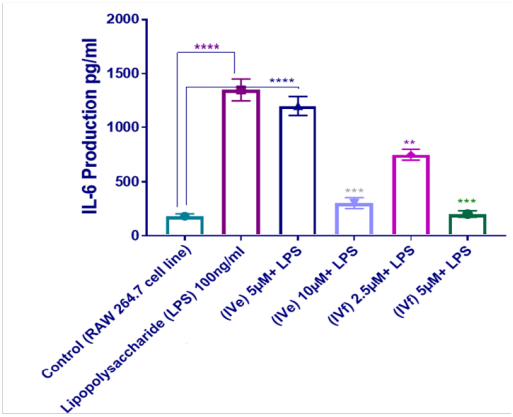

Figure 18: IL-6 production in LPS-stimulated RAW 264.7 cells. Compounds (IV_e and IV_f) inhibited LPS-induced cell inflammation in RAW 264.7 cells.

2.2 µM), respectively. Also compound IV_f at (2.5 and 5µM) reduces the NO level significantly (25 ± 3.1 µM) and (26 ± 2.09 µM), respectively versus LPS alone (59 ± 4.02 ng/mL), as demonstrated in Figure 15.

Determination the Level of Cytokines with Compounds (IV_e and IV_f) in RAW 264.7 Cell Line

Tumor Necrosis Factor (TNF-α)

Based on the anti-inflammatory effects of compounds (IV_e and IV_f) against TNF-α, induced by the LPS, concentration of IV_e (10 µM) and IV_f (5 µM) reduced the mean level of TNF-α in cells (1700 ± 35.51 pg/mL) and (1200 ± 50.08 pg/mL), respectively compared to LPS alone (3100 ± 90.03 pg/mL), as illustrated in Figure 16.

Interleukin IL-1β

Compounds (IV_e and IV_f) reduced the mean level of IL-1β induced by LPS was shown in Figure 17. Highly significant reduction of proinflammatory IL-1β mean was observed in 10 µM of compound IV_e (400 ± 50 pg/mL) and 2.5 µM (500 ± 60 pg/mL), as well as 5 µM (250 ± 40 pg/mL) of compound IV_f versus LPS alone (1100 ± 70 pg/mL) in RAW 264.7 cell line.

Interleukin IL-6

The inhibitory effect of Compounds (IV_e and IV_f) were more potent on IL-6 mean level induced by LPS. Highly significant reduction of IL-6 mean were observed in10 µM of Compound IV_e (300 ± 50 pg/mL) and 2.5 µM (750 ± 50 pg/mL), as well as 5 µM (200 ± 30 pg/mL) of compound IV_f, respectively compared to LPS alone (1350 ± 100 pg/mL), as seen in Figure 18.

CONCLUSION

A series of nine novel synthesized 4,5-dihydro-*1H*-pyrazole-1yl-acetate derivatives were tested *in-vitro* to evaluate their activity as inhibitors for COX enzyme activity using more research is needed as potential inhibitors of PGE2 and NO generation in LPS-induced murine RAW 264.7 macrophages. The collected data indicated that two of the newly synthesized compounds (IV_e and IV_f) observed to reduce COX enzyme fold activity compared to positive control SC560 (selective COX-1) and celecoxib (selective COX-2), respectively and further structural optimization is needed to optimize activity.

CONFLICT OF INTEREST

The authors have no conflicts of interest regarding this investigation.

ACKNOWLEDGMENT

The authors would like to thank Mustansiriyah University (www.uomustansiriyah.edu.iq) Baghdad Iraq for its support to the present work, the authors would like to thank doctor Basma Talib Al-Sudani for her kind help during this study.

REFERENCES

1. Abdulkhaleq L, Assi M, Abdullah R, Zamri-Saad M, Taufiq-Yap Y, Hezmee M. The crucial roles of inflammatory mediators in inflammation: A review. Veterinary world. 2018;11(5):627. doi: 10.14202/vetworld.2018.627-635
2. Coussens LM, Werb Z. Inflammation and cancer. Nature. 2002;420(6917):860-7. doi.org/10.1038/nature01322
3. Qiu H, Johansson A-S, Sjöström M, Wan M, Schröder O, Palmblad J, et al. Differential induction of BLT receptor expression on human endothelial cells by lipopolysacharide, cytokines, and leukotriene B4. Proceedings of the National Academy of Sciences. 2006;103(18):6913-8. doi.org/10.1073/pnas.0602208103
4. Sung B, Prasad S, Yadav VR, Lavasanifar A, Aggarwal BB. Cancer and diet: how are they related? Free radical research. 2011;45(8):864-79. doi.org/10.3109/10715762.2011.582869
5. Lee I-A, Bae E-A, Hyun Y-J, Kim D-H. Dextran sulfate sodium and 2, 4, 6-trinitrobenzene sulfonic acid induce lipid peroxidation by the proliferation of intestinal gram-negative bacteria in mice. Journal of inflammation. 2010;7(1):1-9. doi.org/10.1186/1476-9255-7-7
6. Hochberg MC, editor Changes in the incidence and prevalence of rheumatoid arthritis in England and Wales, 1970–1982. Seminars in arthritis and rheumatism; 1990: Elsevier. doi: 10.1016/0049-0172(90)90052-h.
7. Sastre M, C Richardson J, M Gentleman S, J Brooks D. Inflammatory risk factors and pathologies associated with Alzheimer's disease. Current Alzheimer Research. 2011;8(2):132-41. doi.org/10.2174/156720511795256062
8. Hinz B, Brune K. Cyclooxygenase-2—10 years later. Journal of pharmacology and experimental therapeutics. 2002;300(2):367-75. doi.org/10.1124/jpet.300.2.367
9. Bhardwaj A, Batchu SN, Kaur J, Huang Z, Seubert JM, Knaus EE. Cardiovascular Properties of a Nitric Oxide Releasing Rofecoxib Analogue: Beneficial Anti-hypertensive Activity and Enhanced Recovery in an Ischemic Reperfusion Injury Model. ChemMedChem. 2012;7(8):1365-8. doi.org/10.1002/cmdc.201200234
10. Bhardwaj A, Huang Z, Kaur J, Knaus EE. Rofecoxib Analogues Possessing a Nitric Oxide Donor Sulfohydroxamic Acid (SO2NHOH) Cyclooxygenase-2 Pharmacophore: Synthesis, Molecular Modeling, and Biological Evaluation as Anti-inflammatory Agents. ChemMedChem. 2012;7(1):62-7. doi.org/10.1002/cmdc.201100393
11. Kaur J, Bhardwaj A, Huang Z, Knaus EE. Aspirin Analogues as Dual Cyclooxygenase-2/5-Lipoxygenase Inhibitors: Synthesis, Nitric Oxide Release, Molecular Modeling, and Biological

Evaluation as Anti-Inflammatory Agents. ChemMedChem. 2012;7(1):144-50. doi.org/10.1002/cmdc.201100460

12. Yun H-Y, Dawson VL, Dawson TM. Neurobiology of nitric oxide. Critical Reviews™ in Neurobiology. 1996;10(3-4). DOI: 10.1615/CritRevNeurobiol.v10.i3-4.20
13. Arora RK, Kaur N, Bansal Y, Bansal G. Novel coumarin–benzimidazole derivatives as antioxidants and safer anti-inflammatory agents. Acta Pharmaceutica Sinica B. 2014;4(5):368-75. doi.org/10.1016/j.apsb.2014.07.001
14. Azelmat J, Fiorito S, Taddeo VA, Genovese S, Epifano F, Grenier D. Synthesis and evaluation of antibacterial and anti-inflammatory properties of naturally occurring coumarins. Phytochemistry Letters. 2015;13:399-405. doi.org/10.1016/j.phytol.2015.08.008
15. Wei W, Wu X-W, Deng G-G, Yang X-W. Anti-inflammatory coumarins with short-and long-chain hydrophobic groups from roots of Angelica dahurica cv. Hangbaizhi. Phytochemistry. 2016;123:58-68. doi.org/10.1016/j.phytochem.2016.01.006
16. Srivastava P, Vyas VK, Variya B, Patel P, Qureshi G, Ghate M. Synthesis, anti-inflammatory, analgesic, 5-lipoxygenase (5-LOX) inhibition activities, and molecular docking study of 7-substituted coumarin derivatives. Bioorganic chemistry. 2016;67:130-8. doi.org/10.1016/j.bioorg.2016.06.004
17. Gamal El-Din MM, El-Gamal MI, Abdel-Maksoud MS, Yoo KH, Oh C-H. Design, synthesis, in vitro potent antiproliferative activity, and kinase inhibitory effects of new triarylpyrazole derivatives possessing different heterocycle terminal moieties. Journal of enzyme inhibition and medicinal chemistry. 2019;34(1):1534-43. doi.org/10.1080/14756366.2019.1653292
18. I El-Gamal M, S Abdel-Maksoud M, M Gamal El-Din M, Shin J-S, Lee K-T, Ho Yoo K, et al. Synthesis, in vitro antiproliferative and antiinflammatory activities, and kinase inhibitory effects of new 1, 3, 4-triarylpyrazole derivatives. Anti-Cancer Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Cancer Agents). 2017;17(1):75-84.
19. Marin V, Kaplanski G, Gres S, Farnarier C, Bongrand P. Endothelial cell culture: protocol to obtain and cultivate human umbilical endothelial cells. Journal of immunological methods. 2001;254(1-2):183-90. doi.org/10.1016/S0022-1759(01)00408-2
20. Qusay A, Marie NK, Al-Sudani BT. Utilization of natural stabilizer to prepare liposomal conjugate for the newly developed aptamer. Systematic Reviews in Pharmacy. 2020;11(7):32-50. doi:10.31838/srp.2020.7.07
21. Basma Talib Al-Sudani1* NHM, FHA-S. Redounding of Cuscuta chinensis Lam. on BxPC-3, HepG2, and U2OS Human Cancer Cell Lines. International Journal of Drug Delivery Technology. 2020;10(3):354-9