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Inhibitory effects of triarylpyrazole derivatives on LPS-induced nitric oxide and PGE_2 productions in murine RAW 264.7 macrophages

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ABSTRACT

In this article, a series of 22 triarylpyrazole derivatives were evaluated for *in vitro* antiinflammatory activity as inhibitors of nitric oxide (NO) and prostaglandin E_2 (PGE_2) release induced by lipopolysaccharide (LPS) in murine RAW 264.7 macrophages. The synthesized compounds **1a-h, 2a-f and 3a-h** were first examined for their cytotoxicity for determination of the non-toxic concentration for antiinflammatory screening, so that the inhibitory effects against NO and PGE_2 production were not caused by non-specific cytotoxicity. Compounds **1h** and **2f** were the most active PGE_2 inhibitors with IC_{50} values of 2.94 μM and 4.21 μM, respectively. Western blotting and cell-free COX-2 screening revealed that their effects were due to inhibition of COX-2 protein expression. Moreover, compound **1h** exerted strong inhibitory effect on the expression of COX-2 mRNA in LPS-induced murine RAW 264.7 macrophages.

The inflammatory response is a part of the immune system of higher organisms protecting them from injury and infections.[1] Its activity is to surround and remove both the injurious agent and damaged tissue components allowing the body to start the healing process. Although inflammation is a beneficial mechanism of defense in some cases, it can lead to allergy, autoimmune diseases, and rejection of organs. Chronic inflammatory conditions have been linked to several diseases including cardiovascular diseases,[2] cancer,[3] inflammatory bowel syndrome,[4] arthritis,[5] pulmonary disease, Alzheimer's disease,[6] etc.

Nitric oxide (NO) plays a crucial role in the development of inflammation despite of acting as an anti-inflammatory agent under normal physiological conditions.[7–9] On the contrary, it could be considered as a pro-inflammatory mediator that could induce localized inflammation because of increased secretion in abnormal situations. The inducible nitric oxide synthase (iNOS) secretes NO as an inflammatory mediator resulting in vasodilation at the location of inflammation in chronic inflammation, thus causing edema.[10] So inhibition of NO production through inhibition of iNOS enzyme activity and/or protein expression can be a useful approach for treatment of inflammation. Moreover, cyclooxygenase-2 (COX-2) converts the arachidonic acid into another inflammatory mediator, PGE_2.[11] Similarly, inhibition of PGE_2 production via inhibition of COX-2 protein expression and/or enzyme activity is another potential mechanism of inflammation therapy.

Several triarylpyrazole derivatives have been recently reported as antiinflammatory agents.[12–15] In the present work, we report the inhibitory effects of a series of substituted pyrazole derivatives on LPS-induced NO and PGE_2 productions. They possess structural similarity to

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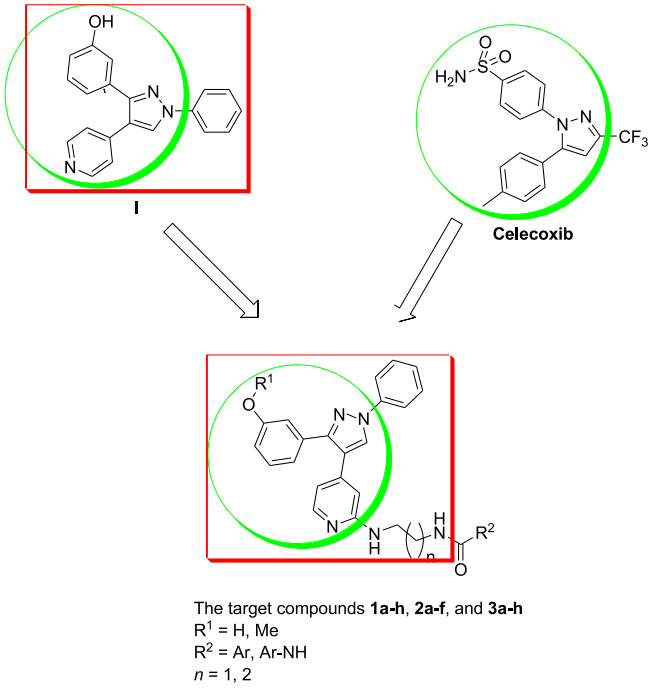


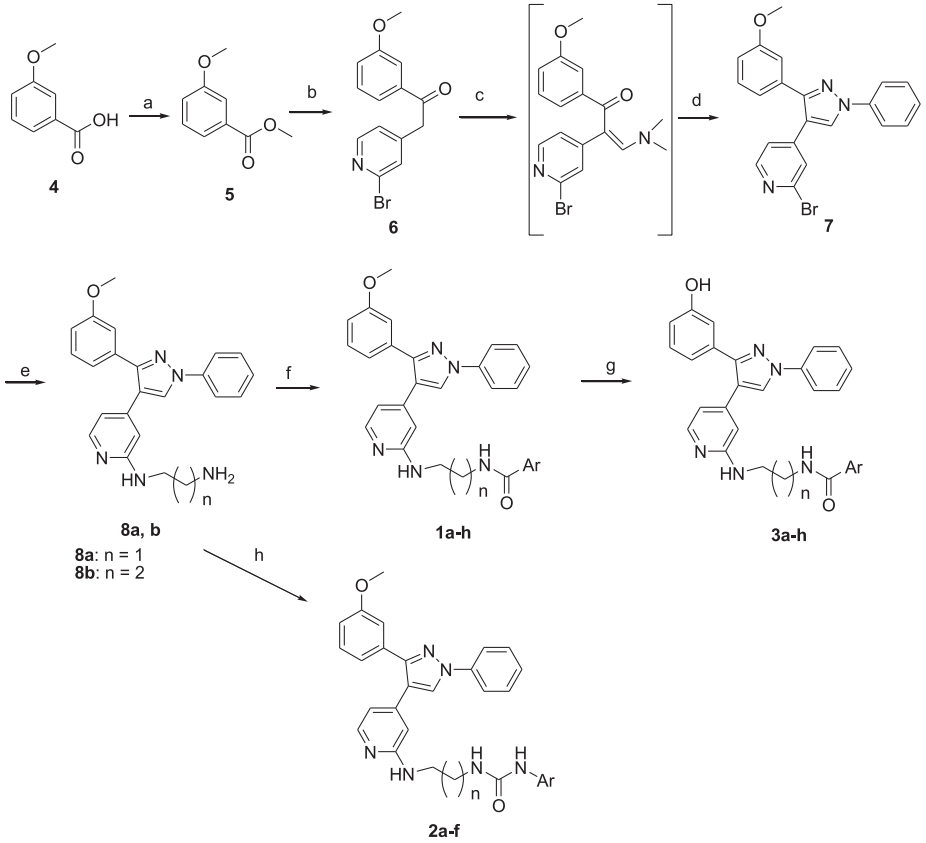
Fig. 1. Chemical structures of the lead compound **I**,[16] celecoxib, and the final target compounds.

the marketed pyrazole anti-inflammatory drug, celecoxib (Fig. 1). In addition, we have reported the *in vitro* anti-inflammatory effects of compound **I** that contains 1,3,4-triarylpyrazole scaffold (Fig. 1).[16] Herein, we decided to test the target compound **1a-h**, **2a-f**, and **3a-h** as inhibitors of NO and PGE_2 productions, and investigated the effect of tether attached to the pyridyl ring on activity. The most promising compounds were further considered at cellular and molecular levels for more understanding of their redundancy mechanisms of action.

Compounds **1a-h, 2a-f**, and **3a-h** were synthesized performing the route illustrated in Scheme 1. 3-methoxybenzoic acid (**4**) was refluxed in methanol with catalytic amount of sulfuric acid to give methyl benzoate ester **5**. The resulted ester was reacted with 2-bromo-4-methylpyridine using the strong base, lithium bis(trimethylsilyl)amide (LiHMDS) to get compound **6**.[17] The intermediate ketide compound 6 was then treated with dimethylformamide dimethylacetal (DMF-DMA), followed by phenylhydrazine to yield the cyclized 1,3,4-triarylpyrazole **7** possessing bromopyridyl moiety. Compound **7** was refluxed with ethylenediamine or 1,3-propylenediamine to get compounds **8a** and **8b**, respectively. The primary NH_2 group of **8a** or **8b** was reacted with the appropriate acid chloride and using triethylamine as a base to give the corresponding amide compounds **1a-h**. Compounds **2a-f** with urea linker were prepared through reacting compound **8a** or **8b** with the appropriate aryl isocyanate derivatives to afford the target aryl urea compounds **2a-f**. Regarding hydroxyl derivatives **3a-h**, they were synthesized by treating compounds **1a-h** with BBr_3 for demethylation of the methoxy group affording the corresponding compounds.[18] The *in vitro* anticancer activity of the target compounds were previously reported.[18] The final target compounds are illustrated in Table 1.

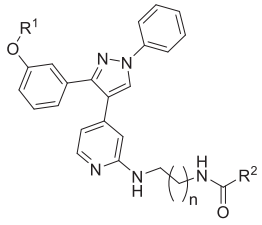
Before conducting the screening tests of NO and PGE_2 production inhibition, it was crucial to detect compounds' cytotoxicity to determine a safe and non-toxic concentration of each compound. After confirmation that 10 μM concentration of each of the target compounds is not cytotoxic against murine RAW 264.7 macrophages, the target compounds were tested at this single-dose concentration for inhibitory effect of various tested compounds on LPS-induced NO and PGE_2 production in murine RAW 264.7 macrophages. The results are summarized in Table 1.

It is noticeable that the targeted compounds are more active as



Scheme 1. Reagents and conditions: a) methanol, Conc. sulfuric acid, overnight, reflux; b) 2-bromo-4-pyridine, lithium bis(trimethylsilyl)amide, tetrahydrofuran, 6 h, −78 °C to rt; c) dimethylformamide dimethylacetal, 3 h, 80 °C; d) $PhNHNH_2$, ethanol, overnight, rt,; e) ethylenediamine or 1,3-propylenediamine, reflux,8h; f) appropriate acid chloride, Et_3N, dichloromethane, overnight, 0 °C; g) boron tribromide, dichloromethane, 3 h, −78 °C to rt; h) appropriate isocyanate, tetrahydrofuran, overnight, rt.

Table 1
Chemical structures of the final compounds **1a-h**, **2a-f**, **and 3a-h**, their effect on cell viability, and inhibitory effects on LPS-induced NO and PGE_2 production in murine RAW 264.7 macrophages.



Compound No.	R^1	R^2	n	% cell viability	NO % inhibition	PGE_2 production
1a	CH_3	C_6H_5	1	131.5	12.8 ± 0.53	36.2 ± 0.54
1b	CH_3	4-F-C_6H_4	1	136.1	15.4 ± 0.89	47.9 ± 0.65
1c	CH_3	3-CF_3-C_6H_4	1	124.7	19.1 ± 0.72	58.1 ± 0.75
1d	CH_3	4-OCH_3-C_6H_4	1	116.5	12.3 ± 0.53	59.6 ± 0.73
1e	CH_3	C_6H_5	2	117.8	18.7 ± 0.64	6.1 ± 0.33
1f	CH_3	4-F-C_6H_4	2	132.9	18.6 ± 0.44	28.9 ± 0.83
1g	CH_3	3-CF_3-C_6H_4	2	115.3	28.8 ± 0.43	73.8 ± 0.89
1h	CH_3	4-OCH_3-C_6H_4	2	112.1	48.8 ± 0.58	75.4 ± 0.74
2a	CH_3	C_6H_5-NH	1	142.6	12.2 ± 0.34	53.9 ± 0.29
2b	CH_3	3-CF_3-C_6H_4-NH	1	165.1	26.1 ± 0.39	69.9 ± 0.43
2c	CH_3	3,4-Cl_2-C_6H_4-NH	1	129.4	22.0 ± 0.45	76.1 ± 0.39
2d	CH_3	C_6H_5-NH	2	143.4	22.2 ± 0.56	56.3 ± 0.12
2e	CH_3	3-CF_3-C_6H_4-NH	2	123.6	31.3 ± 0.65	ND
2f	CH_3	3,4-Cl_2-C_6H_4-NH	2	120.7	38.6 ± 0.34	77.1 ± 0.47
3a	H	C_6H_5	1	104.8	12.2 ± 0.41	20.9 ± 0.36
3b	H	4-F- C_6H_4	1	127.4	20.4 ± 0.37	−1.9 ± 0.26
3c	H	3-CF_3-C_6H_4	1	135.3	18.6 ± 0.31	12.2 ± 0.24
3d	H	4-OH-C_6H_4	1	124.3	34.5 ± 0.35	−56.4 ± 0.23
3e	H	C_6H_5	2	145.1	2.1 ± 0.43	12.6 ± 0.31
3f	H	4-F-C_6H_4	2	120.3	32.6 ± 0.56	63.1 ± 0.35
3g	H	3-CF_3-C_6H_4	2	123.2	ND	11.6 ± 0.43
3h	H	4-OH-C_6H_4	2	126.5	39.7 ± 0.39	31.1 ± 0.89

[a] Values represent means ± S.D. of three independent experiments. Cells were pretreated with tested compounds (10 μM) over 1 h followed by LPS (1 μg/mL) over 24 h for stimulation. Both PGE_2 and NO production were quantified in the culture media applying an enzyme immunoassay (EIA) kit and the Griess assay. Added below data or explain contents. L-NIL (40 μM –31.5 ± 0.38 inhibition %) was used as positive controls for NO and NS-398 (10 nM-59.5 ± 0.96 inhibition %) was used for PGE_2 production.

Table 2
IC_{50} values of the most active compounds as inhibitors of PGE_2 production in LPS-induced murine RAW 264.7 macrophages.

Compound No.	IC_{50} (μM)
1g	5.62 ± 0.94
1h	**2.94 ± 0.66**
2b	5.11 ± 1.21
2c	4.51 ± 0.89
2f	**4.21 ± 0.75**
3f	7.06 ± 1.38

inhibitors of PGE_2 production rather than NO production. The most active compounds were **1g**, **1h**, **2c**, and **2f**. All of them possess 3-methoxyphenylpyrazole ring. The methoxy derivatives **1g** and **1h** are more active than the corresponding hydroxyl analogues **3g** and **3h**. Moreover, the propylene linker is generally more favorable for activity than ethylene. In case of urea derivatives, terminal 3,4-dichlorophenylurea moiety is the best for activity. The most active urea derivatives, **2c** and **2f** possess that moiety.

Based on the preliminary results illustrated in Table 1, the six compounds that exerted more than 60% inhibition of PGE_2 production (compounds **1g**, **1h**, **2b**, **2c**, **2f**, and **3f**) were tested in 4-dose mode to measure their IC_{50} values (Table 2). The most potent compounds are **1h** and **2f**. The inhibitory effects of compounds **1h** and **2f** on PGE_2 production are illustrated in Fig. 2. Compounds **1g**, **1h**, **2b**, **2c**, **2f**, and **3f** are more potent than the lead compound **I** (Fig. 1).[16] The lead compound **I** possesses unsubstituted pyridyl ring, so the tether attached to the pyridyl ring in compounds **1g**, **1h**, **2b**, **2c**, **2f**, and **3f** could contribute to stronger potency.

Furthermore, these most promising compounds **1h** and **2f** were selected for further investigation of their molecular mechanism of action. They were tested for inhibitory effects on COX-2 protein expressions in LPS-induced murine RAW 264.7 macrophages by western blotting (Fig. 3). Compounds **1h** and **2f** showed dose-dependent inhibition of the COX-2 expression, however, compound **1h** was more active. So, the PGE_2 inhibition could occur as a result of inhibition of COX-2 protein expression in a dose-dependent manner.

Compound **1h**, the most potent as PGE_2 production inhibitor, was further tested for its effect on COX-2 mRNA expression in LPS-induced murine RAW 264.7 macrophages (Fig. 4). Compound **1h** strongly inhibited the COX-2 mRNA expression in dose-dependent manner. Thus PGE_2 production inhibition could be a result of inhibition of both COX-2 mRNA expression as well as COX-2 protein expression.

In conclusion, our target triarylpyrazole derivatives were tested as inhibitors of LPS-induced NO and PGE_2 productions in murine RAW 264.7 macrophages. We could get several compounds act as potent inhibitors of PGE_2 production rather than inhibitors of NO release. Among them, we obtained two promising PGE_2 production inhibitors,

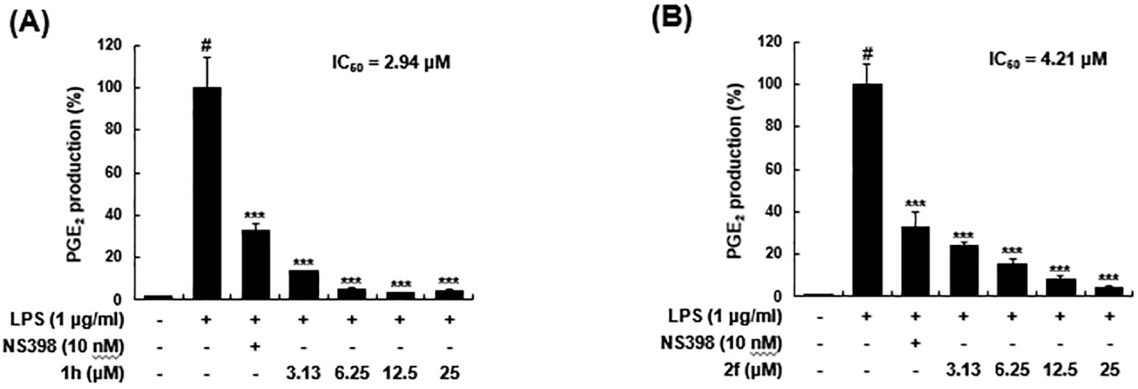


Fig. 2. Effects of compounds **1h** (Fig. 2A) and **2f** (Fig. 2B) on PGE_2 production in LPS-induced murine RAW264.7 macrophages following treatment with compound **1h** or **2f** (3.13–25 μM) for 1 h, cells were stimulated using LPS (1 μg/ml) over 24 h. The amount of PGE_2 in the cell supernatant was estimated using EIA kit. NS-398 was used as a positive control. $^{\#}P < 0.05$ *vs.* the control group;$^{***}P < 0.001$ *vs.* LPS-stimulated cells.

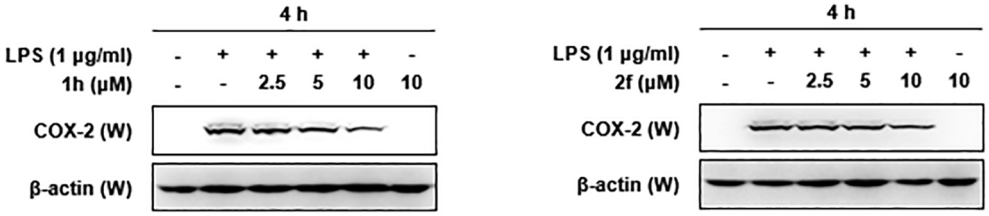


Fig. 3. Inhibitory effects of compounds **1h** and **2f** on LPS-induced COX-2 protein expression in murine RAW 264.7 macrophages. The cellular lysates were prepared from cells pretreated with/without compound **1h** or **2f** over 1 h and then stimulated with LPS (1 μg/mL) over 24 h. Total cellular proteins were resolved by SDS-PAGE, transferred to PVDF membranes, and detected with specific COX-2 antibodies using β-actin as an internal standard.

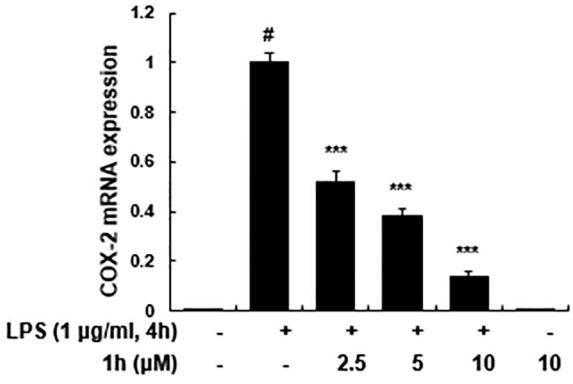


Fig. 4. Effect of compound **1h** on COX-2 mRNA expression in LPS-induced murine RAW 264.7 macrophages. The total RNA was prepared from cells pretreated with/without the compound **1h** over 1 h followed by LPS (1 μg/ml) over 4 h. The levels of COX-2 mRNA were measured by qRT-PCR. $^{\#}P < 0.05$ against the control group;$^{***}P < 0.001$ against LPS-stimulated cells.

1h and **2f** which exerted their effects through inhibition of COX-2 protein expression. The most potent compound, **1h**, demonstrated also dose-dependent inhibition of COX-2 mRNA expression. Compound **1h** possesses propylene spacer, methoxy group on the phenyl ring attached to position 3 of the pyrazole ring, and *p*-methoxybenzamido moieties. It seems that these moieties are favorable for the activity of this series of compounds. Compound **1h** can be a promising lead compound that exerts anti-inflammatory effect through dual inhibition of expression of both COX-2 protein and COX-2 mRNA.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2019.126884.

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