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Original article

Synthesis, anti-inflammatory activity and COX-1/COX-2 inhibition of novel substituted cyclic imides. Part 1: Molecular docking study

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ABSTRACT

A group of cyclic imides (**1**–**13**) was designed for evaluation as selective COX-2 inhibitors and investigated *in vivo* for their anti-inflammatory activities using carrageenan-induced rat paw edema model. Compounds **5b**, **6b**, **11b**, **11c**, **12b** and **12c** were proved to be potent COX-2 inhibitors with IC_{50} range of 0.1–1.0 μM. *In vitro* COX-1/COX-2 inhibition structure–activity studies identified compound **5b** as a highly potent (IC_{50} = 0.1 μM), and an extremely selective [COX-2 (SI) = 400] comparable to celecoxib [COX-2 (SI) > 333.3], COX-2 inhibitor that showed superior anti-inflammatory activity (ED_{50} = 104 mg/kg) relative to diclofenac (ED_{50} = 114 mg/kg). A Virtual screening was carried out through docking the designed compounds into the COX-2 binding site to predict if these compounds have analogous binding mode to the COX-2 inhibitors. Molecular modeling (docking) study showed that the CH_3O substituents of **5b** inserted deep inside the 2°-pocket of the COX-2 active site, where the O-atoms of such group underwent a H-bonding interaction with His^{90} (2.43, 2.83 Å), Arg^{513} (2.89 Å) and Tyr^{355} (3.34 Å). Docking study of the synthesized compound **5b** into the active site of COX-2 revealed a similar binding mode to SC-558, a selective COX-2 inhibitor.

1. Introduction

Inflammation is a normal response to any noxious stimulus that threatens the host and may vary from a localized response to a generalized one [1]. Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used for the treatment of rheumatism diseases, as rheumatoid arthritis, and pain [2,3]. The pharmacological effects of NSAIDs are due to inhibition of a membrane enzyme called cyclooxygenase (COX) which is involved in the prostaglandin biosynthesis [4]. There are two isoforms, COX-1 and COX-2, helped to understand the side-effects of NSAIDs [5]. The constitutive COX-1 is found in healthy populations and has mainly a physiological role in the kidneys and the stomach. In contrast, the mainly inducible COX-2 is involved in the production of prostaglandins mediating pain and supporting the inflammatory process [6,7]. Classical NSAIDs such as flurbiprofen (Fig. 1A) nonselectively inhibit both isoenzymes and cause gastric failure like bleeding and ulcer [8,9]. In order to prevent or decrease these side-effects, a current strategy consists of designing selective COX-2 inhibitors with an improved gastric safety profile [10,11]. The improved safety profile of COX-2 inhibitors may allow the use of these new agents for long-term prophylactic use in certain chronic diseases [4,10,11]. This has led intense efforts in search for potent and selective COX-2 inhibitors which could provide anti-inflammatory drugs with fewer risks. Several classes of compounds having selective COX-2 inhibitory activity have been reported in the literature such as SC-558 [12] and celecoxib [13] (Fig. 1B and C respectively).

On the other hand the cyclic imido group is a common protecting group for amines and is widely used in carbohydrate chemistry. As a known pharmacophore, the phthalimido moiety also has a wide range of activities such as antitumor, [14,15] and anti-inflammatory activities [16,17]. Systematic investigation of phthalimide analogs revealed that derivatives bearing a spherical alkyl group, such as an adamantyl group or a carboranyl group possess potent bi-directional TNF-α (tumor necrosis factor-α) production-regulating activity [18,19]. Recently it is reported that thalidomide directly inhibits COX-1/COX-2 with efficacy comparable to that of the representative drug, aspirin [20,21]. Such earlier work on the COX-inhibiting activity of thalidomide afforded a new scaffold for small-molecular COX inhibitors, such as compound (**D**), which should offer opportunities for various kinds of structural development (Fig. 1).

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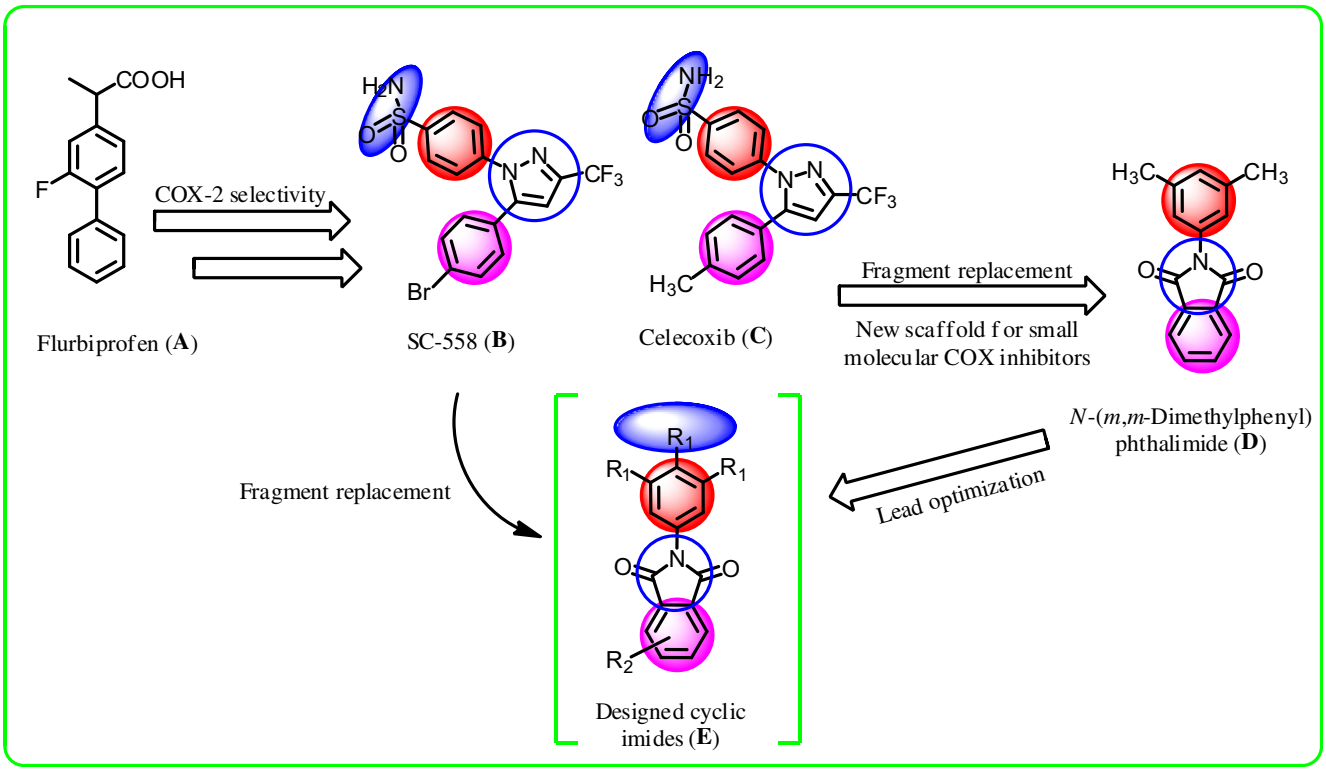


Fig. 1. Representative examples of nonselective (**A**) and selective (**B**, **C** and **D**) COX-2 inhibitors and the designed cyclic imides (**E**).

Thus our main objective is to design novel cyclic imides as specific inhibitors of COX-2 in the hope that these molecules may be further explored as powerful and novel non-ulcerogenic anti-inflammatory lead-candidates. Our strategy is intended to obtain potent anti-inflammatory activity with selective inhibition of COX-2 using traditional medicinal chemistry techniques motivated by the comparative modeling of a COX-1 and -2 complexed with **A** and **B** together with the available pharmacophore.

In view of the previous rationale and in continuation of an ongoing program aiming at finding new structure leads with potential anti-inflammatory activities and selective COX-2 inhibition [22], new series of cyclic imides (**1**–**13**) have been synthesized and evaluated their *in vitro* COX-1/COX-2 inhibition, and *in vivo* assessment as anti-inflammatory activities (Fig. 1). In the present study, the substitution pattern at the *N*-phenyl and the core cyclic imides was selected so as to confer different electronic environment that would affect the lipophilicity, and hence the activity of the target molecules. The objective of forming these hybrids is an attempt to reach an active anti-inflammatory agent with potentiated activity and selectivity toward COX-2. Moreover, in order to investigate the contribution of the imide framework in the anti-inflammatory activity and COX inhibition, the succinimide group was replaced by phthalimide group substituted by a versatile electron repelling or attracting moiety in the derivatives **4**–**10**. Next, we decide to replace the phthalimide ring with other cyclic imides, providing derivatives **2**, **3**, **11**, **12** and **13** (Scheme 1). Drug-likeness and molecular docking methodology were used to identify the structural features required for the COX-2 inhibition properties of these new series. However the results of this molecular docking could support the postulation that our active compounds may act on the same enzyme target where COX-2 inhibitors act confirming the molecular design of the reported class of COX-2 inhibitors.

2. Results and discussion

2.1. Chemistry

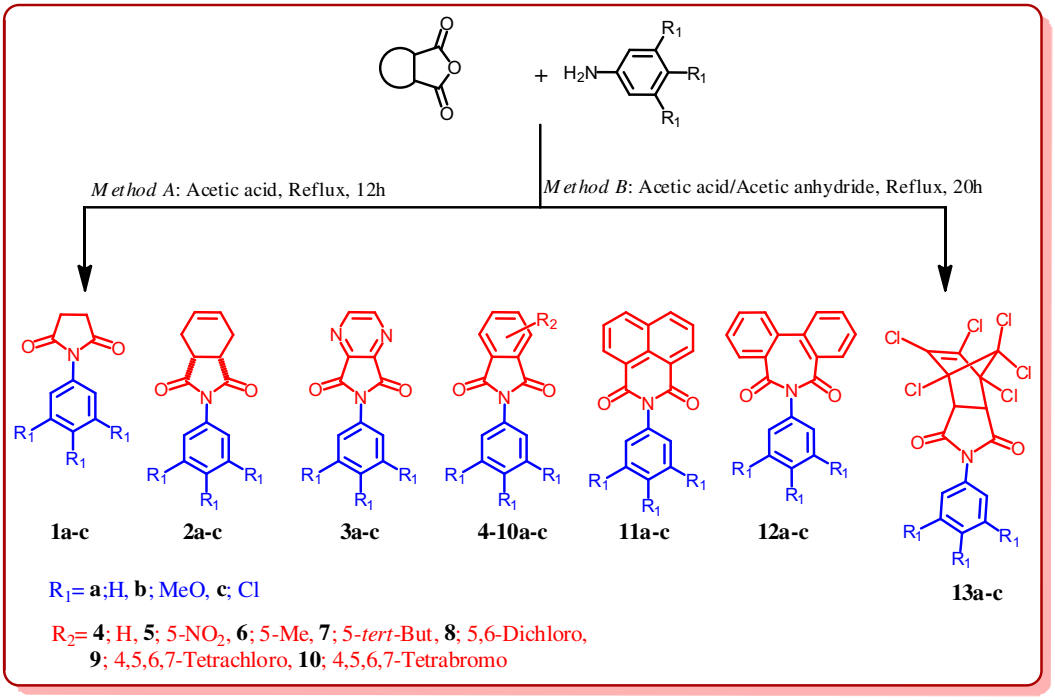
2.1.1. Synthesis of compounds *1*–*13* (Scheme 1)

The preparation of target cyclic imides is shown in Scheme 1. Classical condensation of trisubstituted aniline with acid anhydrides in refluxing acetic acid (method A) afforded these imides in satisfactory yields that could sometimes be increased by adding acetic anhydride or by heating the mixture at 160–200 °C in the absence of solvent (method B). The structures of the isolated products **1**–**13** were established on the basis of their elemental and spectral analyses.

2.2. Biological activity

2.2.1. In vitro COX inhibition

It was found that the structurally simple compound, *m*,*m*-dimethylphenylphthalimide (Fig. 1D), was obtained as a potent and slightly COX-2-selective inhibitor. According to the above rationale, the compounds synthesized in this work are evaluated for their ability to inhibit COX-1 and COX-2 using an ovine COX-1/COX-2 assay kit (Catalog No. 560101, Cayman Chemicals Inc., Ann Arbor, MI, USA). IC_{50} (μM) are determined and are means of two determinations acquired and the deviation from the mean is <10% of the mean value. The selectivity index (SI values) was defined as IC_{50}(COX-1)/IC_{50}(COX-2). In the assay system, the IC_{50} values of celecoxib on COX-1 and COX-2 were determined to be >100 and 0.30 μM respectively, indicating that celecoxib is a selective COX-2 inhibitor [COX-2 (SI) > 333.3]. The results showed that most of the compounds showed potent inhibition against COX-2 (IC_{50} ≅ 0.1–1.0 μM) compared to the inhibition for COX-1 (IC_{50} > 100 μM) as listed in Table 1. Nearly 6 of the compounds (**5b**, **6b**, **11b**, **11c**, **12b** and **12c**)



Scheme 1. Synthesis of the designed cyclic imide derivatives.

were found to be potent and selective similar to celecoxib against COX-2. Compound **5b** was the most potent inhibitor in this series with the activity (IC_{50} = 0.1 μM) 3-fold higher than celecoxib (IC_{50} = 0.3 μM). The effects of substituents introduced into the phthaloyl moiety of compounds **4**–**10** were revealed to be directional, being dependent on the electronic nature of the substituents, that is introduction of a nitro group at the 5-position (**5b**) enhanced both COX-1- and COX-2-inhibiting activity with higher potency for the latter, resulting in a COX-2-selective inhibitor (SI = 400). Moreover introduction of halogen such as chloro or bromo (**8**–**10**) resulted in lowering the COX-2-inhibiting activity, while introduction of an electron-donating methyl group (**6b**) showed just the opposite effects, resulting in a COX-2-selective inhibitor (SI = >250). The activities of some other cyclic imides analogs (**11**–**12**) are shown in Table 1. Among them, compounds **11b** and **12b** are a COX-2-selective inhibitor (SI = >222). No clear-cut structure–activity relationships could be deduced at this stage. The activities of *N*-unsubstituted phenyl cyclic imide derivatives (**1a**–**13a**) are not shown in Table 1 and are inactive. Interestingly methoxy substituents on the *N*-phenyl group play critical roles in the COX-inhibiting activity of the tested compounds. The 3,4,5,trimethoxyphenyl analog (**5b**) showed the most potent activity, being 2.5 and 3 times more active than celecoxib in COX-1 and COX-2-inhibiting activity, respectively. The structure–activity relationship of compounds **1b**,**c**–**13b**,**c** indicates the introduction of 3,4,6-trichloro groups at *N*-phenyl cyclic imides lowered the activity. This result, as well as the previously described effect of the 5-substitution on phthalylimide suggests the importance of electronic effects and spatial structure around the nitrogen atom of the core cyclic imides. Next we investigated the COX-inhibiting activity of non-aromatic succinimide **1** and cyclic imides **2** and **13** which were less active inhibitor as compounds **1** or inactive such as compounds **2** and **13** compared to the phthalimide derivatives **4**–**10** and its analogs **11**–**12**. Diminishment of activity of such compounds **1**, **2** and **13** may be explained on the bases of non-aromatic feature of the imide core indicating the importance of aromatic fragment of imide core for the activity.

2.2.2. *In vivo* anti-inflammatory studies

All the synthesized compounds which have COX-2 inhibitory activity were tested for anti-inflammatory activities including compounds **1b**,**c** and **3**–**12b**,**c**. The results of anti-inflammatory activities against carrageenan-induced rat paw edema and ED_{50} (mg/kg) of all tested compounds were shown in Table 1. Out of 21 compounds, 16 compounds (**3**–**8** and **11**–**12**) were found to possess potent anti-inflammatory activity (60–94% reduction in inflammation) and some of which shown higher inhibition in comparison to reference drug diclofenac. In general, cyclic imides which were substituted with chloro moiety at *N*-phenyl ring (R1 = Cl) have shown lower percentage of inhibition of edema when compared with compounds containing CH_3O group at the same position (R1 = CH_3O). These findings may be attributed to importance of methoxy group in COX receptor binding site. Compounds **5b** (R1 = CH_3O and R2 = NO_2), **6b** (R1 = CH_3O and R2 = CH_3), **7b** (R1 = CH_3O and R2 = *tert*-butyl), **11b** (R1 = CH_3O), **11c** (R1 = Cl) and **12b** (R1 = CH_3O) have shown the maximum percentage of anti-inflammatory activity (94%, 94%, 89%, 91%, 88%, 84% respectively). More interestingly, cyclic imides based on poly halogenated acid anhydrides; such as compounds **8**–**10** and **13**; were shown lower activity compared with non halogenated derivatives this may be attributed to very high lipid solubility and their low bio-availability. ED_{50} of the compounds, which were shown promising degree of anti-inflammatory activity (65–94%), were studied. Compounds **3b**, **5b**, **6b**, **11b**, **11c** and **12b** exhibited better anti-inflammatory activity with ED_{50} of 123, 105, 107, 117, 115 and 120 mg/kg, respectively, as compared to diclofenac (ED_{50} = 114 mg/kg). In the carrageenan-induced rat paw edema assay model, compound **5b** was the most potent anti-inflammatory agent (ED_{50} = 105 mg/kg) within this group of compounds at 2 h postdrug administration (IP). This high *in vivo* anti-inflammatory activity shown by **5b**, relative to other compounds, is attributed to the fact that **5b** inhibits both COX-1 and COX-2 whereas the other compounds inhibit only COX-2. Moreover predictions of ADME properties for all active compounds [23] show that all calculated compounds comply with

Table 1
In vitro COX-1/COX-2 enzyme inhibition assay, *in vivo* anti-inflammatory activity against carrageenan-induced rat paw edema and ED_{50} (mg/kg) of the designed compounds.

Compound No	IC_{50} (μM)[a]		SI[b]	% Inhibition of edema[c]		ED_{50}[d] (mg/kg)
	COX-1	COX-2		After 1 h	After 2 h	
1b	>100	40.00	>2.5	20	40	–
1c	>100	40.00	>2.5	30	33	–
2b	>100	>100	–	–	–	–
2c	>100	>100	–	–	–	–
3b	>100	5.20	>19.2	72	84	123
3c	>100	10.50	>9.5	47	73	175
4b	>100	6.40	>15.6	43	67	185
4c	>100	9.10	>11.0	49	70	172
5b	40.0	0.10	400.0	87	94	105
5c	36.1	40.00	0.90	79	79	122
6b	>100	0.40	>250.0	84	94	107
6c	>100	19.50	>5.1	36	65	183
7b	>100	10.00	>10.0	60	89	146
7c	>100	20.70	>4.8	35	60	195
8b	>100	12.40	>8.1	59	80	186
8c	>100	30.10	>3.3	75	65	187
9b	>100	45.00	>2.2	0	53	212
9c	>100	45.00	>2.2	44	45	229
10b	>100	65.00	>1.5	42	55	213
10c	>100	>100	–	–	–	–
11b	>100	0.45	>222.2	86	91	117
11c	>100	1.00	>100.00	79	88	115
12b	>100	0.45	>222.2	86	84	120
12c	>100	1.00	>100.00	75	72	150
13b	>100	>100	–	36	34	–
13c	>100	>100	–	23	37	–
Diclofenac	–	–	–	88	89	114
Celecoxib	>100	0.30	>333.3	–	–	–

[a] IC_{50} value is the compound concentration required to produce 50% inhibition of COX-1or COX-2 for means of two determinations using an ovine COX-1/COX-2 assay kit (catalog no. 560101, Cayman Chemicals Inc., Ann Arbor, MI, USA) and deviation from the mean is <10% of the mean value.
[b] Selectivity index (COX-1 IC_{50}/COX-2 IC_{50}).
[c] The carrageenan-induced rat paw edema assay was carried out using six animals (male Sprague–Dawley rats)/group following IP of the test compound. The results are expressed as means ± SEM ($n = 4$–6) following a 100 mg/kg IP of the test compounds.
[d] ED_{50} was the effective dose calculated after 2 h.

these rules and even the diclofenac show no violation. Theoretically, these compounds should present good passive oral absorption and differences in their bioactivity can not be attributed to this property.

2.3. Docking studies

Insights into the differences between the binding sites of COX-1 and COX-2 obtained from X-ray crystal structure data [12,24], provided useful guidelines that facilitated the design of the selective COX-2 inhibitors. For example, the COX-2 binding site possesses an additional 2°-pocket that is absent in COX-1, which is highly relevant to the design of selective COX-2 inhibitors. This COX-2 2°-pocket arises due to a conformational change at Tyr^{355} that is attributed to the presence of Ile^{523} in COX-1 relative to Val^{523} having a smaller side chain in COX-2 [24]. It has also been reported that replacement of His^{513} in COX-1 by Arg^{513} in COX-2 plays a key role with respect to the H-bond network in the COX-2 binding site. Access of ligands to the 2°-pocket of COX-2 is controlled by histidine (His^{90}), glutamine (Gln^{192}), and tyrosine (Tyr^{355}) [25]. Interaction of Arg^{513} with the bound drug is a requirement for time dependent inhibition of COX-2 [26].

The level of COX-2 inhibition and anti-inflammatory activities of compound **5b** prompted us to perform molecular docking studies to understand the ligand–protein interactions in detail. All the calculations were performed using MOE 2008.10 software installed on 2.0G Core 2 Duo [27–29]. The crystal structures of COX-2 enzymes complexed with SC-558 [1CX2] were used for the docking [12]. The active site of the enzyme was defined to include residues within a 10.0 Å radius to any of the inhibitor atoms. The automated docking program of MOE 2008.10 was used to dock compound **5b** on the active sites of COX-2 enzymes. The most stable docking model was selected according to the best scored conformation predicted by the MOE scoring function. The complexes were energy-minimized with a MMFF94 force field [30] till the gradient convergence 0.05 kcal/mol was reached. The compound **5b** could dock into the active site of COX-2 successfully (Fig. 2). Compound **5b** produces a deep moving into the hydrophilic pocket of COX-2 with which the methoxy groups are able to reach the hydrophilic pocket and is involved in strong hydrogen bonding with His^{90} (2.43, 2.83 Å) and Arg^{513} (2.89 Å) and weak hydrogen bonding with Tyr^{355} (3.34 Å). Such interactions are almost essential for COX-2 inhibitory activity, as exemplified by the binding interaction of SC-558, an analog of celecoxib cocrystallized in the COX-2 active site [12]. In addition; such interaction forces the imide core to adopt a specific orientation at the top of the channel. This moiety is involved in hydrophobic interaction with Trp^{387}, Tyr^{385}, Val^{523}, Leu^{352} and Phe^{518}. Moreover one of the carbonyl moieties of the imide core and the 5-nitro group were forming weak hydrogen bond with Ala^{527}

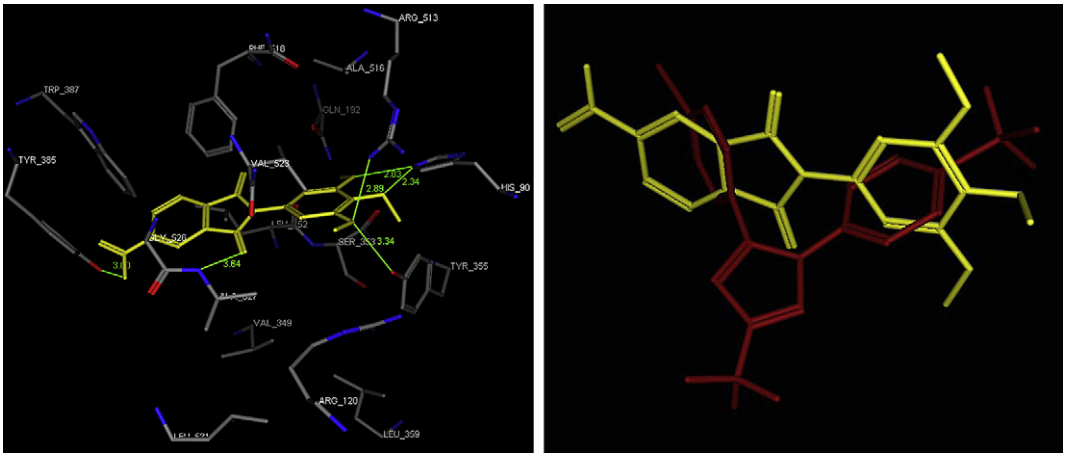


Fig. 2. Left panel showed docking of compound **5b** into the active site of COX-2. Hydrogen bonds are shown in green. Right panel showed alignment of **5b** (yellow) and selective inhibitor SC-558 (red) in the active site of COX-2. (for interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(3.64 Å) and Tyr^{385} (3.00 Å) respectively. The lateral pocket of COX-2 would therefore be responsible for the COX-2 selectivity of **5b** and contributed to stabilize the ligand–enzyme complexes (Fig. 2).

The complex generated by docking studies of **5b** with COX-2 and superimposition with the structure of the selective inhibitor, SC-558, co-crystallized with COX-2, illustrated in Fig. 2, shows that compound **5b** can bind in the active site of this enzyme in approximately similar fashion as the pyrazolic prototype (SC-558). Comparison of the interactions performed by SC-558 in the crystal and the docked structure of **5b** with COX-2 (Fig. 2) shows that the methoxy groups of **5b** hydrogen bonded to the amino acid residue His^{90} and Arg^{513}, similarly to the sulfonyl moiety of the pharmacophoric sulfonamide group pertaining to SC-558. Moreover, an additional hydrogen bond was observed between the carbonyl oxygen and Ala^{527}, while NO_2 hydrogen bonded with Tyr^{385}. Additionally, *N*-phenyl system are positioned in the same region as *p*-sulfonamido-phenyl ring of SC-558, while, the aromatic ring of imide core of compound **5b** is close to the *p*-Br-phenyl ring of SC-558, in the aromatic region of the active site lined by aromatic amino acid residues such as Phe^{518}, Tyr^{385} and Trp^{387}, among others. In short, the described interactions are typical of selective inhibitors of COX-2, confirming the molecular design of the reported class of anti-inflammatory imide derivatives [16,17].

3. Conclusion

Different (substituted) cyclic imide derivatives were synthesized and screened for COX-1/COX-2 inhibition and anti-inflammatory activity. Compounds which showed significant COX-2 inhibition were subjected to anti-inflammatory studies. Compound **5b** exhibit optimal COX-2 inhibitory potency (IC_{50} = 0.1 μM) and selectivity (SI) = 400 comparable with celecoxib and better ED_{50} than diclofenac, so it appears promising. Molecular docking studies further help in understanding the various interactions between the ligands and enzyme active sites in detail and thereby help to design novel potent inhibitors. It is clear that the CH_3O moieties of **5b** insert deep inside the COX-2 2°-pocket and forming strong hydrogen bond with His^{90} (2.43, 2.83 Å) and Arg^{513} (2.89 Å) and weak hydrogen bonding with Tyr^{355} (3.34 Å). This result was corroborated by molecular docking studies with COX-2 inhibition, which showed that this compound presents the pharmacophoric requisites for COX-2 inhibition. Indeed molecular docking studies further supported the strong inhibitory activity of **5b** and further help understanding the various interactions between the ligands and enzyme active sites in detail and thereby help to design novel potent inhibitors. The structure–activity relationships acquired show that appropriately substituted cyclic imides have the necessary geometry to provide potent and selective inhibition of the COX-2 isozyme, and to exhibit excellent anti-inflammatory activities.

4. Experimental

4.1. Chemistry

Melting points (uncorrected) were recorded on Barnstead Electrothermal 9100 melting apparatus. IR spectra were recorded on a FT-IR Perkin–Elmer spectrometer. ^{1}H NMR and ^{13}C NMR were recorded in DMSO-d_6 and/or $CDCl_3$ on a Bruker 500 MHz instrument using TMS as internal standard (chemical shifts in δ ppm). Microanalytical data (C, H, and N) were performed on Perkin–Elmer 240 B analyzer and they agreed with proposed structures within ±0.4% of the calculated values. Mass spectra were recorded on a Perkin–Elmer, Clarus 600 GC/MS and Varian, TQ 320 GC/MS/MS mass spectrometers. Solvent evaporation was performed under reduced pressure using Buchan Rotatory Evaporator unless otherwise stated. Thin layer chromatography was performed on precoated (0.25 mm) silica gel GF_{254} plates (E. Merck, Germany), compounds were detected with 254 nm UV lamp. Silica gel (60–230 mesh) was employed for routine column chromatography separations. Compounds **1a**–**13a**, **1c** and **3b** were prepared following their procedures reported in the literature [15,17,31–44].

4.1.1. General procedure for the synthesis of compounds *1b*–*13b* (method A)

A solution of 3,4,5-trimethoxyaniline (1.83 g, 10 mmol) and acid anhydride (10 mmol) in glacial acetic acid (15 mL) was heated under reflux for 12 h. After the evaporation of the reaction mixture to dryness under reduced pressure, the residue was neutralized by a solution of sodium bicarbonate (4%) until effervescence ceased. The precipitate obtained was washed with water, dried (P_2O_5) and recrystallized from an appropriate solvent.

*4.1.1.1. 1-(3,4,5-Trimethoxyphenyl)pyrrolidine-2,5-dione (**1b**).* Yield, 70%; mp 201–202 °C (Methanol); IR (KBr, cm^{-1}) ν: 1773, 1718 (C=O); ^{1}H NMR (DMSO-d_6); δ 2.77 (s, 4H, CH_2–CH_2), 3.71–3.76 (d, 9H, J = 22.0 Hz, $3CH_3$) 6.58 (s, 2H, Ar-H); ^{13}C NMR (DMSO-d_6): δ 28.91, 56.66, 60.54, 105.97, 128.92, 138.08, 153.39, 177.17; MS m/z (%): 267.2 (12.0, $M^+ + 2$), 266.0 (18.0, $M^+ + 1$), 264.9 (22.0, M^+); $C_{13}H_{15}NO_5$, Cal. C, 58.86; H, 5.70; N, 5.28, Found C, 58.01; H, 6.20; N, 5.88.

*4.1.1.2. cis-2-(3,4,5-Trimethoxyphenyl)-3a,4,7,7a-tetrahydro-1H-isoindole-1,3(2H)-dione (**2b**).* Yield, 69%; mp 141–142 °C (Methanol); IR (KBr, cm^{-1}) ν: 1717, 1663 (C=O); ^{1}H NMR (DMSO-d_6) δ: 1.80 (s, 2H, CH_2), 2.27–2.29 (d, 2H, J = 11.0 Hz, CH_2), 2.43–2.46 (d, 2H, J = 14.5 Hz, CH–CH), 3.68–3.73 (d, 9H, J = 26.0 Hz, $3CH_3$), 5.94 (s, 2H, CH=CH), 6.49 (s, 2H, Ar-H); ^{13}C NMR (DMSO-d_6); δ 23.58, 39.13, 56.57, 60.52, 105.44, 128.04, 128.71, 137.94, 153.38, 179.61; MS m/z (%): 317.8 (2.3, M^+); $C_{17}H_{19}NO_5$, Cal. C, 64.34; H, 6.03; N, 4.41, Found C, 63.99; H, 6.81; N, 4.91.

*4.1.1.3. 2-(3,4,5-Trimethoxyphenyl)isoindoline-1,3-dione (**4b**).* Yield, 88%; mp 241–242 °C (Ethanol); IR (KBr, cm^{-1}) ν: 1766, 1713 (C=O); ^{1}H NMR (DMSO-d_6) δ 3.62 (s, 3H, CH_3), 3.75 (s, 3H, $2CH_3$), 6.79 (s, 2H, Ar-H), 7.08 (s, 2H, Ar-H), 7.37 (s, 2H, Ar-H); ^{13}C NMR (DMSO-d_6); δ 56.64, 60.56, 98.01, 106.39, 123.79, 130.23, 132.00, 135.13, 153.36, 167.48; MS m/z (%): 314.6 (6.0, $M^+ + 1$), 313.8 (18.0, M^+); $C_{17}H_{15}NO_5$, Cal. C, 65.17; H, 4.83; N, 4.47, Found C, 64.89; H, 5.05; N, 5.00.

*4.1.1.4. 5-Nitro-2-(3,4,5-trimethoxyphenyl)isoindoline-1,3-dione (**5b**).* Yield, 87%; mp 258–260 °C (Methanol); IR (KBr, cm^{-1}) ν: 1778, 1728 (C=O); ^{1}H NMR (DMSO-d_6) δ 3.73 (s, 3H, CH_3), 3.77 (s, 3H, $2CH_3$), 6.82 (s, 2H, Ar-H) 8.11–8.14 (t, 1H, J = 7.5 Hz, Ar-H), 8.25–8.26 (d, 1H, J = 7.0 Hz, Ar-H), 8.34–8.36 (d, 1H, J = 7.5 Hz, Ar-H); ^{13}C NMR (DMSO-d_6); δ 56.03, 60.06, 105.76, 122.75, 126.94, 127.05, 128.33, 133.46, 136.42, 137.55, 144.45, 152.89, 162.64, 165.21. MS m/z (%): 358.5 (8.0, M^+). $C_{17}H_{14}N_2O_7$, Cal. C, 56.99; H, 3.94; N, 7.82, Found C, 57.45; H, 4.33; N, 7.64.

*4.1.1.5. 5-Methyl-2-(3,4,5-trimethoxyphenyl)isoindoline-1,3-dione (**6b**).* Yield, 90%; mp 222–223 °C (Methanol/CH_2Cl_2); IR (KBr, cm^{-1}) ν: 1774, 1707 (C=O); ^{1}H NMR (DMSO-d_6) δ 2.53 (s, 3H, CH_3), 3.72 (s, 3H, CH_3), 3.77 (s, 3H, $2CH_3$), 6.79 (s, 2H, Ar-H), 7.70–7.72 (d, 1H, J = 7.5 Hz, Ar-H), 7.78 (s, 1H, Ar-H), 7.83–7.84 (d, 1H, J = 7.5 Hz, Ar-H); ^{13}C NMR (DMSO-d_6); δ 21.39, 56.05, 60.04, 105.69, 123.25, 123.70, 127.58, 129.00, 132.00, 135.04, 137.24, 145.63, 152.82, 167.00; MS m/z (%): 327.0 (9.0, M^+); $C_{18}H_{17}NO_5$, Cal. C, 66.05; H, 5.23; N, 4.28, Found C, 65.65; H, 5.71; N, 4.33.

*4.1.1.6. 5-Tert-Butyl-2-(3,4,5-trimethoxyphenyl)isoindoline-1,3-dione (**7b**).* Yield, 63%; mp 145–147 °C (Methanol/CH_2Cl_2); IR (KBr, cm^{-1})

ν: 1777, 1708 (C=O); ^{1}H NMR (DMSO-d_6) δ 1.38 (s, 9H, 3CH$_3$), 3.78 (s, 3H, CH$_3$), 3.83 (s, 3H, 2CH$_3$), 6.93 (s, 2H, Ar-H), 7.56–7.58 (d, 1H, J = 7.5 Hz, Ar-H), 7.76–7.77 (d, 1H, J = 7.5 Hz, Ar-H), 7.99 (s, 1H, Ar-H); MS m/z (%): 369.1 (22, M$^+$); $C_{21}H_{23}NO_5$, Cal. C, 68.28; H, 6.28; N, 3.79, Found C, 68.59; H, 5.87; N, 4.00.

*4.1.1.7. 5,6-Dichloro-2-(3,4,5-trimethoxyphenyl)isoindoline-1,3-dione (**8b**).* Yield, 95%; mp 197–198 °C (Methanol/DMF); IR (KBr, cm^{-1}) ν: 1779, 1726 (C=O); ^{1}H NMR (DMSO-d_6) δ 3.72 (s, 3H, CH$_3$), 3.77 (s, 3H, 2CH$_3$), 6.80 (s, 2H, Ar-H), 8.29 (s, 2H, Ar-H); ^{13}C NMR (DMSO-d_6); δ 56.06, 60.06, 105.57, 125.47, 127.20, 131.54, 137.48, 152.57, 165.27. MS m/z (%): 385.2 (9.0, M$^+$ + 3), 384.1 (3.0, M$^+$ + 1), 382.6 (24.0, M$^+$); $C_{17}H_{13}Cl_2NO_5$, Cal. C, 53.42; H, 3.43; N, 3.66, Found C, 53.66; H, 3.01; N, 3.90.

*4.1.1.8. 4,5,6,7-Tetrachloro-2-(3,4,5-trimethoxyphenyl)isoindoline-1,3-dione (**9b**).* Yield, 93%; mp >300 °C (Acetic acid); IR (KBr, cm^{-1}) ν: 1782, 1709 (C=O); ^{1}H NMR (DMSO-d_6) δ 3.70 (s, 3H, CH$_3$), 3.76 (s, 3H, 2CH$_3$), 7.06 (s, 2H, Ar-H); ^{13}C NMR (DMSO-d_6); δ 56.76, 60.14, 97.38, 125.47, 128.04, 132.50, 152.79, 170.00. MS m/z (%): 451.4 (11.0, M$^+$); $C_{17}H_{11}Cl_4NO_5$, Cal. C, 45.26; H, 2.46; N, 3.11, Found C, 45.00; H, 2.77; N, 2.85.

*4.1.1.9. 4,5,6,7-Tetrabromo-2-(3,4,5-trimethoxyphenyl)isoindoline-1,3-dione (**10b**).* Yield, 94%; mp >300 °C (Acetic acid); IR (KBr, cm^{-1}) ν: 1706, 1667 (C=O); ^{1}H NMR (DMSO-d_6) δ 3.73 (s, 3H, CH$_3$), 3.77 (s, 3H, 2CH$_3$), 6.68 (s, 2H, Ar-H). MS m/z (%): 628.5 (19.0, M$^+$); $C_{17}H_{11}Br_4NO_5$, Cal. C, 32.47; H, 1.76; N, 2.23, Found C, 32.65; H, 2.00; N, 2.50.

*4.1.1.10. 2-(3,4,5-Trimethoxyphenyl)-1H-benzo[de]isoquinoline-1,3 (2H)-dione (**11b**).* Yield, 89%; mp 175–177 °C (Methanol/DMF); IR (KBr, cm^{-1}) ν: 1784, 1711 (C=O); ^{1}H NMR (DMSO-d_6) δ 3.72 (s, 3H, CH$_3$), 3.75 (s, 3H, 2CH$_3$), 6.98 (s, 2H, Ar-H) 7.90–7.95 (m, 2H, Ar-H), 8.51–8.58 (m, 4H, Ar-H); ^{13}C NMR (DMSO-d_6); δ 55.98, 60.04, 96.73, 106.73, 127.22, 127.55, 130.69, 132.44, 134.41, 135.35, 152.60, 173.95. MS m/z (%): 363.8 (8.0, M$^+$); $C_{21}H_{17}NO_5$, Cal. C, 69.41; H, 4.72; N, 3.85, Found C, 69.13; H, 5.21; N, 4.05.

*4.1.1.11. 6-(3,4,5-Trimethoxyphenyl)-5H-dibenzo[c,e]azepine-5,7 (6H)-dione (**12b**).* Yield, 70%; mp >300 °C (Ethanol); IR (KBr, cm^{-1}) ν: 1695, 1663 (C=O); ^{1}H NMR (DMSO-d_6) δ 3.61 (s, 3H, CH$_3$), 3.72 (s, 3H, 2CH$_3$), 6.92–6.94 (d, 2H, J = 6.5 Hz, Ar-H) 6.97 (s, 2H, Ar-H), 7.23–7.33 (m, 4H, Ar-H), 7.39–7.43 (m, 2H, Ar-H); ^{13}C NMR (DMSO-d_6); δ 55.62, 60.05, 96.73, 126.82, 127.86, 129.01, 133.12, 135.49, 139.59, 152.61, 168.12, 171.85. MS m/z (%): 389.1 (11, M$^+$); $C_{23}H_{19}NO_5$, C, 70.94; H, 4.92; N, 3.60, Found C, 71.36; H, 5.26; N, 3.81.

*4.1.1.12. 4,5,6,7,8,8-Hexachloro-2-(3,4,5-trimethoxyphenyl)-3a,4,7,7a-tetrahydro-1H-4,7-methanoisoindole-1,3(2H)-dione (**13b**).* Yield, 57%; mp 220–221 °C (Methanol/CH_2Cl_2); IR (KBr, cm^{-1}) ν: 1786, 1717 (C=O); ^{1}H NMR (DMSO-d_6) δ 3.71 (s, 3H, CH$_3$), 3.75 (s, 3H, 2CH$_3$), 4.23 (s, 2H, CH–CH), 6.36 (s, 2H, Ar-H); ^{13}C NMR (DMSO-d_6); δ 51.99, 56.03, 60.08, 79.02, 103.64, 104.35, 126.57, 130.65, 137.93, 153.21, 169.61. MS m/z (%): 535.6 (2, M$^+$ − 1), 536.4 (1, M$^+$); $C_{18}H_{13}Cl_6NO_5$, Cal. C, 40.33; H, 2.44; N, 2.61, Found C, 40.64; H, 2.93; N, 3.02.

*4.1.2. General procedure for the synthesis of compounds **1c**–**13c** (method B)*

A solution of 3,4,5-trichloroaniline (1.96 g, 10 mmol) and acid anhydride (10 mmol) in glacial acetic acid (20 mL) was refluxed for 3 h. The solvent was removed under reduced pressure until the volume reached ca. 5 mL. After addition of 10 mL of acetic anhydride, the solution was refluxed again for 20 h. The solvent was removed under reduced pressure. The residue was neutralized by a solution of sodium bicarbonate (4%) until effervescence ceased. The precipitate obtained was washed with water, dried (P_2O_5) and recrystallized from an appropriate solvent.

*4.1.2.1. cis-2-(3,4,5-Trichlorophenyl)-3a,4,7,7a-tetrahydro-1H-isoindole-1,3(2H)-dione (**2c**).* Yield, 61%; mp 159–160 °C (Ethanol/DMF); IR (KBr, cm^{-1}) ν: 1786,1716 (C=O); ^{1}H NMR (DMSO-d_6) δ: 1.85 (s, 2H, CH$_2$), 2.33 (s, 2H, CH$_2$), 2.46 (s, 2H, CH–CH), 5.97 (s, 2H, CH=CH), 8.03–8.04 (d, 2H, J = 3.0 Hz, Ar-H); ^{13}C NMR (DMSO-d_6); δ 23.07, 39.15, 128.82, 128.87, 134.15, 134.42, 135.70, 177.55. MS m/z (%): 330.9 (14.0, M$^+$).

*4.1.2.2. 6-(3,4,5-Trichlorophenyl)-5H-pyrrolo[3,4-b]pyrazine-5,7 (6H)-dione (**3c**).* Yield, 65%; mp >300 °C (Acetic acid); IR (KBr, cm^{-1}) ν: 1798,1745 (C=O); ^{1}H NMR (DMSO-d_6) δ 7.77 (s, 2H, Ar-H), 8.61 (s, 1H, Ar-H), 8.79 (s, 1H, Ar-H); ^{13}C NMR (DMSO-d_6); δ 127.44, 128.13, 132.18, 134.57, 141.27, 145.69, 162.71. MS m/z (%): 328.5 (2.00, M$^+$).

*4.1.2.3. 2-(3,4,5-Trichlorophenyl)isoindoline-1,3-dione (**4c**).* Yield, 81%; mp 291–293 °C (Acetic acid); IR (KBr, cm^{-1}) ν: 1789,1727 (C=O); ^{1}H NMR (DMSO-d_6) δ 8.03 (s, 4H, Ar-H), 8.10–8.11 (m, 2H, Ar-H); ^{13}C NMR (DMSO-d_6); δ 124.44, 128.97, 130.20, 130.61, 132.45, 135.33, 135.86, 173.27. MS m/z (%): 326.1 (11.0, M$^+$).

*4.1.2.4. 5-Nitro-2-(3,4,5-trichlorophenyl)isoindoline-1,3-dione (**5c**).* Yield, 78%; mp 219–220 °C (Methanol/DMF); IR (KBr, cm^{-1}) ν: 1788,1739 (C=O); ^{1}H NMR (DMSO-d_6) δ 8.04 (s, 2H, Ar-H) 8.23–8.26 (t, 1H, J = 7.5 Hz, Ar-H), 8.41–8.42 (d, 1H, J = 7.0 Hz, Ar-H), 8.49–8.50 (d, 1H, J = 7.5 Hz, Ar-H); ^{13}C NMR (DMSO-d_6); δ 122.21, 126.02, 127.43, 128.17, 128.45, 129.10, 130.09, 132.23, 135.24, 136.51, 137.72, 144.93, 160.47, 163.26. MS m/z (%): 371.8 (2.00, M$^+$).

*4.1.2.5. 5-Methyl-2-(3,4,5-trichlorophenyl)isoindoline-1,3-dione (**6c**).* Yield, 67%; mp 279–281 °C (Methanol/DMF); IR (KBr, cm^{-1}) ν: 1786,1727 (C=O); ^{1}H NMR (DMSO-d_6) δ 2.55 (s, 3H, CH$_3$), 7.81–7.83 (d, 1H, J = 7.0 Hz, Ar-H), 7.93 (s, 1H, Ar-H), 7.97–7.99 (d, 1H, J = 7.5 Hz, Ar-H), 8.03 (s, 2H, Ar-H); ^{13}C NMR (DMSO-d_6); δ 21.44, 118.29, 124.31, 124.79, 126.78, 127.45, 128.02, 128.94, 131.01, 135.36, 136.05, 136.13, 147.13, 165.21, 165.35. MS m/z (%): 339.4 (2.00, M$^+$), 339.4 (2.00, M$^+$).

*4.1.2.6. 5-tert-Butyl-2-(3,4,5-trichlorophenyl)isoindoline-1,3-dione (**7c**).* Yield, 58%; mp >300 °C (Methanol/DMF); IR (KBr, cm^{-1}) ν: 1779,1732 (C=O); ^{1}H NMR (DMSO-d_6) δ 1.39 (s, 9H, 3CH$_3$), 8.03–8.11 (m, 5H, Ar-H); ^{13}C NMR (DMSO-d_6); δ 30.65, 35.71, 121.25, 124.32, 126.78, 128.14, 128.95, 130.95, 132.78, 135.37, 136.07, 159.74, 165.07, 165.43. MS m/z (%): 382.9 (14.0, M$^+$).

*4.1.2.7. 5,6-Dichloro-2-(3,4,5-trichlorophenyl)isoindoline-1,3-dione (**8c**).* Yield, 55%; mp >300 °C (Acetic acid); IR (KBr, cm^{-1}) ν: 1787,1727 (C=O); ^{1}H NMR (DMSO-d_6) δ 8.04 (s, 2H, Ar-H), 8.63 (s, 2H, Ar-H); ^{13}C NMR (DMSO-d_6); δ 126.20, 126.73, 129.08, 130.30, 135.19, 136.41, 138.96, 163.42. MS m/z (%): 395.9 (4.00, M$^+$); $C_{14}H_4Cl_5NO_2$ C, 42.52; H, 1.02; N, 3.54.

*4.1.2.8. 4,5,6,7-Tetrachloro-2-(3,4,5-trichlorophenyl)isoindoline-1,3-dione (**9c**).* Yield, 61%; mp 261–263 °C (Acetic acid); IR (KBr, cm^{-1}) ν: 1793,1732 (C=O); ^{1}H NMR (DMSO-d_6) δ 7.94 (s, 1H, Ar-H), 8.05 (s, 1H, Ar-H); ^{13}C NMR (DMSO-d_6); δ 126.64, 129.16, 129.48, 135.19, 139.86, 160.76, 172.04. MS m/z (%): 464.1 (2.00, M$^+$).

*4.1.2.9. 4,5,6,7-Tetrabromo-2-(3,4,5-trichlorophenyl)isoindoline-1,3-dione (**10c**).* Yield, 49%; mp 299–300 °C (Acetic acid); IR (KBr, cm^{-1}) ν: 1778, 1731 (C=O); ^{1}H NMR (DMSO-d_6) δ 8.04 (s, 2H, Ar-H); ^{13}C NMR (DMSO-d_6); δ 120.97, 121.95, 126.12, 126.97, 129.10, 129.93,

135.21, 136.53, 138.22, 140.29, 161.13, 166.59. MS *m/z* (%): 642.3 (6.00, M^+).

*4.1.2.10. 2-(3,4,5-Trichlorophenyl)-1H-benzo[de]isoquinoline-1,3 (2H)-dione (**11c**).* Yield, 70%; mp 216–217 °C (Ethanol/DMF); IR (KBr, cm^{-1}) ν: 1770, 1740 (C=O); ^{1}H NMR (DMSO-d_6) δ 7.91–7.94 (m, 3H, Ar-H), 8.53–8.56 (m, 5H, Ar-H); ^{13}C NMR (DMSO-d_6); 119.01, 127.54, 129.71, 131.36, 132.44, 135.37, 160.68. MS *m/z* (%): 376.9 (3.00, M^+).

*4.1.2.11. 6-(3,4,5-Trichlorophenyl)-5H-dibenzo[c,e]azepine-5,7(6H)-dione (**12c**).* Yield, 61%; mp 283–285 °C (Ethanol/DMF); IR (KBr, cm^{-1}) ν: 1695 (C=O); ^{1}H NMR (DMSO-d_6) δ 6.93–6.94 (d, 2H, J = 6.5 Hz, Ar-H), 7.31–7.38 (m, 6H, Ar-H), 7.43–7.45 (d, 2H, J = 6.5 Hz, Ar-H); ^{13}C NMR (DMSO-d_6); δ 126.87, 127.16, 127.99, 128.16, 129.03, 134.48, 138.13, 138.62, 171.90, 172.51. MS *m/z* (%): 402.2 (4.00, M^+).

*4.1.2.12. 4,5,6,7,8,8-Hexachloro-2-(3,4,5-trichlorophenyl)-3a,4,7,7a-tetrahydro-1H-4,7-methanoisoindole-1,3(2H)-dione (**13c**).* Yield, 47%; mp 256–257 °C (Acetic acid); IR (KBr, cm^{-1}) ν: 1795, 1734 (C=O); ^{1}H NMR (DMSO-d_6) δ 4.80(s, 2H, CH–CH), 8.04 (s, 2H, Ar-H); ^{13}C NMR (DMSO-d_6); δ 51.52, 78.70, 105.03, 125.96, 129.08, 129.18, 131,02, 133.79, 134.15, 136.73, 168.13. MS *m/z* (%): 549.3 (3.00, M^+).

4.2. Pharmacology

4.2.1. In vitro cyclooxygenase (COX) inhibition assay

The ability of the test compounds listed in the Table 1 to inhibit ovine COX-1 and COX-2 (IC_{50} values, μM) was determined using an enzyme immunoassay (EIA) kit (catalog number 560101, Cayman Chemical, Ann Arbor, MI, USA). Cyclooxygenase catalyzes the first step in the biosynthesis of arachidonic acid (AA) to PGH_2. $PGF_{2\alpha}$, produced from PGH_2 by reduction with stannous chloride, is measured by enzyme immunoassay (ACE™ competitive EIA). Stock solutions of test compounds were dissolved in a minimum volume of DMSO. Briefly, to a series of supplied reaction buffer solutions (960 μL, 0.1 M Tris–HCl pH 8.0 containing 5 mM EDTA and 2 mM phenol) with either COX-1 or COX-2 (10 μL) enzyme in the presence of heme (10 μL) were added 10 μL of various concentrations of test drug solutions (0.01, 0.1, 1, 10, 50, and 100 μM in a final volume of 1 mL). These solutions were incubated for a period of 5 min at 37 °C after which 10 μL of AA (100 μM) solution were added and the COX reaction was stopped by the addition of 50 μL of 1 M HCl after 2 min. $PGF_{2\alpha}$, produced from PGH_2 by reduction with stannous chloride was measured by enzyme immunoassay. This assay is based on the competition between PGs and a PG-acetylcholinesterase conjugate (PG tracer) for a limited amount of PG antiserum. The amount of PG tracer that is able to bind to the PG antiserum is inversely proportional to the concentration of PGs in the wells since the concentration of PG tracer is held constant while the concentration of PGs varies. This antibody–PG complex binds to a mouse anti-rabbit monoclonal antibody that had been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's reagent, which contains the substrate to acetylcholine esterase, is added to the well. The product of this enzymatic reaction produces a distinct yellow color that absorbs at 406 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of PG tracer bound to the well, which is inversely proportional to the amount of PGs present in the well during the incubation: Absorbance α [Bound PG Tracer] α1/PGs. Percent inhibition was calculated by the comparison of compounds treated to various control incubations. The concentration of the test compounds causing 50% inhibition (IC_{50}, μM) was calculated from the concentration–inhibition response curve (duplicate determinations).

4.2.2. Anti-inflammatory activity

The test compounds were evaluated using *in vivo* rat carrageenan-induced foot paw edema model reported previously [45]. Male Sprague–Dawley rats (250 g) were fasted with free access to water at least 16 h prior to experiments. Edema was produced by injecting 0.2 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, 1 and 2 h after treatment. The compounds were administered intraperitoneally with a 1 mL suspension of test compounds in vehicle (0.5% methyl cellulose). The percentage was calculated by the following equation: anti-inflammatory activity (%) = (1 − D/C) − 100, where D represents the difference in paw volume before and after compounds was administered to the rats, and C stands for the difference of volume in the control groups.

4.3. Docking methodology

Docking studies have been performed using MOE 2008.10. With this purpose, crystal structure of COX-2/SC-558 (a selective inhibitor) complex (PDB codes: 1CX2) was obtained from the Protein Data Bank in order to prepare the protein for docking studies. Docking procedure was followed using the standard protocol implemented in MOE 2008.10 and the geometry of resulting complexes was studied using the MOE's Pose Viewer utility.

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