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Inhibitory Activities of New Series of 4, 5-diaryl Thiadiazoles Derivatives on Lipopolysaccharide-induced Cox-2 Expression

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Abstract: Recent studies have suggested cyclooxygenase-2 (COX-2) expression as a mechanism involved in carcinogenesis. It has also been suggested that changes in COX-2 expression level can be considered as a possible therapeutic target in tumors. Therefore, it was decided to synthesize a new series of 4, 5-diaryl thiadiazoles (compounds 1-5) as COX-2 inhibitors and evaluate their inhibitory activity on COX-2 expression. The COX-2 expression was induced by lipopolysaccharide (LPS) in bovine aortic endothelial (BAE-1) cells and the protein expression was evaluated by immunocytochemistry (ICC). The inhibitory activity of these compounds has been compared with celecoxib and rofecoxib as selective COX-2 inhibitors and indomethacin as a non-selective COX inhibitor. The cytotoxicity of these compounds was measured in different concentrations (10^{-3}-10^{3} μg mL^{-1}) by trypan blue dye exclusion method. The results showed that the cell viability following exposure to 10^{-2} μg mL^{-1} of each one of compounds 1-4 was about 90%. Among all tested compounds, compound 4 at 10^{-2} μg mL^{-1} showed significant inhibition on COX-2 expression determined by ICC. This effect of compound 4 was comparable to rofecoxib and celecoxib. It is concluded that, the new synthesized compounds and in particular, the compound 4 can be considered for further evaluation as new COX-2 inhibitors.

Key words: Cyclooxygenase-2 (COX-2), lipopolysaccharide (LPS), protein expression, 4, 5-diaryl thiadiazoles, cytotoxicity, immunocytochemistry (ICC)

INTRODUCTION

Cyclooxygenase (prostaglandin G/H synthase) acts as the first step in prostaglandins production. Two isoforms of the cyclooxygenase enzyme have been identified, cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). COX-1 is expressed constitutively in variety of tissues such as platelets, gastrointestinal mucosa and kidney[1]. However COX-2 is an inducible isoform, which its expression is induced by variety of compounds including cytokines, growth factors, lipopolysaccharide (LPSs) and tumor promoters primarily in leukocytes, brain and peripheral tissues[1-3]. Involvement of COX-2 in inflammation, hyperalgia, angiogenesis, neuromodulation, cancer and Alzheimer's disease, gives chance to modulate these conditions using selective COX-2 inhibitors[4].

Many of the currently available non-steroidal anti-inflammatory drugs (NSAIDs) inhibit both COX-1 and COX-2, with a preferential selectivity for COX-1 inhibition. The discovery and identification of COX-2 suggested that selective inhibition of COX-2 would provide a method to avoid the side effects associated with NSAIDs therapy[5]. Moreover recent studies have shown that COX-2 expression may be involved in carcinogenesis[3].

Following the discovery of selective COX-2 inhibitors such as NS-398 and Dup-697, many other selective COX-2 inhibitors have been introduced. All of these compounds can be structurally categorized in three classes: 1) acidic sulfonanilides such as NS-398, 2) tricyclic compounds such as Dup-697, celecoxib and rofecoxib and 3) modified classical NSAIDs such as indomethacin derivatives (Fig. 1)[5].

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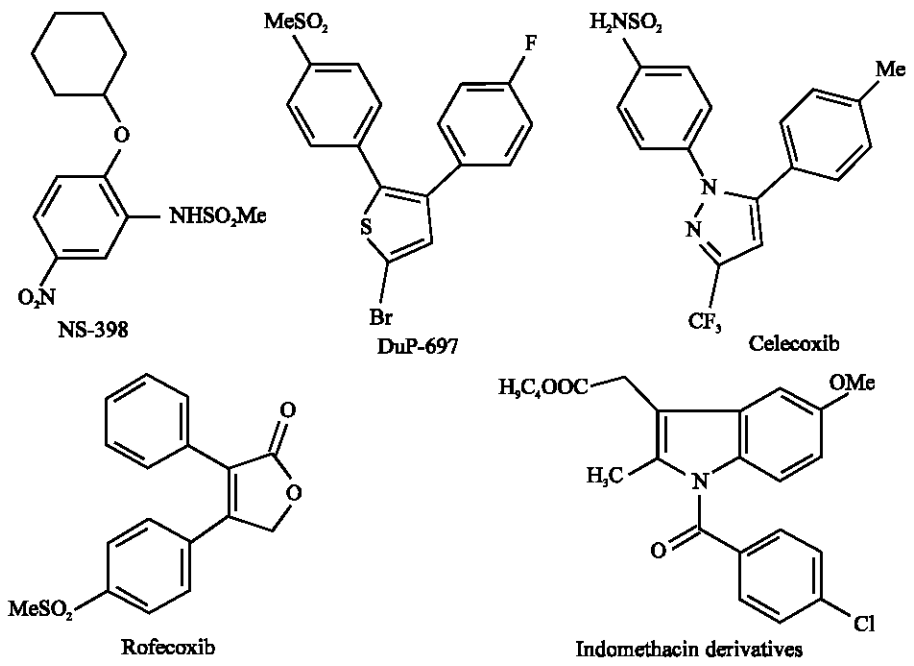
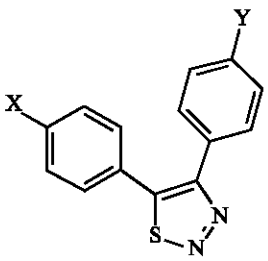


Fig. 1: Original selective COX-2 inhibitors

Table 1: Structure of compound 1-5



Compound	X	Y
1	H	SO_2Me
2	Cl	SO_2Me
3	F	SO_2Me
4	OCH_3	SO_2Me
5	F	SO_2NH_2

Among these groups, tricyclic compounds (class 2) showed an impressive activity with high COX-2 inhibition. Therefore, it is decided to synthesis a new group of 4, 5-diaryl thiadiazoles (compounds 1-5) based on tricyclic structure. The 4-(4-sulfonylmethyl-phenyl)-5-(phenyl)-[1,2,3] thiadiazole (Compound 1), 5-(4-chloro-phenyl)-4-(4-sulfonylmethyl-phenyl)-[1,2,3] thiadiazole (Compound 2), 5-(4-fluoro-phenyl)-4-(4-sulfonylmethyl-phenyl)-[1,2,3] thiadiazole (Compound 3), 4-(4-methansulfonyl phenyl)-5-(4-methoxy-phenyl)-[1,2,3] thiadiazole (Compound 4) and 4-[4-(4-fluorophenyl)-[1,2,3] thiadiazole-5-yl]-benzensulfonamide (Compound 5) were synthesized according to the classical procedures, described in heterocyclic chemistry literatures (Fig. 2, Table 1)[6].

In other study, similar structures with various 5-membered ring were synthesized and IC50s were measured against COX-1 and COX-2. Among these groups thiadiazole derivatives with structures similar to our compounds showed an impressive inhibitory effect on COX-2. They also showed acceptable bioavailability and ED_{50} in animal model[6].

It has also been reported that some of NSAIDs, in addition to direct inhibition of COX-2 enzyme could decrease the COX-2 expression in the cells[7]. In this study, we tried to evaluate the inhibitory activity of compounds 1-5 on LPS-induced COX-2 expression on BAE-1 cells[8] using immunocytochemistry (ICC) method. Also compared the results with known COX-2 inhibitors such as celecoxib and rofecoxib and non-selective COX inhibitors such as indomethacin. The cytotoxicity of these compounds on BAE-1 cells was also measured using trypan blue dye exclusion method to find non-toxic concentration of each compound.

MATERIALS AND METHODS

Chemicals: Celecoxib and rofecoxib were purchased from Sigma (St. Louis, USA) and indomethacin was purchased from Tolid Daru Co. (Tehran, Iran). 4, 5-diaryl thiadiazoles (Compounds 1-5) were synthesized in

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Cell culture: The bovine aortic endothelial (BAE-1) cell line was obtained from Iran Pasteur Institute. BAE-1 cells were grown at 37°C in 5% CO_2 in 25 cm^2 culture flask (Nunc, Denmark) in Dulbecco's Modified Eagle's Medium (DMEM; PAN, Germany) with 20% heat-inactivated fetal bovine serum (FBS; GibcoBRL, USA) and 1% antibiotics (Penicillin-Streptomycin, GibcoBRL, USA). At 90% confluence, cells were subcultured using 0.25% Trypsin-EDTA (GibcoBRL, USA).

Cytotoxicity assay: The cytotoxicity of these compounds was determined on the BAE-1 cells. The cells were cultured in DMEM containing 20% heat-inactivated FBS and 1% antibiotics (Penicillin-Streptomycin). Viable cells were counted using trypan blue dye exclusion method. BAE-1 cells were suspended in appropriate volumes of media and were seeded in 24-well plates at $5x10^4$ cells/well. Plates were incubated at 37°C, 5% CO_2 for 24 h. The stock solution of test compounds (1-5) was prepared in 70% ethanol at concentration of 1 μg mL^{-1}. The test compounds at six different concentrations ranging from 1 μg mL^{-1} to 1 ng mL^{-1} were added to wells of culture plates. Plates were then incubated for 72 h and viable cells were counted using trypan blue dye exclusion method. Each concentration was counted in triplicate wells and each compound was tested in three separate occasions. Celecoxib and rofecoxib as selective COX-2 inhibitors and indomethacin as non-selective COX inhibitor were used as control. Non-cytotoxic concentration of celecoxib, rofecoxib and indomethacin on BAE1 cells was determined experimentally and dilution was made to give final concentration of 1.52, 5.66 and 3.58 μg mL^{-1} in each well, respectively. The trypan blue stained cells were counted on an invert microscope[9].

Immunocytochemistry (ICC) of COX-2 expression: BAE-1 cells were plated in 4-well chamber slides at a density of $5x10^4$ cell cm^{-2}[10]. After 70% confluency, cells were exposed to LPS (500 pg mL^{-1} in PBS) for 24 h[8,11]. Following induction of COX-2 expression, plates were treated with each test compounds for 48 h. The final concentration was 10^{-2} μg mL^{-1} for compounds 1 to 4 and 10^{-4} μg mL^{-1} for compound 5. Control plates were treated with celecoxib, rofecoxib and indomethacin at concentrations of 1.52, 5.66 and 3.58 μg mL^{-1} for 48 h, respectively. At the end of incubation time, cells were rinsed in Phosphate-buffered saline (PBS), fixed in a mixture of acetic acid 2% and absolute ethanol 98% for 5 min, washed again with PBS and permeabilized in 0.2% Triton X-100 in 0.1 M PBS for 20 min. To block endogenous peroxidase activity, the cells were incubated with 1-3 drops of peroxidase block solution in PBS for 5 min. Cells were then blocked with 1-3 drops of serum block (5% normal goat serum) for 20 min, followed by 48 h incubation with primary antibody COX-2 (H-62; Rabbit polyclonal IgG, SC-7951, Santa Cruz, USA) at dilution of 1:80 (2.5 μg mL^{-1}) at 4°C. Then cells were washed with PBS, incubated with 1-3 drops of biotinylated secondary antibody for 30 min, washed with PBS and incubated with 1-3 drops of HRP-Streptavidin complex for 30 min. Antigen-antibody complex were then visualized using diaminobenzidine (DAB) chromogen substrate solution. Stained cells were counted in a minimum of five fields and ratio of COX-2 positive cells to total number per field was calculated at (X 200) magnification[12].

Cell viability: In this study 90% of viability was used in all the experiments. This was assessed using trypan blue dye exclusion method. Cells with round and intact nuclei with clear color were considered as normal and cells with blue cytoplasm were considered as damaged cells.

Statistical analysis: The results presented are the mean±SEM of at least 3 separate experiments. The amount of reduction in the staining of cells was examined by Student's independent t-test. $p \leq 0.05$ were considered statistically significant difference.

RESULTS

Cytotoxicity of test compounds: The cytotoxicity of the new series of 4, 5-diaryl thiadiazoles (compound 1-5) on BAE-1 cells and COX-2 expression were determined. The cytotoxicity of the compounds was determined using trypan blue dye exclusion method. Celecoxib and rofecoxib as selective COX-2 inhibitors and indomethacin as non-selective COX inhibitor were used as control at non-toxic concentrations of 1.52, 5.66 and 3.58 μg mL^{-1}, respectively. The effect of ethanol (1% v/v) as vehicle was also evaluated, which showed no cytotoxicity for these cells, similar to negative control. Based on the results, the non-toxic concentration for compounds 2 (X= Cl and Y= SO_2Me) and 5 (X= SO_2NH_2 and Y= F) were the same (10^{-2} μg mL^{-1}). For compound 3, in which hydrogen was replaced by fluorine, even at concentrations higher than 10^3 μg mL^{-1}, viability was over

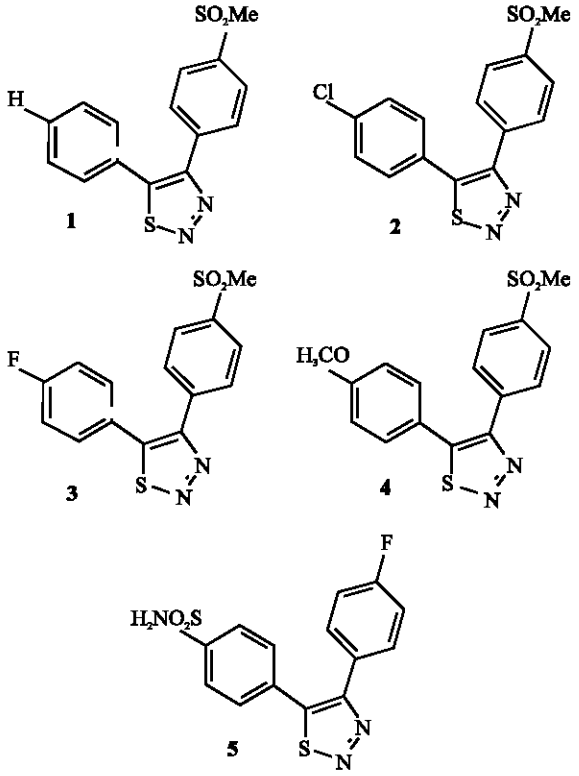

Fig. 2: Structures of compounds (1-5)

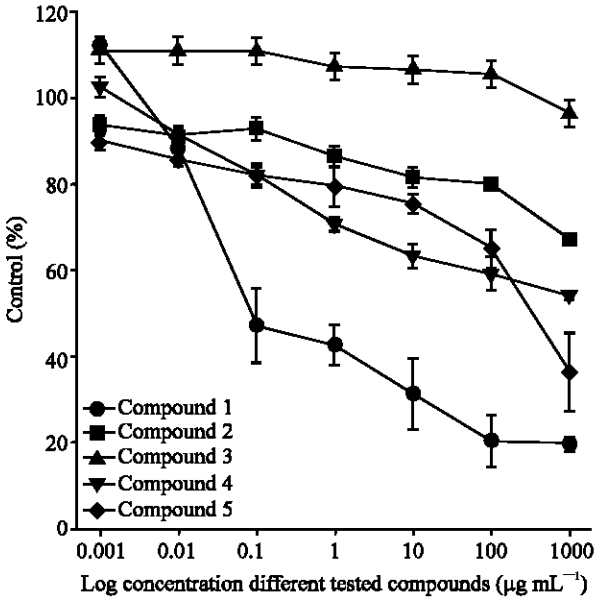

Fig. 3: Cytotoxicity assay of tested compounds on BAE-1cells with dye exclusion method. Each point represents Mean±SEM of 3 samples in 3 separate occasions. Note that except compound 3 all other compounds decrease viability above 1 µg mL^{-1}

90%. Compound 4 (X= OCH_3 and Y= SO_2Me) with concentration of 10^{-3}-10^{-2} µg mL^{-1} did not show any cytotoxicity. However, Compound 1 (X = H and Y= SO_2Me) showed more toxic effect than others and its non-toxic concentration was found to be below 10^{-3} µg mL^{-1} (Fig. 3).

COX-2 expression using immunocytochemistry method: BAE-1 cells incubated in medium without LPS were considered as negative control (Fig. 4a). Cells were incubated with LPS (500 ng mL^{-1}) for 24 h in order to induce expression of COX-2 and considered as positive control (Fig. 4b). Celecoxib (1.52 µg mL^{-1}), rofecoxib (5.66 µg mL^{-1}) and indomethacin (3.58 µg mL^{-1}) were added to designated wells and incubated for 48 h in cells pretreated with LPS (500 ng mL^{-1} for 24 h). Indomethacin, Celecoxib and rofecoxib decreased COX-2 expression compared to positive control (Fig. 4c-e).

In order to determine the inhibitory activity of test compounds on COX-2 expression, cells were treated with LPS (500 ng mL^{-1} for 24 h) followed by exposure to concentration of 10^{-2} µg mL^{-1} for compounds 2 to 5 and 10^{-4} µg mL^{-1} for compound 1 for 48 h. The level of staining was decreased for compound 1 (X= H and Y= SO_2Me) and compound 4 (X= OCH_3 and Y= SO_2Me) (Fig. 5a and d). In cells treated with compound 2 (X= Cl and Y= SO_2Me), 3 (X= F and Y= SO_2Me) and 5 (X=SO_2NH_2 and Y= F), there was no evidence of expression inhibition (Fig. 5 b, c and e). Inhibition of COX-2 expression by compound 1 was less than celecoxib and rofecoxib. Compound 2 did not show any inhibition. Compound 3 showed inhibitory activity less than indomethacin. Compound 4 showed good inhibitory activity as well as broader range of non-toxic concentrations compared to compound 1 (Fig. 6).

DISCUSSION

Previous studies have shown that LPS induces COX-2 expression in different cells[1-3,8-11]. There are increasing evidences that COX-2 is involved in pathogenesis of cancer and Alzheimer's disease[3,4]. Due to the vast effect of COX-2 inhibitors to prevent such diseases and to reduce the side effects of non-selective COX-1 inhibitors, numerous COX-2 selective inhibitors have been introduced[3-6]. The major known activity of these compounds is to inhibit enzyme in inflamed tissues. Although the major mechanism of action of these compounds is inhibition through their direct action on COX enzyme, some of known compounds showed extra feature of inhibition of COX-2 gene/protein expression[7].

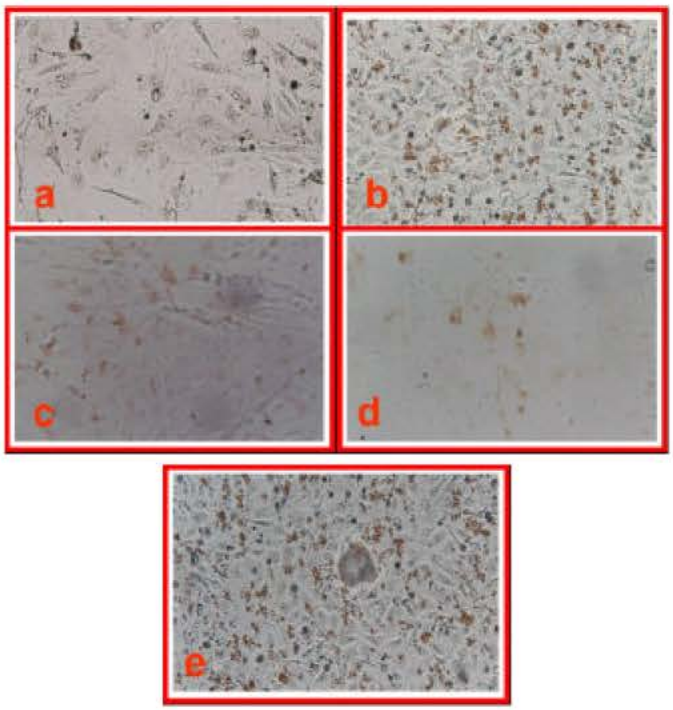

Fig. 4: Immunocytochemical staining of BAE-1 cells exposed to Celecoxib, Rofecoxib and Indomethacin. (a) Negative control, (b) positive control, (c) Celecoxib, (d) Rofecoxib, (e) Indomethacin (X200)

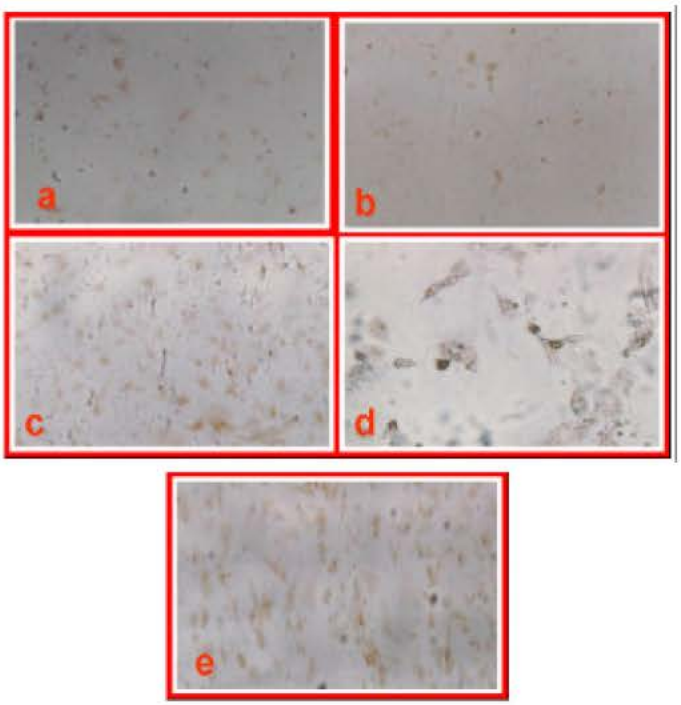

Fig. 5: Immunocytochemical staining of BAE-1 cells exposed to new synthesized compouds. (a) Compound 1, (b) Compound 2, (c) Compound 3, (d) Compound 4 and (e) Compound 5 (X200)

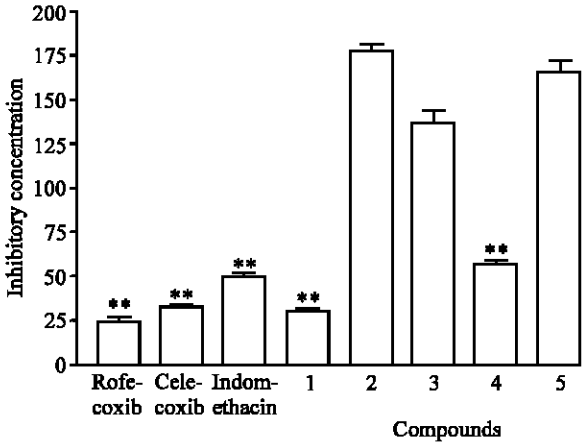

Fig. 6: Quantative data of COX-2 expression inhibition by controls and new synthesized compounds (1-5) using immunocytochemistry assay

This feature possibly shows extra activity of these compounds to reduce induction of COX-2 expression in inflamed cells. In this study a new series of 4, 5-diaryl thiadiazoles (compounds 1-5) has been synthesized to evaluate COX-2 expression inhibitory activity (Fig. 2). In cytotoxicity assay compound 1-4 shows 90% viability in nontoxic concentrations. On the other hand compound 1 and 4 show good COX-2 expression inhibition. Although compound 1 shows best activity, it is more cytotoxic than compound 4. The significant inhibitory activity of compound 1 on COX-2 expression may be due to mechanism other than COX-2 enzymatic inhibition such as general non-specific cytotoxicity and perhaps the effect is mediated through effect on transcription-translation mechanism, such as inhibition of protein production. According to the results compound 4 demonstrates the most powerful inhibition among the test compounds. Although compounds with sulfonylmethyl and sulfonamide structures have shown good COX-2 enzyme inhibition[5,6]. This study proposed that these structures could have suitable inhibition of COX-2 gene expression and can be introduced as inhibitors of COX-2 gene expression. Based on our results, compound 4 with sulfonylmethyl moiety has the best inhibitory activity.

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