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Original article

Synthesis and Effects of 4,5-Diaryl-2-(2-alkylthio-5-imidazolyl) Imidazoles as Selective Cyclooxygenase Inhibitors

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Abstract

Objective(s)
In recent years highly selective COX-2inhibitors were withdrawn from the market because of an increased risk of cardiovascular complications. In this study we were looking for potent compounds with moderate selectivity for cox-2. So, four analogues of 4, 5-diaryl-2-(2-alkylthio-5-imidazolyl) imidazole derivatives were synthesized and their anti-inflammatory and anti-nociceptive activities were evaluated on male BALB/c mice (25-30 g). Molecular modeling and *in vitro* COX-1 and COX-2 isozyme inhibition studies were also performed.

Materials and Methods
2-(2-Alkylthio-5-imidazolyl)-4,5-diphenylimidazole compounds were obtained by the reaction of benzyl with 2-alkylthio-1-benzylimidazole-5-carbaldehyde, in the presence of ammonium acetate. Spectroscopic data and elemental analysis of compounds were obtained and their structures elucidated. Anti-nociception effects were examined using writhing test in mice. The effect of the analogues (7.5, 30, 52.5 and 75 mg/kg) against acute inflammation were studied using xylene-induced ear edema test in mice. Celecoxib (75 mg/kg) was used as positive control.

Results
All four analogues exhibited anti-nociceptive activity against acetic acid induced writhing, but did not show significant analgesic effect ($P< 0.05$) compared with celecoxib. It was shown that analogues injected 30 min before xylene application reduced the weight of edematic ears. All analogues were found to have less selectivity for COX-2 in comparison to celecoxib.

Conclusion
Injected doses of synthesised analogues possesses favorite anti-nociceptive effect and also has anti-inflammatory effects, but comparing with celecoxib this effect is not significantly different. On the other hand selectivity index for analogues is less than celecoxib and so we expect less cardiovascular side effects for these compounds.

Keywords: Analgesic, Anti-inflammatory, Imidazoles

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Introduction

The use of nonsteroidal anti-inflammatory drugs (NSAIDs) for the treatment of inflammation and pain is often accompanied by adverse gastrointestinal and renal side effects. Their anti-inflammatory activity results from inhibition of cyclooxygenases (COXs), which catalyzes the bioconversion of arachidonic acid to prostaglandins. However, inhibition of COXs may lead to undesirable side effects. Nowadays, it is well established that there are at least two COX isozymes, COX-1 and COX-2 (1, 2). The constitutive COX-1 isozyme is produced in a variety of tissues and appears to be important to the maintenance of physiological functions such as gastroprotection and vascular homeostasis (3). Alternatively, the COX-2 isozyme is induced by mitogenic and proinflammatory stimuli linking its involvement to inflammatory processes (4). Thus, selective inhibition of COX-2 over COX-1 is useful for the treatment of inflammation and inflammation-associated disorders with reduced gastrointestinal toxicities when compared with NSAIDs. In addition to role of COX-2 in rheumatoid arthritis and osteoarthritis, it is also implicated in colon cancer and angiogenesis (5, 6). Recent studies have shown that the progression of Alzheimer's disease is reduced among some users of NSAIDs. Chronic treatment with selective COX-2 inhibitors may therefore slow the progress of Alzheimer's disease without causing gastrointestinal damage (7). So more selective COX-2 inhibitors were developed as a new generation of NSAIDs with diminished GI side effects. However, highly selective rofecoxib and valdecoxib were withdrawn from the market because of an increased risk of cardiovascular complications. COX-2 mediates the biosynthesis of prostacyclin, a vasodilator and inhibitor of platelet aggregation, the inhibition of prostacyclin production by selective COX-2 inhibitors might account for their adverse cardiovascular effects (8, 9). So we are looking for compounds with moderate selectivity for cox-2 to diminish possible cardiovascular side effects.

Diarylheterocycles, and other central ring pharmacophore templates, have been extensively studied as selective COX-2 inhibitors. All these tricyclic molecules possess 1,2 diaryl substitution on a central hetero- or carbocyclic ring system (see structures 1–5 in Figure 1) (10-14). Synthesis and analgesic activities of tetracyclic systems, 2-aryl-4,5-diphenylimidazole derivatives (see structures 6 in Figure 1), has also been reported previously (15).

As part of our program to design new types of tetracyclic moderate selective COX-2 inhibitors, we now report synthesis, anti-inflammatory, and anti-nociceptive activity of a group of 4,5-diaryl-2-(2-alkylthio-5-imidazolyl) imidazole derivatives. The target 4,5-diaryl-2-(2-alkylthio-5-imidazolyl) imidazole derivatives [8a-d] were synthesized via the route outlined in Figure 2. Accordingly, an appropriate imidazole carbaldehyde [7a-d] was treated with benzil in the presence of ammonium acetate to give the target 4,5-diaryl-2-(2-alkylthio-5-imidazolyl) imidazole derivatives [8a-d].

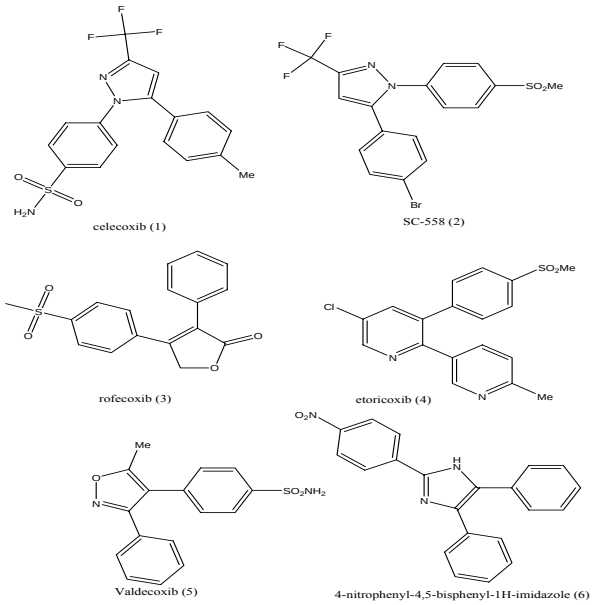

Figure 1. Representative examples of COX-2inhibitors.

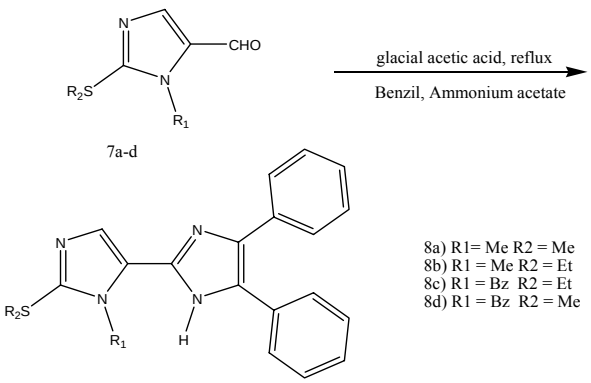

Figure 2. Synthesis of compounds 8a-d from appropriate imidazole aldehyde 7a-d.

Materials and Methods

Chemical methods

Melting points were determined on Electrothermal Capillary apparatus and are uncorrected. The IR spectra were obtained using a Perkin-Elmer Model 1000. ^{1}H NMR were obtained on Bruker Ac-80 spectrophotometer and chemical shifts (δ) are in ppm relative to internal tetramethylsilane. C, H, N analyses were within ±0.4% of theoretical values. Compounds 7a-d were prepared as described previously (16).

General procedure for preparation of 4,5-diaryl-2-(2-alkylthio-5-imidazolyl) imidazoles (8a-d).

Benzil (0.01 mol) and imidazole-5-carbaldehyde [7] were reacted with ammonium acetate (0.08 mol) in glacial acetic acid and refluxed for 6 hr.

4,5-Diphenyl-2-(1-methyl-2-methylthio-5-imidazolyl) imidazole [*8a*].

This compound was obtained in 66% (71.5%) yield; IR (KBr): 3050 cm^{-1} (NH); ^{1}H NMR ($CDCl_3$): 8.43 (bs,1H, NH), 7.7-7.6 (m, 4H, arom), 7.45-7.3 (m,7H, arom), 4.25 (s,3H, NCH_3), 2.50 (s,3H, CH_3S); Anal. Calcd. for $C_{20}H_{18}N_4S$: C, 69.34; H, 5.24; N, 16.17. Found: C, 68.33; H, 5.31; N, 15.97.

4,5-Diphenyl-2-(1-methyl-2-ethylthio-5-imidazolyl) imidazole [8b].

This compound was obtained in 56% (63%) yield; mp 189-190 ^{o}C; IR (KBr): 3050 cm^{-1} (NH); ^{1}H NMR ($CDCl_3$): 8.43 (bs,1H, NH), 7.7-7.6 (m,4H,arom), 7.45-7.3 (m,7H,arom), 4.25 (s,3H, NCH_3), 3.50 (q, 2H, CH_2S), 1.46 (t, 3H,CH_3); Anal. Calcd. For $C_{21}H_{20}N_4S$: C, 69.97; H, 5.59; N, 15.54. Found: C, 70.11; H, 5.41; N, 15.34.

4,5-Diphenyl-2-(1-benzyl-2-ethylthio-5-imidazolyl) imidazole [8c].

This compound was obtained in 66% (79%) yield; mp 91.5-92.9 ^{o}C; IR (KBr): 3050 cm^{-1} (NH); ($CHCl_3$): 3100 (NH), 1650 cm^{-1} (CO); ^{1}H NMR ($CDCl_3$): 8.43 (bs,1H,NH), 7.5-6.8 (m, 16H, arom), 5.6 (s, 2H, CH_2N), 3.50 (q, 2H, CH_2S), 1.46 (t, 3H,CH_3): Anal. Calcd. For $C_{27}H_{24}N_4S$: C, 74.28; H, 5.54; N, 12.83. Found: C, 74.12; H, 5.39; N, 12.53.

4,5-Diphenyl-2-(1-benzyl-2-methylthio-5-imidazolyl) imidazole [8d]

This compound was obtained in 53% yield; mp 185-186 ^{o}C; IR ($CHCl_3$): 3100 cm^{-1} (N-H); 1H NMR ($CDCl_3$): 8.43 (bs, 1H, NH), 7.5-6.8 (m, 16H, arom), 5.6 (s, 2H, CH_2N), 2.50 (s, 3H, CH_3S); Anal. Calcd. For $C_{26}H_{22}N_4S$: C, 73.90; H, 5.25; N, 13.26. Found: C, 73.73; H, 5.14; N, 13.55.

Modeling and docking studies

Similarity search of COX-2 against PDB bank using BLAST program in EXPASY server was turned up with many results in which PGH-2 of mice encoded as Q05769 having 604 amino acids was chosen as the best candidate for docking study due to quite high identity, 86%. In such case we did not need to model the human enzyme and the mice one was used directly instead.

The ligand molecules were constructed using Chem 3D 2003 and Hyperchem 6 and were energy minimized for 1000 iterations reaching a convergence of 0.01 kcal/mol and finally saved as PDB files for further docking procedure. Meanwhile, the protein PDB file was downloaded from RCSB site as code: 1PXX followed by water molecules removal using viewerlite program. The final corrected PDB file of the protein and designed ligands were submitted to AutoDock tools to run doking process. Furthermore, a selective COX-2 inhibitor named SC-588 which has been co-crystalized with murine COX-2 was obtained from RCSB site.

The purpose of docking is to search for favorable binding configuration between the small flexible ligands and the rigid protein. Docking studies were performed using Autodock software Version 3.0 (17). Searching was conducted within a specified 3D docking box using annealing based on the Monte Carlo method and MMFF94 molecular mechanics force field for 8000 iterations. The size of the box was set 40 angstrom in all aspects. Essential pdbqs, gpf and dpf files of both protein and ligands were produced in

AutoDock tools while further docking was performed applying Autodock3 using related commands in Linux. Docked structures looked very similar to the minimized structures obtained initially and the quality of the docked structures was evaluated by measuring the intermolecular energy of the ligand-enzyme assembly. The results will be discussed later in this article.

Biological evaluation

In vitro cyclooxygenase (COX) inhibition assay

The ability of the test compounds 8a-d to inhibit ovine COX-1 and COX-2 was determined using a colorimetric COX (ovine) inhibitor screening assay which utilizes the peroxidase component of cyclooxygenase. The peroxidase activity is assayed colorimetrically by monitoring the appearance of oxidized N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) at 590 nm.

Pharmacological methods

The experiments were performed under the Animals (scientific procedures) Act of 1986 and conform to the National Institutes of Health guidelines for the use of experimental animals. This study was carried out on male BALB/c mice (25-30 g) from Razi Institute, Mashhad, Iran. Animals were housed in plastic cages in an animal room maintained at 21±2 °C on a 12 hr dark cycle and supplied with food and water ad libitum.

The maximum non- fatal doses

Different doses of compounds were dissolved in normal saline containing 0.5% CMC and injected intraperitoneally (i.p.) into groups of six mice and the number of deaths was counted at 48 hr after treatment.

Writhing test

Anti-nociceptive effects of compounds were examined using writhing test in mice. One hour after administration of the compounds (7.5, 30, 52.5 and 75 mg/kg) and celecoxib (75 mg/kg) to the male BALB/c mice weighing 25–30 g, the mice were given an intraperitoneal injection of 0.7% (v/v) acetic acid solution (volume of injection 0.1 ml/10 g). The number of writhes produced in these animals was counted for 30 min (18). Celecoxib was used as positive and normal saline as negative control.

Xylene-induced ear edema

The effect of the analogues against acute inflammation was studied using xylene-induced ear edema test in mice. Thirty minutes after i.p. injection of the compounds (7.5, 30, 52.5 and 75 mg/kg) and celecoxib (75 mg/kg), 0.03 ml of xylene was applied to the anterior and posterior surfaces of the right ear. The left ear was considered as control. Two hours after xylene application the mice were sacrificed and both ears were removed. Circular sections were taken using a cork borer with a diameter of 7 mm, and weighed. The increase in weight caused by the irritant was measured by subtracting the weight of the untreated left ear section from that of the treated right ear sections (18).

Statistical analysis

The data were expressed as ED_{50} of compounds and tested with analysis of variance followed by the multiple comparison test of Tukey–Kramer.

Results

In vitro cyclooxygenase (COX) inhibition assay of test compounds 8a-d showed that none of compounds to be more selective than celecoxib. Selectivity index for test compounds was found to be between 1.66-1.75 and that of celecoxib was 3.22 (Table 1).

Table 1. *In vitro* COX-1 and COX-2 enzyme inhibition data.

compound	IC_{50}[a](nM)		COX-2 SI[b]
	COX-1	COX-2	
8a	339.3	195	1.74
8b	350	200	1.75
8c	280.5	170	1.65
8d	290.5	175	1.66
celecoxib	483	150	3.22

[a] Values are mean values of two determinations acquired using an ovine COX-1/COX-2 assay kit, where the deviation from the mean is <10% of the mean value.
[b] *In vitro* COX-2 selectivity index (COX-1 IC_{50}/COX-2 IC_{50}).

The maximum non fatal doses of compounds were 75 mg/kg. Injection of four doses of analogues (7.5, 30, 52.5 and 75 mg/kg) in mice showed analgesic effect in writhing test. Celecoxib (75 mg/kg) was used as positive control. All four analogues exhibited anti nociceptive activity against acetic acid induced writhing, but did not show significant (P> 0.05) analgesic effect compared with celecoxib. Values of ED_{50} of compounds and positive control in writhing test have been shown in Table 2.

In xylene-induced ear edema test, all four analogues exhibited anti-inflammatory activity but did not show significant effect compared with celecoxib (P> 0.05). Values of ED_{50} of compounds and positive control in xylene-induced ear edema test have been shown in Table 3.

Four designed structures were docked into the active site of murine COX-2 as well as celecoxib, indometacin, diclofenac and sc558 where the last one was directly derived from an X-Ray crystallography file. As seen in Figure 3 all mentioned structures fill the same cavity of the enzyme and show more or less the same size.

Table 2. Values of ED_{50} of compounds in writhing test.

Compound	ED_{50} (mg/kg)	Statistics (comparison with celecoxib)
8a	56.82±0.95	n.s.
8b	51.26±0.13	n.s.
8c	40.06±0.60	n.s.
8d	42.91±0.62	n.s.
Celecoxib	10.2±0.21	*

n.s. P> 0.05; * P< 0.05

Table 3. Values of ED_{50} of compounds in xylene-induced ear edema test.

Compound	ED50 (mg/kg)	Statistics (comparison with celecoxib)
8a	121.66±0.82	n.s.
8b	111.22±0.40	n.s.
8c	87.74±0.47	n.s.
8d	96.89±0.59	n.s.
Celecoxib	11.54±0.56	*

n.s. P> 0.05; * P< 0.05

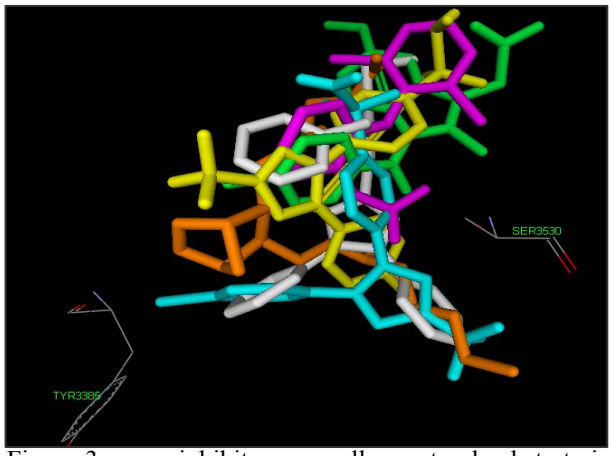

Figure 3. some inhibitors as well as natural substrate in the active site of murine COX-2. Indometacine= green. Diclofenac= pink. Celecoxib= Yellow. Sc558= Blue. An inhibitor of ours= white. Prostaglandin= Orange.

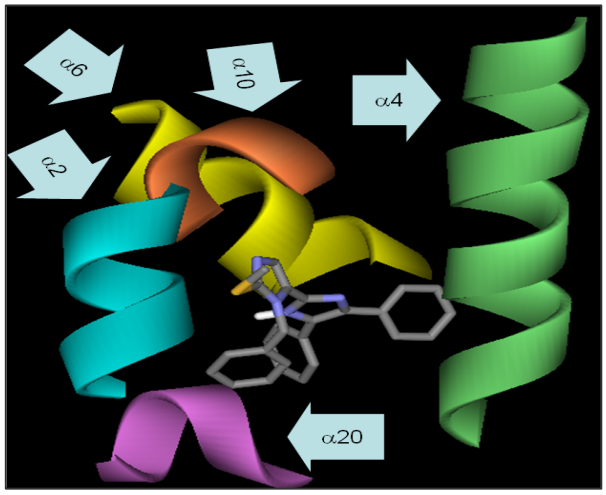

Figure 4. Compound 8d docked in the active site of murine COX-2 isozyme. Five helices have made the active site.

Two important residues, TYR385 and SER530 are highlighted in Figure 1. Changing these residues will lead to loss of complete action of the enzyme (19). Five helices surround the ligands in the active site. This has been clearly indicated in Figure 4.

Investigating the docking results clears that no hydrogen binding is involved in ligand-protein interaction while hydrophobe-hydrophobe interactions instead is clearly seen. Ligplot diagrams (20) confirm this conclusion where bulky amino acids, TRP, TYR and PHE surround the ligands in the active site (Figure 5). Another interesting result is consideration of a big hydrophobic pocket at the distant terminal of helix 6 where accommodates bulky substitutions on ligands such as bis-phenyl and naphthalene.

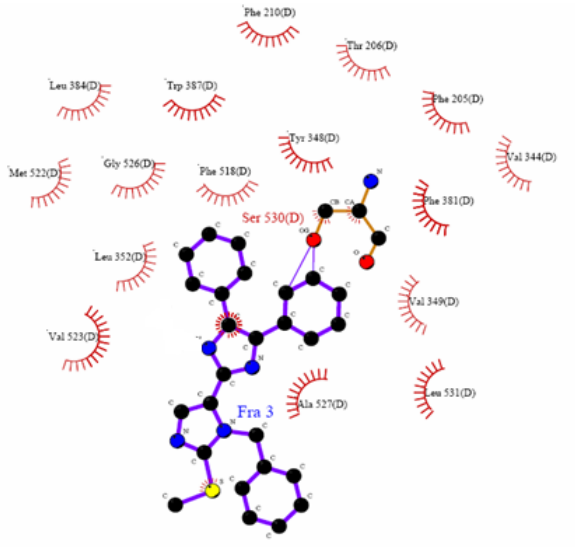

Figure 5. The ligplot diagram of compound 8d in the active site. Many hyrophobe and bulky residues have surrounded the ligand. No hydrogen binding is being seen.

Discussion

In vitro biological evaluations showed that the synthesized compounds exhibited moderate selectivity, less than that of celecoxib, but their IC_{50} for inhibition of COX-2 was not significantly different from that of celecoxib (Table 1). All the synthesized compounds were docked in the active site of murine COX-2 isozyme and their mode of interaction compared with that of known COX-2 inhibitors like celecoxib (Figure 3). Ligplot diagrams showed, in contrast to celecoxib no hydrogen binding was involved in ligand-protein interaction while hydrophobe-hydrophobe interactions instead was clearly seen (Figure 5). It may be the reason for less selectivity of our compounds in comparison to celecoxib. Although selective COX-2 inhibitors had a significantly lower incidence of gastrointestinal adverse effects, but increasing selectivity also increases the potential for adverse cardiovascular thrombotic events by tipping the balance of prostacyclin/thromboxane in favor of thromboxane, a prethrombotic eicosanoid (8), so our compounds with moderate selectivity may have less cardiovascular side effects (9).

In vivo comparison of the anti-nociceptive and anti-inflammatory activity of compounds (Table 2 and 3) also had no significant difference with celecoxib. These results were in agreement with *in vitro* results. But it was found that compounds having benzyl substituent on imidazole ring (8c, d) were more potent than those having methyl substituent [8a, b]. *In vitro* results showed the same order. Investigation of docking results showed the presence of a big hydrophobic pocket at the distant terminal of helix 6, where may accommodates bulky substitutions on ligands such as benzyl substituent (Figure 4). This may describe the higher potency of compounds (8c, d) with benzyl substituent.

Conclusion

It is concluded that injected doses of synthesised analogues possess favourite anti-nociceptive effect and also have anti-inflammatory effect, but comparing with celecoxib this effect is not significantly different. On the other hand selectivity index for analogues is less than celecoxib and so we expect less cardiovascular side effects for these compounds.

Acknowledgment

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