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Design & synthesis of novel oxazolone & triazinone derivatives and their biological evaluation as COX-2 inhibitors

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ABSTRACT

A new series of oxazolones and triazinones were designed and synthesized and evaluated against both COX-1 and COX-2 enzymes. Full structure elucidation of the new derivatives was performed using micro-analyses, IR, 1H NMR, 13C NMR and mass spectra. Most of the derivatives showed good inhibitory activity against COX-2 enzyme specifically compounds IIIc, IIIe, IVd and IVg with IC50 values 0.024, 0.019, 0.011 and 0.014 μM compared to celecoxib as reference drug with IC50 value of 0.05 μM. Altogether, these results indicate that these derivatives can be effective anti-inflammatory agents.

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) nonselective non-steroidal anti-inflammatory drugs (nsNSAIDs) and selective cyclooxygenase 2 NSAIDs (COXIBs) are some of the most widely prescribed drugs in the world, commonly used to treat fever, pain and inflammation.

But NSAIDs produce serious adverse effects, the most important being gastric injury up to gastric ulceration and renal damage [1]. After the discovery of two COX isoforms, it was recognized that selective inhibitors of the inducible form COX-2 expressed mainly in inflammatory cells could provide anti-inflammatory agents devoid of the undesirable effects associated with classical, nonselective NSAIDs [2].

Celecoxib (Celebrex)™ was the first selective COX-2 inhibitor (coxibs) that appeared on the world markets in 1999 as a safer replacement for NSAIDs (non-selective COX-1/COX-2 inhibitors) as it causes less gastrointestinal complications [3]. Many other compounds were used in the treatment of pain and inflammation, such as rofecoxib, valdecoxib and indomethacin [4] Fig. 1.

Oxazolones are important intermediates for the synthesis of several compounds such as amino alcohols, amides [5], amino acids [6,7], dyes [7,8]. Many oxazolones were found to have potent COX-2 inhibitory activity as the diaryl derivative **(1)** where the methyl sulfone group on the 4-phenyl ring was replaced by a sulfonamide moiety resulting in compounds with superior *in vivo* anti-inflammatory properties [9]. On the other hand, Oxaprozin **(2)** is an oxazole derivative that blocks prostaglandin synthesis by non-selective inhibition of both COX-1 & COX-2 [10]. Moreover, A series of 4,5-diphenyl-2-oxo-3H-1,3-oxazole derivatives (**3**) were prepared as selective cyclooxygenase-2 (COX-2) inhibitors [11] Fig. 2.

Triazine derivatives have been reported to possess a broad spectrum of biological activities including antifungal, anti-HIV, anticancer, anti-inflammatory, analgesic and anti-hypertensive [12]. New1,2,4-triazine derivatives bearing hydrazone compounds **(4)** were synthesized and exhibited good anti-inflammatory effect in carrageenan-induced rat paw edema [13]. Also, a series of 5-Aryl-6-(4-methylsulfonyl)-3-(metylthio)-1,2,4-triazine derivatives (**5**) which were evaluated for their COX-1/COX-2 inhibitory activity and showed strong inhibition of COX-2 over COX-1 [14]. Moreover, 5, 6–diphenyl–1,2,4–triazin–3(2H)–one derivatives bearing 5–substituted 1,3,4–oxadiazole **(6)** were found to possess potent COX-2 inhibitory activity [15].

From the above findings and for design purpose it was useful to build on well-established structural features of selective COX-2 inhibitors based on oxazolone and triazinone

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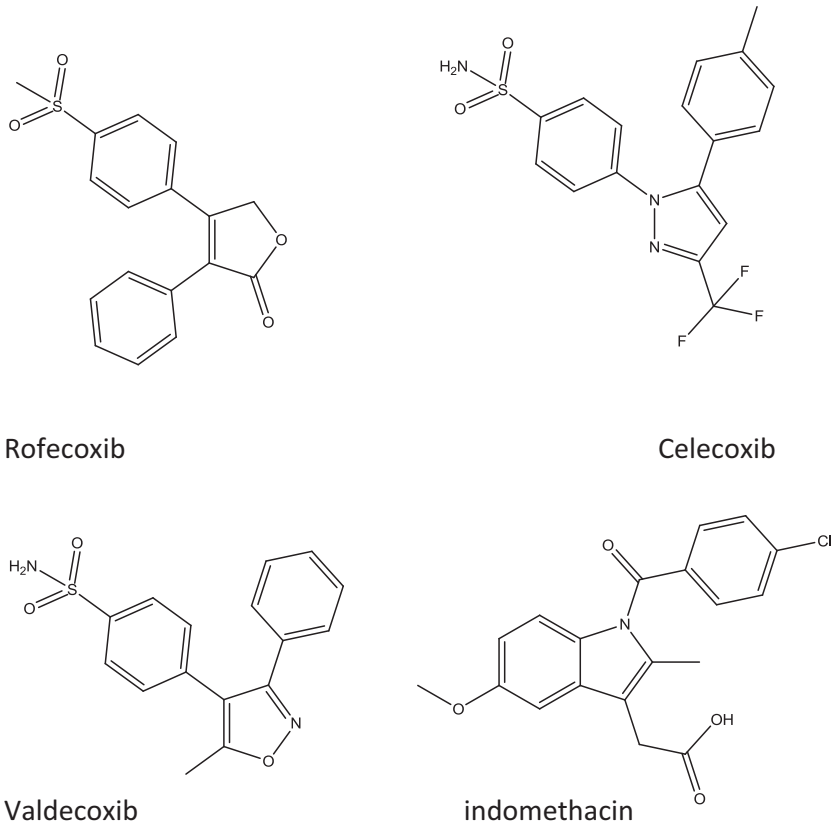


Fig. 1. Structure of some NSAIDs and COX inhibitors.

moiety and mimicking the structure of well-known COX-2 inhibitors.

2. Results and discussion

2.1. Chemistry

The target compounds **IIIa-g and IVa-g** were obtained as the reaction sequence outlined in Scheme 1 starting with nicotinic acid which was converted to nicotinoyl chloride using thionyl chloride by a previously reported procedure [16]. Nucleophilic substitution of chlorine atom in nicotinoyl chloride **I** with glycine in presence of triethylamine in chloroform provided 2-(nicotinamido) acetic acid (**II**) in quantitative yield. The second reaction was accompanied (as an one pot synthesis) by condensation with the appropriate aldehyde in presence of fused sodium acetate in acetic acid for 2 h producing **IIIa-g** in moderate to good yield, the structure was proven by IR spectral bands of CO at ranges from 1781 to 1681 cm^{-1}. In addition, the disappearance of OH band, moreover, 1HNMR spectra showed significant signal of =CH proton at range 7.5–7.8 ppm as a singlet signal. Finally, treatment of the oxazolone derivatives **IIIa-g** with phenyl hydrazine in the presence of fused sodium acetate in acetic acid resulted in the formation of the expected triazine derivatives **IVa-g** which showed the appearance of NH band in IR at 3400–3200 cm^{-1} and in 1HNMR at 3.4–6.16 ppm.

2.2. COX-1&2 inhibitory activities

Compounds **IIIa-g and IVa-g** were evaluated for their inhibitory activities towards COX-1 and COX-2 enzymes (Table 1).

Both series were substituted by an aldehyde bearing an aromatic moiety of different substitutions to examine their effect on activity in order to establish the structure activity relationship. The main difference in the structure of both COX-1 and COX-2 enzymes is larger active site of COX-2 [17] and hence the COX-2 selective drugs have bulky structure which makes the molecules too large to fit into the COX-1 active site but still able to fit the COX-2 active site [18]. Moreover, it was reported that optimal activity against COX-2 enzyme is achieved by tricyclic structure compounds bearing unsaturated heterocyclic ring with attached two aromatic rings [19]. In this paper, the two new synthesized series showed an improved inhibitory activity against COX-2 better than inhibiting COX-1owing to structural resemblance to reported COX-2 inhibitors Fig. 3, the trizine derivatives showed better inhibitory activity than the oxazolone derivatives on both COX-1 and COX-2 enzymes and this may be attributed to the larger size which make it fit better to COX-2 enzyme. Compound **IVd** was more active as COX-2 inhibitor than COX-1 owing to the bulky *p*-methoxy group with IC50 value of 0.011 μM followed by **IVg** a pyridine substituted triazine with IC50 value of 0.014 μM against COX-2 compared to 0.084 μM against COX-1. On the other hand, **IVf** an *o*-substituted hydroxyl triazine showed good inhibitory activity against COX-2 over COX-1 with IC50 values of 0.017 and 0.085 μM respectively. Compounds **IVc, IVe** and **IIIe** have similar inhibitory activity on COX-2 with IC50 value of 0.019 μM where they inhibited COX-1 with IC50 value of 0.096, 0.116 and 0,08 μM. **IIIc, IVb** and **IVa** showed moderate inhibitory activity towards COX-2 but still better than celecoxib with IC 50 values 0.024, 0.034 and 0.035 μM compared to 0.05 μM of celecoxib. Compounds **IIIa,b,d,g,f** though showed inhibitory activity against COX-2 better than COX-1 but their effect was less than celecoxib with IC 50 values 0.059, 0.075, 0.077, 0.085 and 0.106 u μM.

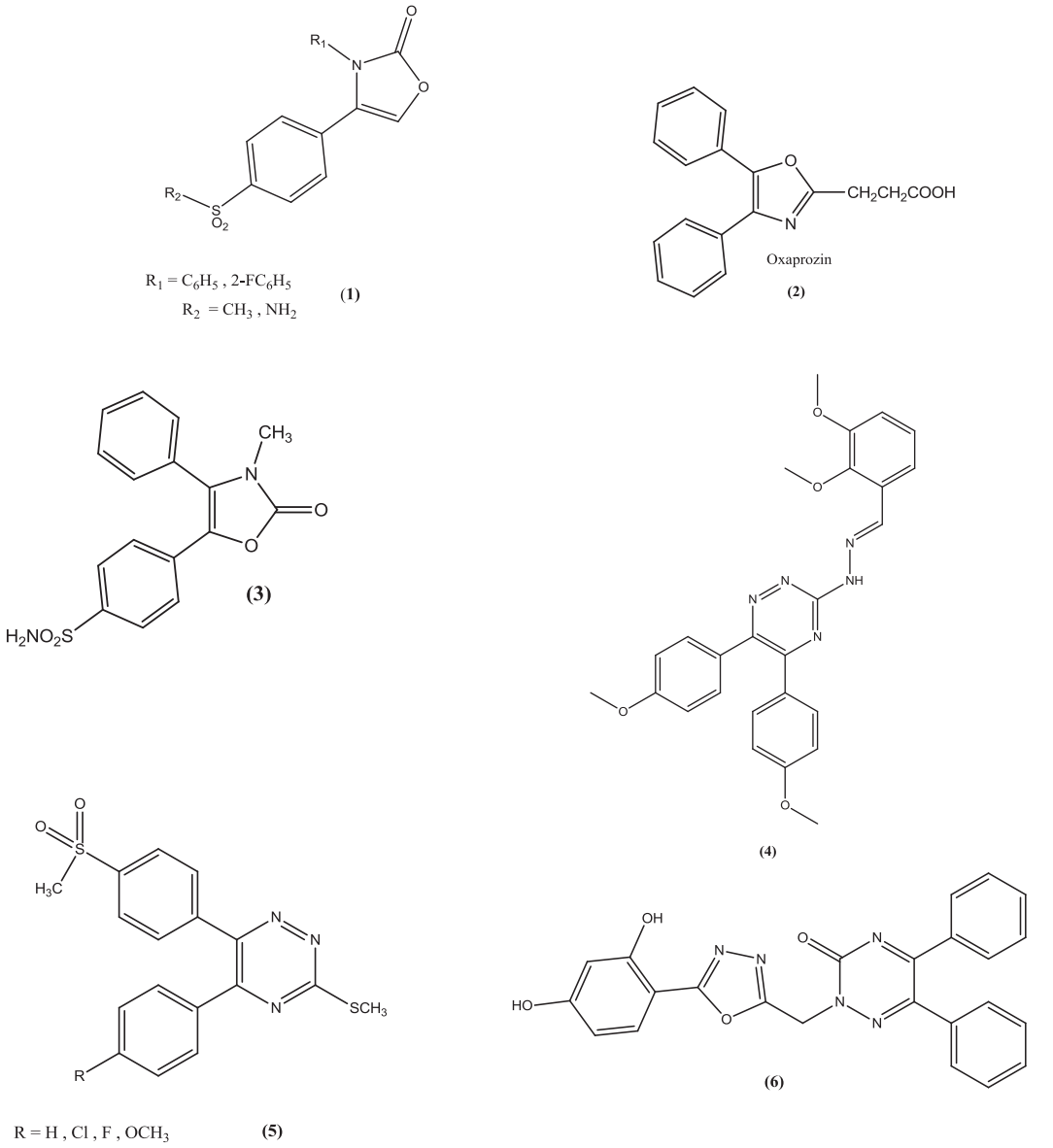


Fig. 2. Structure of some reported biologically active COX inhibitors.

From the above it was clear that the bulkier derivatives showed better activity than the less bulky ones.

3. Conclusion

The synthesis of the new oxazolones and triazinones was performed with the aim of finding structure-activity relationship for COX-2 inhibitory activity. The most promising inhibitors were found in the triazinone series especially compound **IVd** with IC50 value of 0.011 um μM for COX-2 and due to size related potency and to added H-bond binding to amino acids of the receptor by the methoxy derivative and NH of **IVd.**

4. Materials and methods

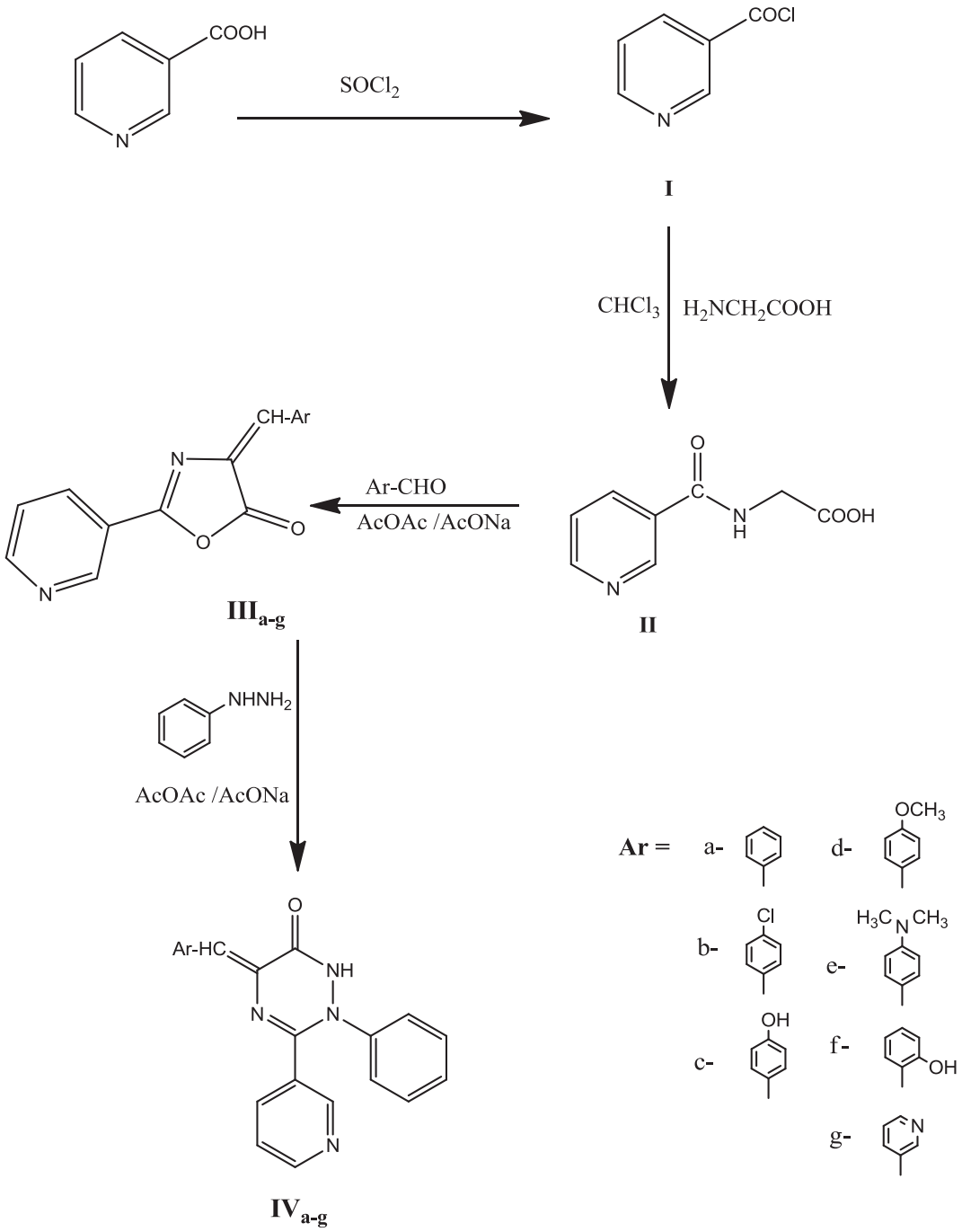
4.1. Chemistry

Melting points were determined on Stuart apparatus and the values given were uncorrected.

IR spectra were determined on Shimadzu IR 435 spectrophotometer at the Faculty of Pharmacy, Cairo University, Egypt. Using KBr discs (values were represented in cm^{-1}). ^1H NMR spectra were recorded on Varian Gemini 300 MHz spectrophotometer at National Research Centre (NRC) Labs., Egypt and Bruker 400 MHz at Faculty of Pharmacy, Cairo University, Egypt. Using TMS as internal standard. Chemical shift values were recorded in ppm on δ scale.

^{13}C NMR spectra were recorded on Varian Gemini 300 MHz spectrophotometer at National Research Centre (NRC) Labs, Egypt. Using TMS as internal standard. Chemical shift values were recorded in ppm on δ scale. Mass spectra were recorded on EI-MS Hewlett Packard 5988 spectrometer at National Research Centre (NRC) Labs., Egypt. Elemental analyses were carried out at the Micro analytical Centre, Alazhar University, Egypt. Progress of the reactions was monitored using TLC aluminum sheets precoated with UV fluorescent silica gel (Merck 60F 254) using $CHCl_3$/Ethanol (9:1) and were visualized using UV lamp Nicotinoyl Chloride **(I)** was synthesized according to reported procedure [16].

Synthesis protocol. General synthesis of compounds IIIa-g & IVa-g.



Scheme 1. Synthesis protocol. General synthesis of compounds **IIIa-g & IVa-g.**

4.1.1. General procedure for the synthesis of 2-(nicotinamido) acetic acid (II)

In a dry flask Nicotinoyl chloride (0.142 mol, 20 g) was added portion wise to a solution of glycine (0.142 mol, 10.6 g) and triethylamine (0.5 mL)in dry chloroform (100 mL). The reaction mixture was stirred at 50 °C for 7 h, filtered, washed with chloroform, dried and crystallized from chloroform.

Mp 238–240 °C, yield: 12.5 g (83%), IR (KBr) cm^{-1}: 3417 (NH), 3201 (COOH), 3055 (CH aromatic), 2927, 2900 (CH aliphatic), 1716 (C=O). ^{1}H NMR 400 MHz (DMSO-d_6): 3.90 (s, 2H, CH_2), 7.60 (t, 1H, Pyridine C5H), 7.72 (s, 1H, NH, D_2O exchangeable), 8.31 (d, *j = 3.45*, 2H, Pyridine C4), 8.70 (d, *j = 2.6*, 2H, Pyridine C6H), 9.10 (s, 1H, Pyridine C2H), 9.50 (s, 1H, COOH, D_2O exchangeable) ppm. *m/z* (% abundance): 181 (M^++1, 20%), 79 (C_5H_4N, 100%). Anal. Calcd for $C_8H_8N_2O_3$: C, 53.33; H, 4.48; N, 15.55. Found: C, 53.51; H, 4, 54; N, 15.79.

4.1.2. General procedure for the synthesis (III_{a-g})

In a dry flask a mixture of an appropriate aldehyde (0.010 mol), (**II**) (0.011 mol, 2 g), and fused sodium acetate (0.012 mol, 1 g) in acetic anhydride (3 mL) was heated under reflux for 2 h. After completion of reaction, ethanol (4 mL) was added and the reaction mixture was kept at room temperature for 18 h. The solid formed

Table 1
The IC50 values of celecoxib, the target compounds **IIIa-g and IVa-g** against COX-1 and COX-2 enzymes.

Compound	IC50 COX-1 μmol	IC50 COX-2 μmol
Celecoxib	14.8	**0.05**
IIIa	0.44	0.077
IIIb	0.45	0.106
IIIc	0.27	**0.024**
IIId	0.21	0.059
IIIe	0.08	**0.019**
IIIf	0.44	0.085
IIIg	0.34	0.075
IVa	0.15	0.035
IVb	0.16	0.034
IVc	0.096	**0.019**
IVd	0.108	**0.011**
IVe	0.116	**0.019**
IVf	0.085	**0.017**
IVg	0.084	**0.014**

was filtered, washed with ethanol, dried and crystallized from ethanol.

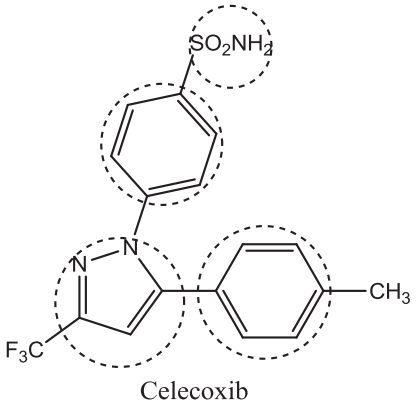
4.1.2.1. 4-Benzylidene-2-(pyridin-3-yl) oxazol-5(4H)-one (IIIa). Mp 167 °C, yield: 68%, IR (KBr) cm^{-1}: 3055 (CH aromatic), 2927 (CH aliphatic), 1735 (C=O), 1624 (C=C aliphatic). ^{1}H NMR 400 MHz (DMSO-d_6): 7.11 (m, 5H, Phenyl), 7.50 (s, 1H, =CH), 8.22 (t, 1H, Pyridine C5H), 8.70 (t, 2H, Pyridine C4, 6H), 9.0 (s, 1H, Pyridine C2H). *m/z* (% abundance): 250 (M^+, 1.4%), 79 (C_5H_5N, 100%). Anal. Calcd for $C_{15}H_{10}N_2O_2$: C, 71.99; H, 4.03; N, 11.19. Found: C, 72.13; H, 4,62; N, 11.34.

4.1.2.2. 4-(4-Chlorobenzylidene)-2-(pyridin-3-yl)oxazol-5(4H)-one (IIIb). Mp 172 °C, yield: 70%, IR (KBr) cm^{-1}: 3051 (CH aromatic), 2981 (CH aliphatic), 1681 (C=O), 1650 (C=C aliphatic). ^{1}H NMR 400 MHz (DMSO-d_6) 7.52 (m, 4H, Phenyl), 7.58 (s, 1H, =CH), 7.89 (t,1H, Pyridine C5H), 7.91 (t,2H, Pyridine C4,6H), 7.95 (s,1H, Pyridine C2H). Anal. Calcd for $C_{15}H_9ClN_2O_2$: C, 63.28; H, 3.19; N, 9.84. Found: C, 63.47; H, 3.31; N, 10.07.

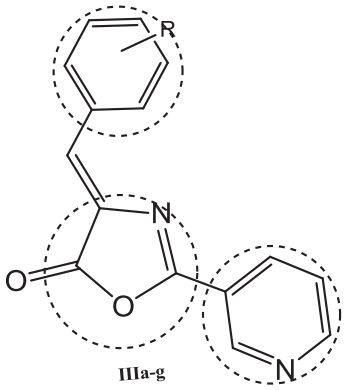
4.1.2.3. 4-(4-Hydroxybenzylidene)-2-(pyridin-3-yl)oxazol-5(4H)-one (IIIc). Mp 184 °C, yield: 60%, IR (KBr) cm^{-1}: 3410 (OH), 3070 (CH aromatic), 2927 (CH aliphatic), 1705 (C=O), 1682 (C=C aliphatic). ^{1}H NMR 400 MHz (DMSO-d_6): 6.79 (s, 1H, OH, D_2O exchangeable), 7.16 (d, *j = 2.13*, 2H, Phenyl), 7.43 (d, *j = 2.14*, 2H, Phenyl), 7.60 (s, 1H, =CH), 8.18 (t, 1H, Pyridine C5H), 8.65 (t, 2H, Pyridine C4, 6H), 9.01 (s, 1H, Pyridine C2H). *m/z* (% abundance): 266 (M^+, 10%), 78 (C_5H_4N, 100%). Anal. Calcd for $C_{15}H_{10}N_2O_3$: C, 67.67; H, 3.79; N, 10.52. Found: C, 67.79; H, 3.74; N, 10.72.

4.1.2.4. 4-(4-Methoxybenzylidene)-2-(pyridin-3-yl)oxazol-5(4H)-one (IIId). Mp 184 °C, yield: 60%, IR (KBr) cm^{-1}: 3093 (CH aromatic), 2854 (CH aliphatic), 1716 (C=O), 1651 (C=C aliphatic). ^{1}H NMR 400 MHz (DMSO-d_6): 3.30 (s, 3H, OCH_3), 6.74 (d, *j = 2.12*, 2H, Phenyl), 7.01 (d, *j = 2.13*, 2H, Phenyl), 7.60 (s, 1H, =CH), 8.20 (t, 1H, Pyridine C5H), 8.72 (t, 2H, Pyridine C4, 6H), 9.02 (s, 1H, Pyridine C2H). ^{13}C NMR (DMSO-d_6-100 MHz, ppm): 39.94 (OCH_3), 124.50 (Phenyl C3,5), 127.34 (C=C—N aliphatic), 130.00 (C=C—N aliphatic), 138.12 (Phenyl C1,2,6), 150.15 (Pyridine C3,4,5), 153.08 (Pyridine C6, PhenylC4), 166.55 (Pyridine C2), 170.00 (Oxazole C=N), 172.5.56 (C=O). Anal. Calcd for $C_{16}H_{12}N_2O_3$: C, 68.56; H, 4.32; N 9.99. Found: C, 68.41; H, 4.32; N, 10.09.

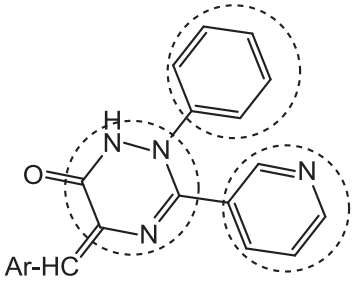
4.1.2.5. 4-(4-(Dimethylamino)benzylidene)-2-(pyridin-3-yl)oxazol-5 (4H)-one (IIIe). Mp 192 °C, yield: 62%, IR (KBr) cm^{-1}: 3077 (CH aromatic), 2897, 2862 (CH aliphatic), 1706 (C=O), 1643 (C=C



Celecoxib



IIIa-g



IVa-g

Fig. 3. Structure resemblance of new derivatives **IIIa-g IVa-g** to celecoxib.

aliphatic). ^{1}H NMR 400 MHz (DMSO-d_6): 3.09 (s, 6H, $2CH_3$), 6.70 (d, *j = 2.6*, 2H, Phenyl), 7.20 (d, *j = 2.4*, 2H, Phenyl), 7.51 (s, 1H, =CH), 8.11 (t, 1H, Pyridine C5H), 8.40 (t, 2H, Pyridine C4, 6H), 9.40 (s, 1H, Pyridine C2H). ^{13}C NMR (DMSO-d_6-100 MHz, ppm): 40.55 ($2CH_3$), 121.48 (Phenyl C3,5), 127.25 (C=C—N aliphatic), 135.04 (C=C—N aliphatic),148.63 (Phenyl C1,2,6), 148.64 (Pyridine C3,4,5), 152.11 (Pyridine C6, PhenylC4), 158.22 (Pyridine *C*2), 167.94 (Oxazole C=N), 170.92 (C=O). MS: *m*/*z* (% abundance): 293 (M^+, 45%), 159 ($C_9H_5NO_2$, 100%). Anal. Calcd for $C_{17}H_{15}N_3O_2$: C, 69.61; H, 5.15; N, 14.33. Found: C, 69.78; H, 5.17; N, 14.71.

4.1.2.6. 4-(2-Hydroxybenzylidene)-2-(pyridin-3-yl)oxazol-5(4H)-one (IIIf). Mp 182 °C, yield: 67%, IR (KBr) cm^{-1}: 3320 (OH), 3140 (CH aromatic), 2954 (CH aliphatic), 1716 (C=O), 1635 (C=C aliphatic). ^{1}H NMR 400 MHz (DMSO-d_6):5.55 (s, 1H, OH, D_2O exchangeable), 7.19 (m, 4H, Phenyl), 7.80 (s, 1H, =CH), 8.54 (t, 1H, Pyridine C5H), 8.89 (t, 2H, Pyridine C4, 6H), 9.10 (s, 1H, Pyridine C2H). Anal. Calcd for $C_{15}H_{10}N_2O_3$ C, 67.67; H, 3.79; N, 10.52. Found: C, 67.89; H, 3.77; N, 10.69.

4.1.2.7. 2-(pyridin-3-yl)-4-(pyridin-3-ylmethylene)oxazol-5(4H)-one (IIIg). Mp 172 °C, yield: 55%, IR (KBr) cm^{-1}: 3055 (CH aromatic), 2856 (CH aliphatic), 1718 (C=O), 1670 (C=C aliphatic). ^{1}H NMR 400 MHz (DMSO-d_6):7.70 (s, 1H, =CH), 7.80 (t, 2H, Pyridine C5, 5′H), 8.51 (m, 4H, Pyridine C4, 4′, 6, 6′H), 9.11 (d, *j = 3.6*,2H, Pyridine *C*2, 2′H). Anal. Calcd for $C_{14}H_9N_3O_2$: C, 66.93; H, 3.61; N, 16.73. Found: C, 66.98; H, 3.67; N, 16.88.

4.1.3. General procedure for the synthesis (IV_{a-g})

In dry flask a solution of oxazolone **III_{a-g}** (0.016 mol) in acetic acid (20 mL) was treated with phenyl hydrazine (0.016 mol, 1.72 g) and heated under reflux for 6 h in presence of fused anhydrous sodium acetate (2.44 mmol, 0.2 g). The reaction mixture was cooled, filtered, dried and crystallized from benzene.

4.1.3.1. 5-Benzylidene-2-phenyl-3-(pyridin-3-yl)-1,2-dihydro-1,2,4-triazin-6(5H)-one (IVa). Mp 224 °C, yield: 68%, IR (KBr) cm^{-1}: 3352 (NH), 3055 (CH aromatic), 2850 (CH aliphatic), 1712 (C=O), 1641 (C=C aliphatic). ^{1}H NMR 400 MHz (DMSO-d_6): 7.40 (m, 5H, Phenyl), 7.81 (m, 5H, Phenyl), 7.89 (s, 1H, =CH), 8.62 (t, 1H, Pyridine C5H), 8.90 (t, 2H, Pyridine C4, 6H), 9.11 (s, 1H, Pyridine C2H), 9.15 (s, 1H, NH, D_2O exchangeable), Anal. Calcd for $C_{21}H_{16}N_4O$: C, 74.10; H, 4.74; N, 16.46. Found: C, 74.19; H, 4.80; N, 16.57.

4.1.3.2. 5-(4-Chlorobenzylidene)-2-phenyl-3-(pyridin-3-yl)-1,2-dihydro-1,2,4-triazin-6(5H)-one (IVb). Mp 231 °C, yield: 70%, IR (KBr) cm^{-1}: 3200 (NH), 3091 (CH aromatic), 2837 (CH aliphatic), 1685 (C=O), 1641 (C=C aliphatic). ^{1}H NMR 400 MHz (DMSO-d_6): 6.63 (m, 5H, Phenyl), 7.47 (d, *j = 5.16*, 2H, Phenyl), 7.61 (s, 1H, =CH),7.85 (t, 1H, Pyridine C5H), 7.89 (d, *j = 5.16*, 2H, Phenyl), 8.15 (t, 2H, Pyridine C4, 6H), 8.30 (s, 1H, Pyridine C2H), 9.60 (s, 1H, NH,D_2O exchangeable), Anal. Calcd for $C_{21}H_{15}ClN_4O$: C, 67.29; H, 4.03; N, 14.95. Found: C, 67.37; H, 4.05; N, 15.06.

4.1.3.3. 5-(4-Hydroxybenzylidene)-2-phenyl-3-(pyridin-3-yl)-1,2-dihydro-1,2,4-triazin-6(5H)-one (IVc). Mp 252 °C, yield: 62%, IR (KBr) cm^{-1}: 3445 (OH), 3205 (NH), 3093 (CH aromatic), 2929 (CH aliphatic), 1716 (C=O), 1666 (C=C aliphatic). ^{1}H NMR 400 MHz (DMSO-d_6): 3.40 (s, 1H, OH,D_2O exchangeable), 6.63 (m, 5H, Phenyl),7.11 (s, 1H, =CH), 7.53 (m, 4H, Phenyl),8.25 (t, 1H, Pyridine C5H), 8.77 (t, 2H, Pyridine C4, 6H), 9.06 (s, 1H, Pyridine C2H), 9.07 (s, 1H, NH, D_2O exchangeable), Anal. Calcd for $C_{21}H_{16}N_4O_2$: C, 70.77; H, 4.53; N, 15.72. Found: C, 70.98; H, 4.62; N, 15.89.

4.1.3.4. 5-(4-Methoxybenzylidene)-2-phenyl-3-(pyridin-3-yl)-1,2-dihydro-1,2,4-triazin-6(5H)-one (IVd). Mp 234 °C, yield: 73%, IR (KBr) cm^{-1}: 3238.48 (NH), 3055 (CH aromatic), 2854 (CH aliphatic), 1716 (C=O),1666 (C=C aliphatic). ^{1}H NMR 400 MHz (DMSO-d_6): 3.30 (s, 3H, OCH_3), 6.64 (m, 5H, Phenyl),7.06 (s, 1H, =CH), 7.89 (m, 4H, Phenyl), 8.63 (t, 1H, Pyridine C5H), 8.95 (t, 2H, Pyridine C4, 6H),9.16 (s, 1H, Pyridine C2H), 9.26 (s, 1H, NH, D_2O exchangeable), 13C NMR (DMSO-d_6-100 MHz, ppm): 39.59 (OCH_3), 124.61 (C=C—N aliphatic, Phenyl C2, 3′, 4′, 5, 6′), 127.99 (Phenyl C1, 2, 3′, 5, 6), 140.44 (C=C—N aliphatic, Pyridine C 3, 4, 5),148.15 (Pyridine C2, 6, Phenyl C1′, 4′), 151.09 (triazine C=N), 166.1 (C=O). **MS**: *m*/*z* (% abundance): 370 (M^+, 1%), 82 (C_3H_2N2O, 100%). Anal. Calcd for $C_{22}H_{18}N_4O_2$: C, 71.34; H, 4.90; N, 15.13. Found: C, 71.49; H, 4.95; N, 15.30.

4.1.3.5. 5-(4-(Dimethylamino) benzylidene)-2-phenyl-3-(pyridin-3-yl)-1,2-dihydro-1,2,4-triazin-6(5H)-one (IVe). Mp 239 °C, yield: 65%, IR (KBr) cm^{-1}: 3352 (NH), 3055 (CH aromatic), 2854 (CH aliphatic), 1716 (C=O), 1635 (C=C aliphatic). ^{1}H NMR 400 MHz (DMSO-d_6): 3.09 (s, 6H, $2CH_3$), 6.69 (m, 5H, Phenyl), 6.80 (d, *j = 8.72*,2H, Phenyl), 7.18 (s, 1H, =CH), 7.20 (d, *j = 6.72*, 2H, Phenyl), 8.24 (t, 1H, Pyridine C5H), 8.70 (t, 2H, Pyridine C4, 6H), 9.22 (s, 1H, Pyridine C2H), 9.35 (s, 1H, NH,D_2O exchangeable), Anal. Calcd for $C_{23}H_{21}N_5O$: C, 72.04; H, 5.52; N, 18.26. Found: C, 72.13; H, 5.56; N, 18.53.

4.1.3.6. 5-(2-Hydroxybenzylidene)-2-phenyl-3-(pyridin-3-yl)-1,2-dihydro-1,2,4-triazin-6(5H)-one (IVf). Mp 246 °C, yield: 62%, IR (KBr) cm^{-1}: 3414 (OH), 3400 (NH), 3095 (CH aromatic), 2926 (CH aliphatic), 1716 (C=O), 1635 (C=C aliphatic). ^{1}H NMR 400 MHz (DMSO-d_6): 6.16 (s, 1H, OH, D_2O exchangeable), 6.65 (m, 5H, Phenyl),7.15 (m, 3H, Phenyl),7.70 (t, 1H, Phenyl), 7.74 (s, 1H, =CH), 8.44 (t, 1H, Pyridine C5H), 8.86 (t, 2H, Pyridine C4, 6H), 9.11 (s, 1H, Pyridine C2H), 9.75 (s, 1H, NH, D_2O exchangeable). Anal. Calcd for $C_{21}H_{16}N_4O_2$: C, 70.77; H, 4.53; N, 15.72. Found: C, 70.89; H, 4.62; N, 15.88.

4.1.3.7. 2-Phenyl-3-(pyridin-3-yl)-5-(pyridin-3-ylmethylene)-1,2-dihydro-1,2,4-triazin-6(5H)-one (IVg). Mp 230 °C, yield: 60%, IR (KBr) cm^{-1}: 3381 (NH), 3055 (CH aromatic), 2854 (CH aliphatic), 1714 (C=O), 1653 (C=C aliphatic). 1H NMR 400 MHz (DMSO-d_6): 6.69 (m, 5H, phenyl), 7.11 (s, 1H, =CH), 8.30 (m, 2H, Pyridine C5, 5′H), 8.80 (m, 4H, Pyridine C4, 4′, 6, 6′H), 9.10 (d, *j = 1.6*, 2H, Pyridine *C*2, 2′H), 9.65 (s, 1H, NH, D_2O exchangeable). Anal. Calcd for $C_{20}H_{15}N_5O$: C, 70.37; H, 4.43; N, 20.52. Found: C, 70.54; H, 4.47; N, 20.71.

4.2. Biological evaluation

The *in vitro* COX-1 and COX-2 inhibition by compounds **IIIa-g** and **IVa-g** was evaluated through Cayman's colorimetric COX (ovine) inhibitor screening assay measures the Peroxidase component of COX 1 & 2. The Peroxidase activity is assayed calorimetrically by monitoring the appearance of oxidized N,N,N′,N′-tetra methyl -p-phenylenediamine (TMPD) at 590 nm. The colorimetric COX (ovine) inhibitor screening assay kit includes both ovine COX-1 and COX-2 enzymes in order to screen isozyme-specific inhibitors.

4.2.1. Pre-Assay preparation

Dilute 3 mL of assay buffer concentrate with 27 mL of HPLC - grade water. This final assay buffer (0.1 M Tris-HCl, pH 8) should be used for dilution of heme and COX enzymes prior to assaying. This vial contains a solution of heme in dimethylsulphoxide. Dilute 88 μL of heme with 1.912 mL of diluted assay buffer prior to use. A

vial contains a solution of ovine COX-1 and should be kept on ice when thawed. Dilute 200 μL of enzyme with 400 μL of diluted assay buffer and store on ice. A vial contains a solution of ovine COX-2 and should be kept on ice when thawed. Dilute 200 μL of enzyme with 400 μL of diluted assay buffer and store on ice. A vial contains a solution or arachidonic acid in ethanol. Transfer 100 μL of the supplied substrate to another vial, add 100 μL of potassium hydroxide (item no. 760115), vortex, and dilute with 1.8 mL of HPLC - grade water to achieve a final concentration of 1.1 mM. Use the prepared arachidonic acid solution within 30 min. A 20 μL aliquot will yield a final concentration of 100 μM in the wells. A vial contains 0.1 M potassium hydroxide (KOH). A vial contains a solution of TMPD.

4.2.2. Performing the assay

Background wells: add 160 μL of assay buffer, and 10 μL of heme to three wells.

100% Initial Activity wells: add 150 μL assay buffer, 10 μL of heme, and 10 μL of enzyme (either COX-1 or COX-2) to three wells. Inhibitor wells: add 150 μL of assay buffer, 10 μL of heme, and 10 μL of enzyme (either COX-1 or COX-2) to three wells. Add 10 μL of inhibitor to the inhibitor wells and 10 μL of solvent (methanol, dimethylsulphoxide or ethanol) to the 100% Initial Activity wells and background wells, this process was repeated three times. Carefully shake the plate for a few seconds and incubate for five minutes at 25 °C. Add 20 μL of the colorimetric substrate solution to all the wells that you are using. Add 20 μL of arachidonic acid to all the wells you are using. Carefully shake the plate for few seconds and incubate for five minutes at 25 °C. Read the absorbance at 590 nm using a plate reader.

4.2.3. Data analysis

Determine the average absorbance of all the samples. Subtract the absorbance of the background wells from the Initial Activity sample, then divide by the 100% Initial Activity sample, and multiply by 100 to give the percent inhibition. Graph the percent inhibition and determine the IC_{50} value by using the three results obtained. (Concentration at which there was 50% inhibition).

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