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Design, synthesis, and biological evaluation of substituted 2-alkylthio-1,5-diarylimidazoles as selective COX-2 inhibitors

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Abstract—A new type of 1-aryl-5-(4-methylsulfonylphenyl)imidazoles, possessing C-2 alkylthio (SMe or SEt) substituents, were designed and synthesized for evaluation as selective cyclooxygenase-2 (COX-2) inhibitors with in vivo anti-inflammatory activity. The compound, 1-(4-bromophenyl)-5-(4-methylsulfonylphenyl)-2-methylthioimidazole (**11g**), was the most potent and selective COX-2 inhibitor (COX-2 $IC_{50} = 0.43$ μM with no inhibition of COX-1 up to 25 μM) relative to the reference drug celecoxib (COX-2 $IC_{50} = 0.21$ μM with no inhibition of COX-1 up to 25 μM) and also showed very good anti-inflammatory activity compared to celecoxib in carrageenan-induced rat paw edema assay.

1. Introduction

The differential tissue distribution of cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) provides a rationale for the development of selective COX-2 inhibitors as anti-inflammatory-analgesic agents that lack the GI side effects exhibited by traditional nonsteroidal anti-inflammatory drugs (NSAIDs).[1–4] However, emerging evidence suggests that adverse reactions such as GI irritations or ulceration and renal liabilities are associated with prolonged use of COX-2 selective inhibitors. The adverse reactions have been attributed, at least in part, to COX-1 inhibition occurring with long-term exposure or at higher doses.[5] Furthermore, a precautionary concern regarding the use of COX-2 inhibitors in patients at risk for an adverse cardiovascular event such as myocardial infarction has been raised. For example, the clinical use of rofecoxib and valdecoxib was recently terminated due to adverse cardiovascular side effects associated with their use. One plausible explanation for this increased incidence of a prothrombic episode is attributed to a lower level of the vasodilator and platelet aggregation inhibitor prostacyclin (PGI_2) in conjunction with a higher level of the platelet activator and aggregator thromboxane A_2 (TxA_2).[6] Accordingly, there is still a need for the design of COX-2 inhibitors with a greater safety profile for the treatment of arthritis.

To achieve good activity and selectivity, the diarylheterocycle COX-2 selective inhibitors require the presence of a 4-methylsulfonylphenyl group attached to an unsaturated (generally five-membered) ring in which an additional vicinal lipophilic moiety is present (Fig. 1).[7–10] The methylsulfonyl group can be replaced by a SO_2NH_2 group, whereas the lipophilic pocket is usually occupied by an optionally substituted phenyl ring or a bulky alkoxy substituent. The nature of the central scaffold is crucial for the activity, although in many instances it does not establish well-defined electrostatic interactions with any of the amino acid residues, as shown either in the crystal structure of COX-2 inhibitor complexes or in different calculations.[11] Presumably, the highly lipophilic active site requires a given (low) polarity of the central scaffold.

Keywords: Cyclooxygenase-2 (COX-2) inhibitor; 1,5-Diarylimidazoles; Alkylthio; Celecoxib.
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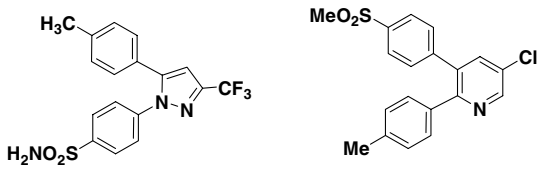


1 (Celecoxib) 2 (Etoricoxib)

Figure 1. Representative examples of selective tricyclic COX-2 inhibitors.

Regioisomeric imidazoles have been explored previously as replacements of the central heterocyclic core. The 4,5-diarylimidazoles **3** showed diminished potency versus **1**,[12] while the 1,2-diarylimidazoles **4** were shown to be potent COX-2 selective inhibitors only when a trifluoromethyl group was present in the 4-position.[13] 1,5-Diarylimidazoles in which the 4-sulfonylphenyl moiety is attached in a 1,3-relationship relative to the halo or methyl substituent of the imidazole **5**–**6** showed remarkable potency and selectivity.[14] Also, different 4,5-di (halo, alkyl or alkoxy substituted phenyl)-2-thioimidazole derivatives **7** exhibited anti-inflammatory activity.[15] Furthermore, novel classes of 6-alkylthio-substituted six-membered lactone (pyrane-2-one) rings **8** and 3-alkylthio-substituted 1,2,4-triazoles have been designed and exhibited very good in vitro COX-2 inhibitory potency and selectivity.[16,17] Considering the above results and as a part of our ongoing program to design novel selective COX-2 inhibitors, we describe herein the structure–activity relationship (SAR) of new 1,5-diarylimidazoles **11** (Fig. 2), in which a defined alkylthio substituent in position 2 confers appropriate polarity and charge distribution for good activity.

2. Chemistry

The synthetic reactions used for the synthesis of 2-alkylthio-5-(4-methylsulfonylphenyl)-1-phenylimidazoles (**11a**–**j**) are outlined in Scheme 1.

Starting from 4-methylsulfonylphenacylamine hydrochloride **9**, title compounds **11a**–**j** were prepared. Reaction of **9** with appropriate aryl isothiocyanate in the presence of Et_3N and subsequent cyclization, followed by alkylation in basic media using alkyl iodide, afforded 2-alkylthio-5-(4-methylsulfonylphenyl)-1-phenylimidazoles (**11a**–**j**) in good yields.[18]

3. Results and discussion

The effect of alkylthio substituents on central five-membered imidazole ring on COX-2 selectivity and potency was determined (Table 1).[19]

In vitro enzyme inhibition studies for **11a**–**j** exhibited no inhibition of COX-1 up to 25 μM and good COX-2 inhibition. In general, for these compounds, COX-2 selectivity and potency was dependent upon steric properties of C-2 alkylthio substituent on the central imidazole ring and electronic properties of C-5 phenyl ring substituent at *para* position.

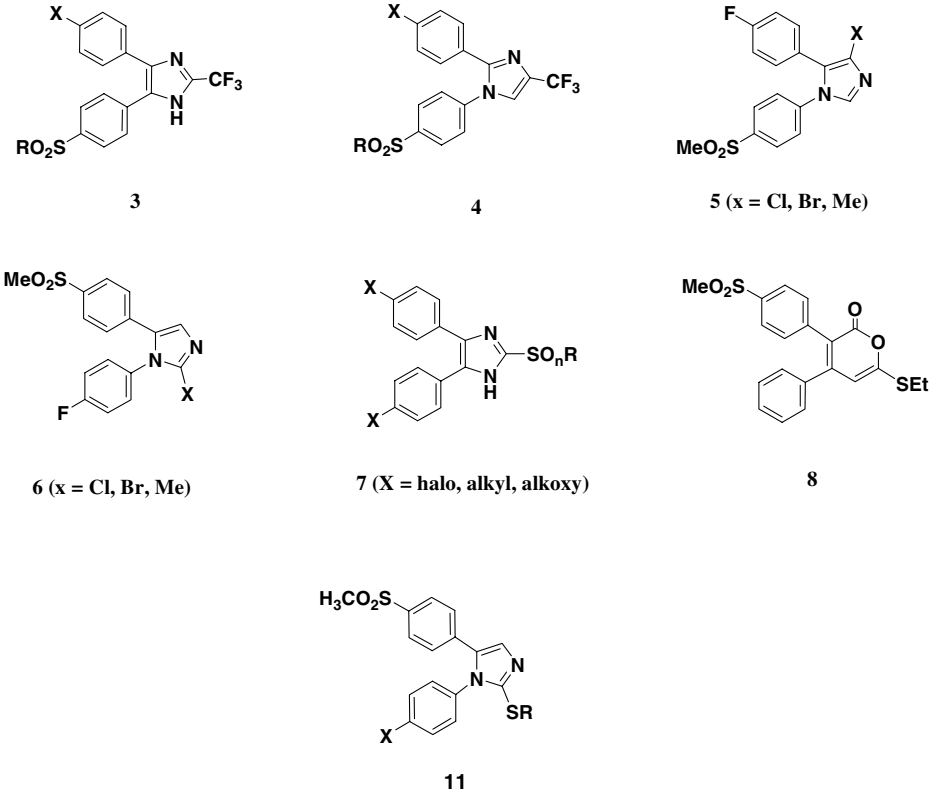
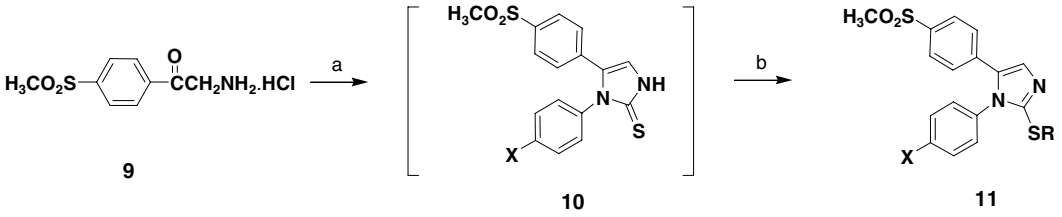


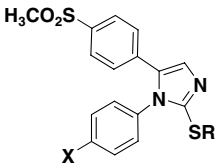
Figure 2. Representative examples of some imidazoles and pyran-2-one as COX-2 inhibitors.



X: H, F, Cl, Br, OMe
R: Me, Et

Scheme 1. Reagents and conditions: (a) Ar—N=C=S, Et_3N, EtOH, reflux, 4 h; (b) RI, KOH, EtOH, reflux, 3 h.

Table 1. IC_{50} values and anti-inflammatory activities for **11a–j**



Compound	X	R	IC_{50} (μM)		AI activity	
			COX-1[a]	COX-2[a]	% Inhibition at 3 h [d]	% Inhibition at 5 h[d]
11a	H	CH_3	>25[b]	0.58	74.12 ± 5.8	61.6 ± 2.5
11b	H	CH_2CH_3	>25[b]	0.58	89.68 ± 5.2	51.11 ± 7.2
11c	F	CH_3	>25[b]	0.52	40.0 ± 12.4	18.7 ± 5.9
11d	F	CH_2CH_3	>25[c]	0.50	65.0 ± 2.5	43.0 ± 2.3
11e	Cl	CH_3	>25[b]	0.47	59.47 ± 7.1	42.7 ± 11.1
11f	Cl	CH_2CH_3	>25[b]	0.82	53.8 ± 9.2	52.7 ± 7.7
11g	Br	CH_3	>25[b]	0.43	90.67 ± 2.6	88.0 ± 2.2
11h	Br	CH_2CH_3	>25[b]	>4	66.0 ± 5.9	59.0 ± 9.5
11i	OMe	CH_3	>25[b]	2.61	41.32 ± 6.5	34.3 ± 4.9
11j	OMe	CH_2CH_3	>25[b]	0.51	n.t.[e]	n.t.[e]
Celecoxib			>25[b]	0.21	70.53 ± 4.7	50.0 ± 2.5

[a] Values are means of two determinations and deviation from the mean is <10% of the mean value.
[b] They showed no inhibition of COX-1 up to 25 μM.
[c] Compound **11d** showed 21% inhibition of COX-1 at 0.5 μM and only 29% inhibition of COX-1 at 25 μM.
[d] Inhibitory activity on carrageenan-induced rat paw edema. The results are expressed as means ± SEM (n = 4–6) following a 50 mg/kg oral dose of the test compound.
[e] n.t., not tested.

In the presence of C-4 phenyl chloro or bromo substituent (**11e–h**), the size of C-2 alkylthio substituent had effect on COX-2 inhibition. Decreasing the size led to an increase in COX-2 potency. In the presence of smaller C-4 substituents (H and F) **11a–d**, increasing the size of alkylthio did not affect the COX-2 inhibitory potency. In C-4 methoxy substituted compounds **11i** and **11j**, increasing the size of alkylthio substituent improved COX-2 inhibition.

In addition, among different substituents on MeS substituted derivatives (**11a**, **11c**, **11e**, **11g**, and **11i**), lipophilic substituents (**11e** and **11g**) were slightly preferred at the C-4 phenyl ring over electron-rich or electron-deficient ones. Thus, the most potent COX-2 inhibitor was **11g** (COX-2 IC_{50} = 0.43 μM with no inhibition of COX-1 up to 25 μM), relative to the reference drug celecoxib (COX-2 IC_{50} = 0.21 μM with no inhibition of COX-1 up to 25 μM).

The orientation of the potent and selective COX-2 inhibitor, 1-(4-bromophenyl)-5-(4-methylsulfonylphenyl)-2-methylthioimidazole (**11g**), in the COX-2 active site was examined by a flexible docking experiment.[20] Figure 3 shows the joined binding pockets containing

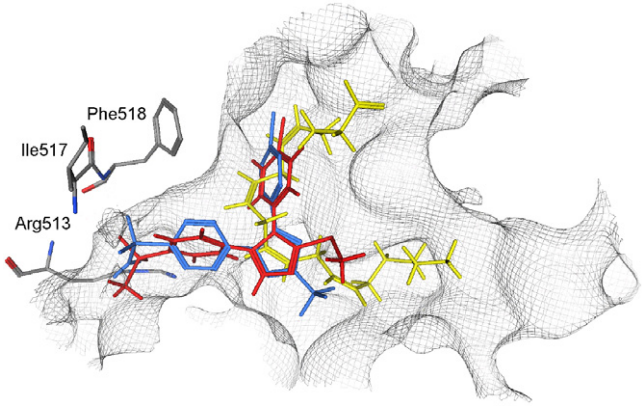


Figure 3. Superposed structures of **11g** (red), SC-558 (blue), and arachidonic acid (yellow) inside the binding pocket (mesh). Three amino acids which make hydrogen bonds to **11g** are also displayed.

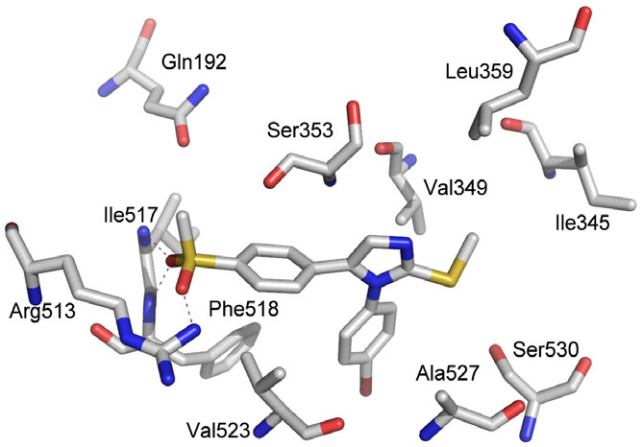


Figure 4. Interaction of **11g** with amino acids of COX-2 binding pocket.

structures of **11g** (red), SC-558 (blue; superposed from 1cx2.pdb), and arachidonic acid which mainly fills the primary pocket (yellow; superposed from 1cvu.pdb). The three amino acids which form hydrogen bonds with **11g** are displayed outside the pocket. Overall, **11g** seems to bind similarly but not identically to SC-558. The SO_2Me moiety in **11g** interacts with the secondary pocket amino acid residues Phe^{518}, Gln^{192}, Arg^{513}, Ser^{353}, Ile^{517}, and Val^{523}. One of the O-atoms of the SO_2Me substituent forms a hydrogen bond with the amine hydrogen (guanidine group) of Arg^{513} (O—H: 1.7 Å) and the other oxygen forms hydrogen bonds with backbone amide hydrogens of Phe^{518} (O—H: 2.2 Å) and Ile^{517} (O—N: 2.1 Å) as shown in Figure 4. Interestingly, the C-2 MeS-substituent is oriented toward hydrophobic region formed by Val^{349}, Leu^{359}, Leu^{345}, Ser^{530}, Ala^{527}, with the S-atom weakly interacting with side-chain hydroxyl group on Ser^{530} (S—H: 3.5 Å).

These observations provide a good explanation for the potent and selective inhibitory activity of **11g**.

In vivo pharmacological evaluation of **11a**–**i** was carried out to assess their potential anti-inflammatory activity.[21] Qualitative structure–activity relationship data, acquired using the anti-inflammatory rat paw edema assay, showed that this group of C-2 alkylthio substituted 1,5-diarylimidazoles exhibit anti-inflammatory activity with moderate to good activity range (40–91% inhibition) (Table 1).

In this series, 1-(4-bromophenyl)-5-(4-methylsulfonylphenyl)-2-methylthioimidazole (**11g**) was the most active anti-inflammatory agent (91% and 88% reduction in inflammation at 3 and 5 h postdrug administration, respectively) for a 50-mg/kg oral dose as compared to celecoxib (70% and 50% reduction in inflammation at 3 and 5 h postdrug administration, respectively).

4. Conclusion

The results of this investigation show (i) a C-2 SR substituent (**11g**) in this 1,5-diarylimidazole class of diarylheterocycles provides potent and selective inhibition of the COX-2 isozyme, (ii) molecular modeling studies indicate the SO_2Me moiety inserts deep into the COX-2 secondary pocket and the C-2 SR sulfur atom forms a weak hydrogen bond with OH atom of Ser.[530]

5. Experimental

Melting points were determined with a Reichert–Jung hot-stage microscope and are uncorrected. Infrared spectra were recorded on a Nicolet Magna 550-FT spectrometer. ^1H NMR (400 MHz) spectra were measured on a Varian Unity plus 400 spectrometer in $CDCl_3$ or DMSO-d_6 with TMS as the internal standard, where J (coupling constant) values are estimated in Hertz. Spin multiples are given as s (singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quartet), m (multiplet), and br (broad). Mass spectra were obtained with a Finnigan Mat TSQ-70 spectrometer. Elemental microanalyses were carried out with a Perkin-Elmer 240-C apparatus and were within ±0.4% of the theoretical values for C, H, and N. All solvents and reagents were purchased from the Fluka, Aldrich or Merck Chemical Company. Male Sprague–Dawley rats, used in the anti-inflammatory screens, were purchased from Pasteur Institute (Karaj, Iran), and experiments were carried out using protocols approved by the Ethics Committee of Tehran University of Medical Sciences.

5.1. General procedure for the preparation of 2-alkylthio-5-(4-methylsulfonylphenyl)-1-(4-substituted phenyl)imidazoles (11a–j)

Triethylamine (0.2 g, 2 mmol) was added dropwise to a stirred suspension of 4-methylsulfonylphenacylamine hydrochloride **9** (0.5 g, 2 mmol) and respective phenyl isothiocyanate (2 mmol) in ethanol (10 mL). The mixture was refluxed for 2–4 h. After cooling, KOH (0.11 g, 2 mmol) dissolved in water (0.6 mL) and alkyl iodide (2 mmol) were added. Purification by flash chromatography, eluting with CH_3Cl/MeOH (20:1), and crystallization from suitable solvent gave **11a**–**j**.

5.1.1. 5-(4-Methylsulfonylphenyl)-2-methylthio-1-phenylimidazole (11a). Yield, 65%; mp 162–164 °C (*n*-butanol); IR (KBr): 1278, 1151 (SO_2) cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.75 (d, J = 8.8 Hz, 2H), 7.6–7.3 (m, 3H), 7.44 (s, 1H), 7.25–7.15 (m, 4H), 3.02 (s, 3H), 2.46 (s, 3H); MS *m*/*z* (%) 344 (M^+, 100), 313 (15), 310 (16), 235 (51), 192 (14), 191 (23), 164 (10), 157 (10), 137 (7), 91 (72), 78 (39), 63 (37).

Anal. calcd for $C_{17}H_{16}N_2O_2S_2$: C, 59.28; H, 4.68; N, 8.13. Found: C, 59.36; H, 4.89; N, 8.01.

5.1.2. 2-Ethylthio-5-(4-methylsulfonylphenyl)-1-phenylimidazole (11b). Yield, 60%; mp 147–149 °C (*n*-butanol); IR (KBr): 1308, 1142 (SO_2) cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.75 (d, J = 8.4 Hz, 2H), 7.55–7.45 (m, 3H), 7.43 (s, 1H), 7.26–7.19 (m, 4H), 3.18 (q, J = 7.6 Hz, 2H), 3.02 (s, 3H), 1.38 (t, J = 7.6 Hz, 3H); MS *m*/*z* (%) 358 (M^+,

63), 329 (46), 254 (33), 249 (100), 191 (33), 170 (18), 105 (70), 77 (15).

Anal. calcd for $C_{18}H_{18}N_2O_2S_2$: C, 60.31; H, 5.06; N, 7.81. Found: C, 60.50; H, 4.91; N, 7.98.

5.1.3. 1-(4-Fluorophenyl)-5-(4-methylsulfonylphenyl)-2-methylthioimidazole (11c). Yield, 58%; mp 185–187 °C (*n*-butanol); IR (KBr): 1289, 1149 (SO_2) cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.77 (d, $J = 8.8$ Hz, 2H), 7.41 (s, 1H), 7.27-7.15 (m, 6H), 3.03 (s, 3H), 2.63 (s, 3H); MS *m/z* (%) 362 (M^+, 79), 329(10), 284 (37), 235 (65), 209 (18), 196 (16), 184 (18), 149 (13), 109 (100), 78 (45), 63 (40).

Anal. calcd for $C_{17}H_{15}FN_2O_2S_2$: C, 56.34; H, 4.17; N, 7.73. Found: C, 56.52; H, 4.06; N, 7.59.

5.1.4. 2-Ethylthio-1-(4-fluorophenyl)-5-(4-methylsulfonylphenyl)imidazole (11d). Yield, 47%; mp 145–147 °C (*n*-butanol); IR (KBr): 1306, 1148 (SO_2) cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.78 (d, $J = 8$ Hz, 2H), 7.42 (s, 1H), 7.26–7.12 (m, 6H), 3.19 (q, $J = 7.2$ Hz, 2H), 3.03 (s, 3H), 1.39 (t, $J = 7.6$ Hz, 3H); Mass *m/z* (%) 376 (M^+, 68), 347 (22), 268 (15), 249 (47), 209 (19), 197 (14), 135 (13), 123 (60), 83 (49), 78 (81), 63 (100).

Anal. calcd for $C_{18}H_{17}FN_2O_2S_2$: C, 57.43; H, 4.55; N, 7.44. Found: C, 57.59; H, 4.69; N, 7.23.

5.1.5. 1-(4-Chlorophenyl)-5-(4-methylsulfonylphenyl)-2-methylthioimidazole (11e). Yield, 40%; mp 190–192 °C (*n*-butanol); IR (KBr): 1299, 1141 (SO_2) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.79 (d, $J = 8$ Hz, 2H), 7.47(d, $J = 8$ Hz, 2H), 7.42 (s, 1H), 7.23 (d, $J = 8.4$ Hz, 2H), 7.19 (d, $J = 8.4$ Hz, 2H), 3.03 (s, 3H), 2.64 (s, 3H); MS *m/z* (%) 380 (M^+, 3), 378 (9), 351 (37), 206 (5), 196 (100), 150 (12), 125 (14), 105 (13), 78 (12).

Anal. calcd for $C_{17}H_{15}ClN_2O_2S_2$: C, 53.89; H, 3.99; N, 7.39. Found: C, 53.68; H, 3.82; N, 7.52.

5.1.6. 1-(4-Chlorophenyl)-2-ethylthio-5-(4-methylsulfonylphenyl)imidazole (9f). Yield, 45%; mp 226–228 °C (methanol); IR (KBr): 1299, 1141 (SO_2) cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.84 (d, $J = 8.4$ Hz, 2H), 7.44-7.34 (m, 3H), 7.23 (d, $J = 8.8$ Hz, 2H), 7.07 (d, $J = 8.8$ Hz, 2H), 3.07 (s, 3H), 3.02 (q, $J = 7.6$ Hz, 2H), 1.29 (t, $J = 7.6$ Hz, 3H); MS *m/z* (%) 394 (M^+, 8), 392 (24), 365 (20), 198 (100), 194 (32), 179 (12), 125 (10), 104 (16), 78 (19), 65 (10).

Anal. calcd for $C_{18}H_{17}ClN_2O_2S_2$: C, 55.02; H, 4.36; N, 7.13. Found: C, 55.21; H, 4.22; N, 7.01.

5.1.7. 1-(4-Bromophenyl)-5-(4-methylsulfonylphenyl)-2-methylthioimidazole (11g). Yield, 30%; mp 193–195 °C (*n*-butanol); IR (KBr): 1298, 1141 (SO_2) cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.78 (d, $J = 8.4$ Hz, 2H), 7.60 (d, $J = 8.4$ Hz, 2H), 7.41 (s, 1H), 7.23 (d, $J = 8.4$ Hz, 2H), 7.11 (d, $J = 8.4$ Hz, 2H), 3.03 (s, 3H), 2.64 (s, 3H); MS *m/z* (%) 424 (M^+, 80), 422 (80), 389 (15), 310 (17), 270 (3), 248 (10), 236 (13), 235 (100), 190 (23), 171 (81), 169 (80), 156 (14), 89 (9), 78 (73), 63 (70).

Anal. calcd for $C_{17}H_{15}BrN_2O_2S_2$: C, 48.23; H, 3.57; N, 6.62. Found: C, 48.45; H, 3.75; N, 6.78.

5.1.8. 1-(4-Bromophenyl)-2-ethylthio-5-(4-methylsulfonylphenyl)imidazole (11h). Yield, 35%; mp 170–172 °C (methanol); IR (KBr): 1311, 1151 (SO_2) cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.85 (d, $J = 8.4$ Hz, 2H), 7.59–7.51 (m, 3H), 7.23 (d, $J = 8.8$ Hz, 2H), 7.01 (d, $J = 8.8$ Hz, 2H), 3.07 (s, 3H), 3.03 (q, $J = 7.4$ Hz, 2H), 1.29 (t, $J = 7.4$ Hz, 3H); MS *m/z* (%) 438 (M^+, 10), 436 (10), 260 (12), 229 (100), 213 (10), 184 (19), 152 (13), 51 (26).

Anal. calcd for $C_{18}H_{17}BrN_2O_2S_2$: C, 49.43; H, 3.92; N, 6.40. Found: C, 49.28; H, 3.79; N, 6.59.

5.1.9. 1-(4-Methoxyphenyl)-5-(4-methylsulfonylphenyl)-2-methylthioimidazole (11i). Yield, 66%; mp 215–217 °C (*n*-butanol); IR (KBr): 1299, 1144 (SO_2) cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.75 (d, $J = 8.4$ Hz, 2H), 7.42 (s, 1H), 7.25 (d, $J = 8.4$ Hz, 2H), 7.15 (d, $J = 8.4$ Hz, 2H), 6.96 (d, $J = 8.4$ Hz, 2H), 3.87 (s, 3H), 3.02 (s, 3H), 2.63 (s, 3H); MS *m/z* (%) 374 (M^+, 43), 234 (22), 179 (13), 178 (25), 140 (12), 121 (100), 120 (32), 78 (3), 63 (5).

Anal. calcd for $C_{18}H_{18}N_2O_3S_2$: C, 57.73; H, 4.84; N, 7.48. Found: C, 57.59; H, 4.99; N, 7.26.

5.1.10. 2-Ethylthio-1-(4-methoxyphenyl)-5-(4-methylsulfonylphenyl)imidazole (11j). Yield, 50%; mp 151–153 °C (*n*-butanol); IR (KBr): 1307, 1144 (SO_2) cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.75 (d, $J = 8.4$ Hz, 2H), 7.42 (s, 1H), 7.25 (d, $J = 8.4$ Hz, 2H), 7.14 (d, $J = 8.4$ Hz, 2H), 6.96 (d, $J = 8.4$ Hz, 2H), 3.86 (s, 3H), 3.17 (q, $J = 7.6$ Hz, 2H), 3.02 (s, 3H), 1.37 (t, $J = 7.6$ Hz, 3H); MS *m/z* (%) 388 (M^+, 52), 361 (18), 359 (43), 279 (11), 248 (38), 223 (15), 207 (11), 179 (8), 170 (9), 135 (100), 77 (12).

Anal. calcd for $C_{19}H_{20}N_2O_3S_2$: C, 58.74; H, 5.19; N, 7.21. Found: C, 58.88; H, 5.02; N, 7.06.

5.2. In vitro cyclooxygenase (COX) inhibition assay

IC_{50} values for the inhibition of purified mouse COX-2 (63 nM) or ovine COX-1 (22 nM) by test compounds were determined by a thin layer chromatography (TLC) assay.[19] Hematin-reconstituted COX-2 (125 nM) or COX-1 (44 nM) in 100 mM Tris–HCl, pH 8.0, containing 500 μM phenol was treated with several concentrations of inhibitors (0–25 μM) at 25 °C for 20 min. Since the recombinant COX-2 had specific activity lower than that of ovine COX-1, the protein concentrations were adjusted such that the percentages of total products obtained following arachidonic acid oxygenation by the two isozymes were comparable. The cyclooxygenase reaction was initiated by the addition of [1-^{14}C]arachidonic acid (50 μM) at 37 °C for 30 s. Reactions were terminated by solvent extraction in Et_2O/CH_3OH/1 M citrate, pH 4.0 (30:4:1), and the organic phase was spotted on a 20 × 20 cm TLC plate (EM-11798-7, VWR). The plate was developed in EtOAc/CH_2Cl_2/glacial AcOH (75:25:1), and radiolabeled prostaglandins were quantitated with a radioactivity scanner (Bioscan, Inc., Washington, DC). The

percentage of total products observed at different inhibitor concentrations was divided by the percentage of products observed for protein samples preincubated with DMSO. Control experiments in the absence of inhibitor indicated ~25–30% conversion of arachidonic acid to products, which was sufficient for assessing the inhibitory properties of all compounds described in this study.

5.3. Anti-inflammatory assay

The test compounds were evaluated using in vivo rat carrageenan-induced foot paw edema model reported previously.[21] Male Sprauge–Dawley rats (120–150 g) were fasted with free access to water at least 16 h prior to experiments. Edema was produced by injecting 0.1 mL of a solution of 1% λ-carrageenan in the hind-paw. Paw volume was measured by water displacement with a plethysmometer (UGO BASILE) before, 3 and 5 h after treatment. The compounds were administered orally with a 1 mL suspension of test compound in vehicle (0.5% methyl cellulose and 0.025% Tween 20), after being hydrated with H_2O (5 mL). The percentage was calculated by the following equation: anti-inflammatory activity (%) = $(1 - D/C) \times 100$, where D represents the difference in paw volume before and after drug was administered to the rats, and C stands for the difference of volume in the control groups.

5.4. Molecular modeling (docking) studies

Docking studies were performed using our in-house docking algorithm (U-Dock 1.0) which was designed in SVL language as implemented in MOE software version 2006.02 (CCG Inc.)[20] and runs on a cluster of 12 Pentium IV processors. The 2.4 Å resolution coordinates of arachidonic acid bound to the murine COX-2 enzyme were obtained from the RCSB Protein Data Bank (1CVU), hydrogen atoms were added, and the structure was optimized using MMFF94s[22,23] with Born solvation model.[24] Ligand conformers were constructed using the Builder module and systematic conformer search method and non-redundant conformers up to 7 kcal from global minima conformer were retained (24 conformers).

Random orientations of all conformers of the ligand were generated inside a virtual box (the docking box) which was large enough to accommodate arachidonic acid or selective inhibitor SC-558 as they appear in corresponding pdb structures (1CX2 and 1CVU), therefore covering both binding pockets and giving the ligand a non biased opportunity to explore these pockets. U-Dock uses a layered-shell model to simulate flexibility of the protein while optimizing performance. As shown in Figure 5, the first shell or S1 was defined to include full residues of all amino acids having at least one atom closer then 4.5 Å to default hypothetical ligand (merged structure of arachidonic acid and SC-558 as described). The second shell or S2 was defined as full residues of all amino acids having at least one atom closer than 9 Å to same virtual ligand, excluding those already defined in S1. The rest of the atoms were deleted and the broken bonds were capped with hydrogen atoms which made

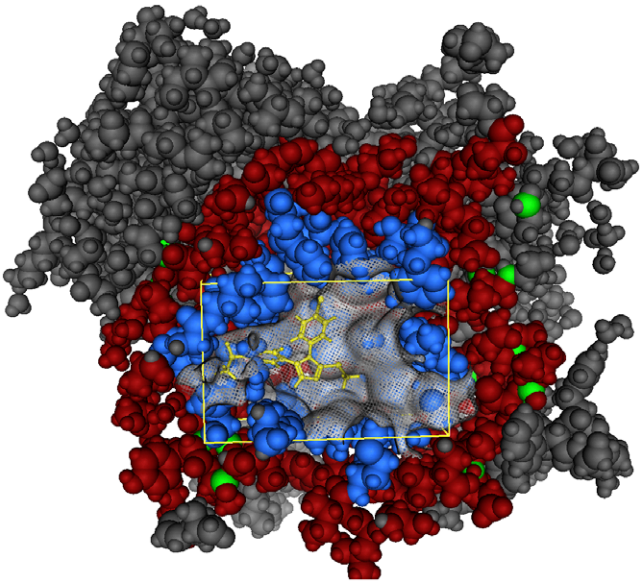


Figure 5. Cross section of the simplified 'layered shell' model S1 (blue), S2 (red), and S3 (green). The rest of protein (gray) was deleted during docking. Ligand (**11g**) is displayed in yellow, and the binding pocket is rendered as mesh. The virtual 'docking box' is also displayed in yellow.

the third shell or S3. Thus, S1 included all the active site atoms, containing 35 amino acids (607 atoms), S2 included 123 amino acids (2202 atoms), and S3 contained only 60 atoms. During the docking, after assigning a random orientation to each ligand conformer, the complex was energy minimized while S3 was kept fixed, S2 was semi-flexible, and S1 and ligand were relaxed normally (fully flexible). Semi-flexibility was simulated using 'tether constants' as implemented in MOE, which are a vector of quadratic forces applied to selected atoms that reduce the repositioning of the atoms during minimization. A tether constant of 30 was used for S2 (a value of 300 is roughly equal to a chemical bond). Born solvation model was turned off during docking, since deletion of amino acids beyond S3 creates a new front for the model which is unrealistically close to active site and other internal amino acids which construct S1 and S2. U-Dock uses a special pruning method to save the calculation time, which is based on prediction of evolution of energy of the randomly generated complexes during minimization and abortion of calculations on those which are unlikely to lead to a local minimum energy complex. Each conformer of ligand was used to create up to 200 complexes. The procedure took about 48 h (2 h per conformer). We use the force field energies of the fully flexible part of the complex (ligand plus S1) to rank the optimized complexes. While the energy plot and geometries of top complexes verify the convergence of method, the lowest energy structure was interpreted as optimum binding mode, and described above.

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