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Design, synthesis and biological evaluation of substituted 9-oxo-1,2-dihydropyrrolo[2,1-b]quinazolin-3(9H)-ylidene)methyl)piperidine-1-carboxamide derivatives as dual COX-2 and (sEH) inhibitor

Vijaykumar Nimbarte

Department of Medicinal Chemistry, National Institute of Pharmaceutical Education and Research (NIPER) Hyderabad, Balanagar, Hyderabad, India

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ABSTRACT

A series of novel dual inhibitor of substituted 9-oxo-1,2-dihydropyrrolo[2,1-b]quinazolin-3(9H)-ylidene)methyl)piperidine-1-carboxamide Derivatives as a pharmacophore lead for Potent antiinflammatory and sEH inhibition have been designed, synthesized and evaluated as novel analogues to act as selective COX-2 Inhibitors over COX-1 which prevent blood pressure elevation by acting as sEH inhibitors in addition. The synthesized compounds ***10e*** *and* ***10g*** *exhibit varying degrees COX-2 selectivity and inhibition of sEH enzyme displaying* IC_{50} *values of* ***0.124±0.011µM*** *and* ***0.110±0.01µM*** *for in-vitro sEH inhibition respectively.*

Keywords: Anti inflammation, Hypertension, Inflammation, sEH inhibition etc.

INTRODUCTION

Isaindigotone derivatives have been synthesized and evaluated for their biological a wide spectrum of pharmacologic activities such as antiinflammatory and analgesic activity [1]. Isaindigotone derivatives are neither polycyclic nor macrocyclic, they have a unique asymmetric unfused aromatic chromophore with an aliphatic five-member ring in the center core due to that free rotation around the single bond takes place and allows the possible twisted or coplanar conformations of the aryl groups [2].

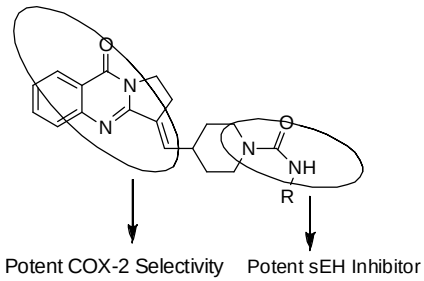


Figure 1: Figure of Potent dual COX-2 and sEH Inhibitor

Various synthetic Isaindigotone derivatives have been synthesized by incorporating terminal amine side chains these analogues show multiple binding with the active site, midgorge recognition site and the PAS of the enzyme. Moreover they act as AChE and BuChE inhibitors. Structure–activity relationships reveal that the length of side chain is an important factor for the inhibitory activity [3] potential side effect of selective COX-2 Inhibition is

elevation in blood pressure which can be overcome by including pharmacophore for sEH Inhibition i.e piperidine ring substituted urea side chain.

The sEH is also involved in the metabolism of arachidonic acid, 4 linoleic acid [4] and other lipid epoxides, some which are endogenous chemical mediators. Epoxides arachidonic acid (epoxyeicosatrienoic acids or EETs) are known effectors of blood pressure [5] and modulators vascular permeability [6, 7]. Hydrolysis of the epoxides by sEH diminishes this activity [8]. It is already reported in that treatment with selective sEH inhibitors significantly reduces the blood pressure of spontaneous hypertensive rats (SHRs) or angiotensin II induced hypertension in rats [9, 10] in addition, male knockout sEH mice have significantly lower blood pressure than wild-type mice, further supporting the role of sEH in blood pressure regulation. sEH hydrolysis of EETs also regulates their incorporation into coronary endothelial phospholipids mediates a regulation of endothelial function by sEH [11, 12]. The EETs have also demonstrated anti-inflammatory properties in endothelial cells [13, 14]. In contrast, diols derived from epoxylinoleate (leukotoxin) perturb membrane permeability and calcium homeostasis [15], which is responsible for results in inflammation that is modulated by nitric oxide synthase and endothelin-1 [16]. Micro molar concentrations of leukotoxin reported in association with inflammation and hypoxia[17] depress mitochondrial respiration in vitro [18] and cause mammalian cardiopulmonary toxicity in vivo [19, 20, and 21]. Leukotoxin toxicity presents symptoms suggestive of multiple organ failure and acute respiratory distress syndrome (ARDS) [22]. In both cellular and organismal models, leukotoxin-mediated toxicity is dependent upon epoxide hydrolysis, suggesting a role for sEH in the regulation of inflammation. The bioactivity of these epoxy-fatty acids suggests that inhibition of *vic*-dihydroxy-lipid biosynthesis may have therapeutic value, making sEH a promising pharmacological target.

All analogues were screened by using In *vitro* Cayman's fluorescence-based sEH inhibitor screening assay kit containing mammalian recombinant sEH enzyme and analogues which showed greater than 80% inhibition were taken and IC_{50} values were determined for them [23]. The obtained results were showing that the compounds are sEH inhibition. Further these synthesized compounds selectivity towards COX-2 over COX-1 inhibition activity has been evaluated by using In *vitro* Cayman's fluorescence-based COX inhibitor screening assay kit [24].

MATERIALS AND METHODS

General Materials and Methods

Reagents and solvents were purchased from commercial suppliers (Across, Sigma–Aldrich, Avra) and used as provided, unless indicated otherwise. All the solvents used for reactions were analytical grade and used as provided. Reactions were carried out in oven-dried glassware under nitrogen atmosphere and stirred at room temperature, unless indicated otherwise. AUDA was purchased from Prolab.

^{1}H NMR was recorded on either a Varian Unity 500 or Bruker Avance 300 MHz .The samples were made in $CDCl_3$ and/or DMSO-d_6 using TMS (tetramethylsilane) as the internal standard. The chemical shifts are expressed as d values in parts per million (ppm), using the residual solvent peaks (chloroform: ^{1}H, 7.26 ppm; DMSO: ^{1}H, 2.50 ppm) as a reference. Coupling constants are given in Hertz (Hz). The peak patterns are indicated by the following abbreviations: brs = broad singlet, s = singlet, d = doublet, t = triplet, q = quartet and m = multiple.

High resolution mass spectra were recorded on Micro Mass VG-7070H Mass spectrometer for ESI. Infrared spectra were recorded on Perkin-Elmer Infrared spectrophotometer with NaCl optics. Spectra were calibrated against the polystyrene absorption at 1610 cm^{-1}. Samples were scanned in neat KBr pellets. Melting points were determined on an SMP3 Stuart melting point apparatus and are uncorrected.

For TLC, precoated aluminum sheets were used (Merck, Silica Gel 60 F_{254}). The spots were visualized by UV light. Column chromatography was performed using silica gel (60-120, 100-200 mesh) and the column was usually eluted with EtoAc/Hexanes. All evaporation of solvents was carried out under reduced pressure on Heidolph Laborota-4000 rotary evaporator below 45 ^{o}C. All compounds were characterized by NMR and MS.

Starting material: The isonipecotic acid **1** was commercially obtained from Avra lab and used as provided.

tert-Butoxycarbonyl) piperidine-4-carboxylic acid (2). To a solution of acid **1** (1 g, 7.75 mmol) in THF (5 mL) was added dropwise 1N NaOH (20 mL) at 0 ^{o}C. To this was added $(Boc)_2O$ (4 mL, 16.7 mmol) drop-wise over a period of 5 min with vigorous stirring. After 30 min, the mixture was brought to room temperature and allowed to stir overnight. The resulting mixture was concentrated to half of the original volume and then neutralized with dilute HCl (pH 5-6). The precipitated compound was filtered, washed with water and air dried to give the titled compound **2** (1.73 g, 97%) as a white color solid; mp 144-147 ^{o}C; TLC: Methanol, R_f ~ 0.7; ^{1}H NMR ($CDCl_3$, 300 MHz): δ

4.02 (d, 2H, *J*=12.0 Hz), 2.85 (t, 2H, *J*=12.0 Hz), 2.54 – 2.43 (m, 1H), 1.9 (d, 2H, *J* = 13.2), 1.71-1.56 (m, 2H), 1.45 (s, 9H). MS (ESI): m/z 252 $[M+23]^+$.

tert-butyl 4-formylpiperidine-1-carboxylate (3).

A. ***N, N*-Dimethylchloromethylenammonium chloride** A 500-mL, three-necked, round-bottomed flask is equipped with a magnetic stirring bar, a thermometer, and a three-way stopcock fitted with a drying tube containing anhydrous calcium chloride and a rubber septum. The flask is charged with 50 mL of dichloromethane and 3.07 (42 mmol) of *N*,*N*-Dimethylformamide added through the septum from a syringe, and cooled in an ice bath. To the cooled mixture is slowly added 5.23 mL (60 mmol) of oxalyl chloride by means of a syringe. The addition is accompanied by gas evolution and formation of a white precipitate. The reaction mixture is stirred for an additional hour at 0°C. Excess oxalyl chloride and solvent are removed under reduced pressure by first using a water aspirator and then a rotary pump at room temperature through the drying tube. The white solid remaining in the flask is ***N, N*-Dimethylchloromethylenammonium chloride** which is used directly in Part B. The reaction mixture was allowed to warm to room temperature and stirred for overnight. The resulting mixture was quenched with cool water and acidified to pH 5-6 with 5M citric acid. It was extracted with EtoAc/hexanes (1:1) (2 x 100 mL, then 2 x 50 mL) and the combined organic extracts were washed with brine and dried over anhydrous $NaSO_4$.

The drying tube is removed and the flask is flushed with nitrogen. A nitrogen atmosphere is maintained throughout the subsequent reaction. A dropping funnel is attached and charged with 7.45 g (32.53 mmol) of tert-Butoxycarbonyl) piperidine-4-carboxylic acid, 3.32 g of pyridine, and 80 mL of tetrahydrofuran, which are mixed well by shaking. The flask is charged with 45 mL of acetonitrile and 80 mL of tetrahydrofuran and cooled (methanol–liquid nitrogen) to −30°C. The contents of the funnel are added to the flask at −30°C over 30 min. The reaction mixture is stirred at −30°C for an additional hour and at −20°C for 30 min. After the mixture is cooled to −90°C, 34 mL (46 mmol) of a 1.35 *M* solution of lithium tri(*tert*-butoxy) aluminium hydride in tetrahydrofuran is injected through the septum by means of a syringe over 30 min, while the internal temperature is kept below −85°C. Stirring is continued for an additional 30 min at −90°C. To the flask is added 50 mL of 2 *M* hydrochloric acid solution, and the cooling bath is immediately removed. The organic layer is separated and the aqueous layer is extracted with three 50-mL portions of ether. The combined organic extracts are washed with two 50-mL portions of saturated sodium hydrogen carbonate solution and 50 mL of brine, dried over anhydrous sodium sulfate, and filtered. The solvent is removed with a rotary evaporator and the residual liquid is distilled under reduced pressure to yield 5.78–6.35 g (85–93%) of tert-butyl 4-formylpiperidine-1-carboxylate (3). as a white solid, mp 85–90°C **(3)**; TLC: EtoAc/ Hexanes (3:7), $R_f \sim 0.5$; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 9.50 (s, 1H), 4.18-4.11 (m, 2H), 2.79-2.62 (m, 2H), 2.34-2.27 (m, 1H), 2.07-2.03 (m, 3H), 1.58 – 1.52 (m, 2H), 1.45 (s, 9H). MS (ESI): m/z 214 $[M+1]^+$.

2,3-dihydropyrrolo[2,1-b]quinazolin-9(1H)-one (6)

To a stirred suspension of pyrrolidin-2-one (0.86 g, 10 mmol) and 2-amino benzoic acid (1.37 g, 10 mmol) in 300 mL anhydrous toluene, $POCl_3$ (4 mL) was added drop wise at room temperature and the mixture was refluxed for 8 h. The reaction product was poured onto ice, and then sat. Sodium bicarbonate was added to make the solution neutral. The precipitate was separated from water by filtration, and the filtrate was extracted with 3 × 100 mL portions of ethyl acetate. The combined organic dried over sodium sulfate, and concentrated to dryness, purified by flash chromatography with ethyl acetate/hexane (20:80) to afford compound **14** (1.5 g, 80%); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.20 (d, 1H, J = 7.5 Hz), 7.61-7.63 (m, 1H), 7.57 (d, 1H, *J* = 7.5 Hz), 7.40-7.35 (m, 1H), 4.14 (t, 2H, *J* = 7.5 Hz), 3.12 (t, 2H, *J* = 7.5 Hz), 2.27-2.15 (m, 2H); MS (ESI): *m/z* 187 $[M+1]^+$.

***(E)*-tert-butyl 4-((9-oxo-1,2-dihydropyrrolo[2,1-b]quinazolin-3(9H)-ylidene)methyl)piperidine-1-carboxylate (7).** A mixture of 2,3-dihydropyrrolo[2,1-b]quinazolin-9(1H)-one (**14**) (500 mg, 2.86 mmol), tert-butyl 4-formylpiperidine-1-carboxylate (642mg, 2.86 mmol), and Ac_2O (10 mL) was heated at reflux temperature for 24 h. After cooling, the solvent was removed under reduced pressure, and the crude product was purified by column chromatography on silica gel using EtOAc/hexane (30:70) as eluent to give compound **7** (700mg, 84%). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.28 (d, 1H, J = 7.5 Hz), 7.86-7.82 (m, 1H), 7.74 (d, 2H, *J* = 3.0 Hz), 7.57 (d, 2H, *J* = 8.3 Hz), 7.47-7.41 (m, 1H), 7.17 (d, 2H, *J* = 8.3 Hz), 4.3 (t, 2H, *J* = 7.5 Hz), 2.79-2.62 (m, 2H), 2.34-2.27 (m, 1H), 2.07-2.03 (m, 2H), 1.58 – 1.52 (m, 2H), 1.45 (s, 9H). MS MS (ESI): *m/z* 382 $[M+1]^+$.

General procedure for the synthesis of (*E*)-3-(piperidin-4-ylmethylene)-2,3-dihydropyrrolo[2,1-b]quinazolin-9(1H)-one (8) To a solution of compound 4a/4b (2.7 g, 7.0866 mmol) in ethanol (10 ml) was added HCl (0.5 ml) and stirred vigorously for about 4 h at room temperature. The solvent was evaporated under reduced pressure and the obtained residue was washed with Methanol: EtoAc (1:9) (2 x 30 ml) to give compound **8.**

(*E*)-3-(piperidin-4-ylmethylene)-2,3-dihydropyrrolo[2,1-b]quinazolin-9(1H)-one (8). White solid (2.0 g, 97% yield) from compound **7** (2.0 g, 7.4 mmol) by the general procedure detailed above; mp 183-187 ^{o}C. TLC:

Methanol, R_f ~ 0.2; ^{1}H NMR ($CDCl_3$, 300 MHz): ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.28 (d, 1H, J = 7.5 Hz), 7.86-7.82 (m, 1H), 7.74 (d, 2H, *J* = 3.0 Hz), 7.57 (d, 2H, *J* = 8.3 Hz), 7.47-7.41 (m, 1H), 7.17 (d, 2H, *J* = 8.3 Hz), 4.3 (t, 2H, *J* = 7.5 Hz), 2.79-2.62 (m, 2H), 2.34-2.27 (m, 1H), 2.2 (s, 1H), 2.07-2.03 (m, 2H), 1.58 – 1.52 (m, 2H); MS (ESI): m/z 282.0 $[M+1]^+$.

General procedure for synthesis of the piperidine amide disubstituted Isaindigotone (10a-g). To a solution of compound **8** (0.4 g, 1.2 mmol) in THF (15 mL) was added Et_3N (2 mL) and stirred for about 20 min. To the resulting mixture, the corresponding acid chloride was added at 0 ^{o}C and allowed to come to room temperature and stirred for 3 h. The solvent was evaporated under vacuum to give respective amide derivative which was further purified by column chromatography using EtoAc/hexanes as an eluent.

(*E*)-N-cyclohexyl-4-((9-oxo-1,2-dihydropyrrolo[2,1-b]quinazolin-3(9H)-ylidene)methyl)piperidine-1-carboxamide (10a). The titled compound was prepared in 95% yield as a White solid by treating compound **8** with cyclohexyl isocyanate using the procedure detailed above; mp 82-84 ^{o}C; TLC: EtoAc/ Hexanes (9:1), R_f ~ 0.4. ^{1}H NMR (DMSO-d_6, 300 MHz): δ 8.28 (d, 1H, J = 7.5 Hz), 7.86-7.82 (m, 1H), 7.74 (d, 2H, *J* = 3.0 Hz), 7.57 (d, 2H, *J* = 8.3 Hz), 7.47-7.41 (m, 1H), 7.17 (d, 2H, *J* = 8.3 Hz), 4.3 (t, 2H, *J* = 7.5 Hz), 3.50 - 3.42 (m, 1H), 2.93 - 2.85 (m, 1H), 2.63 (t, 2H, *J* 12.5 Hz), 2.23 (s, 3H), 1.83 - 1.67 (m, 5H), 1.61 - 1.43 (m, 4H), 1.29 - 1.12 (m, 3H);MS (ESI): m/z 407 $[M+1]^+$;HRMS (ESI *m/z*) Calcd for $C_{24}H_{30}N_4O_2$:407.1687 and found: 407.1689; Purity: 96.47% (R_t = 11.665).

(*E*)-4-((9-oxo-1,2-dihydropyrrolo[2,1-b]quinazolin-3(9H)-ylidene)methyl)-N-(p-tolyl)piperidine-1-carboxamide (10b). The titled compound was prepared in 93 % yield as a White solid by treating compound **8** with p-tolyl isocyanate using the procedure detailed above; mp 196-198 ^{o}C; TLC: EtoAc/ Hexanes (9:1), R_f ~ 0.4; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 8.28 (d, 1H, J = 7.5 Hz), 7.86-7.82 (m, 1H), 7.74 (d, 2H, *J* = 3.0 Hz), 7.57 (d, 2H, *J* = 8.3 Hz), 7.47-7.41 (m, 1H), 7.17 (d, 2H, *J* = 8.3 Hz), 4.3 (t, 2H, *J* = 7.5 Hz), 6.89 – 6.82 (m, 1H), 6.1 (s, 1H), 4.26 (d, 2H, *J* =12.86 Hz,), 2.38 – 2.36 (m, 1H), 2.26 (s, 5H), 1.89 – 1.83 (m, 2H), 1.66 – 1.61 (m, 2H), 1.38 – 1.32 (m, 2H); MS (ESI): m/z 415 $[M+1]^+$; HRMS (ESI *m/z*) Calcd for $C_{25}H_{26}N_4O_2$: 415.1253 and found: 415.1258; Purity: 99.62% (R_t = 12.863).

(*E*)-N-(4-chlorophenyl)-4-((9-oxo-1,2-dihydropyrrolo[2,1-b]quinazolin-3(9H)-ylidene)methyl)piperidine-1-carboxamide (10c). The titled compound was prepared in 96 % yield as a White solid by treating compound **8** with *p*-Chloro phenyl isocyanate using the procedure detailed above; mp 197-199 ^{o}C; TLC: EtoAc/ Hexanes (9:1), R_f ~ 0.3; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 8.28 (d, 1H, J = 7.5 Hz), 7.86-7.82 (m, 1H), 7.74 (d, 2H, *J* = 3.0 Hz), 7.57 (d, 2H, *J* = 8.3 Hz), 7.47-7.41 (m, 1H), 7.17 (d, 2H, *J* = 8.3 Hz), 4.3 (t, 2H, *J* = 7.5 Hz), 4.26 (d, 2H, *J* =12.8 Hz), 3.15 – 3.12 (m, 2H), 3.09 – 3.06 (m, 1H), 2.26 (s, 3H), 1.89 – 1.83 (m, 2H), 1.66 – 1.61 (m, 2H); MS (ESI): m/z 435 $[M+1]^+$; HRMS (ESI *m/z*) Calcd for $C_{24}H_{23}ClN_4O_2$: 435.18414 and found: 435.18; Purity: 99.22% (R_t = 12.60).

(4-methoxyphenyl)(4-(3-methyl-1-((methylsulfonyl)phenyl)-1H-pyrazol-5-yl)piperidin-1-yl)methanone (10d). The titled compound was prepared in 96% yield as a white solid by treating compound **4a** with p-methoxyphenyl isocyanate using the procedure detailed in 4.1.6; mp 96-98 ^{o}C; TLC: EtoAc/ Hexanes (9:1), R_f ~ 0.3; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 8.28 (d, 1H, J = 7.5 Hz), 7.86-7.82 (m, 1H), 7.74 (d, 2H, *J* = 3.0 Hz), 7.57 (d, 2H, *J* = 8.3 Hz), 7.47-7.41 (m, 1H), 7.17 (d, 2H, *J* = 8.3 Hz), 4.3 (t, 2H, *J* = 7.5 Hz), 3.716-3.580 (m,1H), 3.13 (s, 3H), 3.08-2.98 (m, 2H), 2.31(s, 3H), 1.64-1.51 (m, 5H); MS (ESI): m/z 401 $[M+1]^+$; HRMS (ESI *m/z*) Calcd for $C_{24}H_{24}N_4O_2$: 401.1841 and found: 437.18414; Purity: 96.53% (RT = 11.752).

(*E*)-*N*-butyl-4-((9-oxo-1,2-dihydropyrrolo[2,1-b]quinazolin-3(9H)-ylidene)methyl)piperidine-1-carboxamide (10e). The titled compound was prepared in 95% yield as a white solid by treating compound **4a** with butyl isocyanate using the procedure detailed above ; mp 191-193 ^{o}C; TLC: EtoAc/ Hexanes (9:1), R_f ~ 0.4; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 8.28 (d, 1H, J = 7.5 Hz), 7.86-7.82 (m, 1H), 7.74 (d, 2H, *J* = 3.0 Hz), 7.57 (d, 2H, *J* = 8.3 Hz), 7.47-7.41 (m, 1H), 7.17 (d, 2H, *J* = 8.3 Hz), 4.3 (t, 2H, *J* = 7.5 Hz), 3.18 (s, 3H), 3.14 - 3.03 (m, 2H), 2.99 - 2.85 (m, 1H), 2.68 (t, 2H, *J* = 12.3 Hz), 1.80 (d, 2H, *J* = 12.1 Hz), 1.59 - 1.37 (m, 2H), 1.38 - 1.22 (m, 2H), 0.903 (t, 3H, *J* = 6.8 Hz); MS (ESI): m/z 381 $[M+1]^+$; HRMS (ESI *m/z*) Calcd for $C_{22}H_{28}N_4O_2$: 381.1533 and found: 469.15366; Purity: 97.44% (R_t = 11.204).

(*E*)-4-((9-oxo-1,2-dihydropyrrolo[2,1-b]quinazolin-3(9H)-ylidene)methyl)-*N*-propylpiperidine-1-carboxamide (10f). The titled compound was prepared in 95% yield as a white solid by treating compound **8** with Propyl isocyanate using the procedure detailed above; mp 157-159 ^{o}C; TLC: EtoAc/ Hexanes (9:1), R_f ~ 0.4; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 8.28 (d, 1H, J = 7.5 Hz), 7.86-7.82 (m, 1H), 7.74 (d, 2H, *J* = 3.0 Hz), 7.57 (d, 2H, *J* = 8.3 Hz), 7.47-7.41 (m, 1H), 7.17 (d, 2H, *J* = 8.3 Hz), 4.3 (t, 2H, *J* = 7.5 Hz), 3.91 (d, *J* = 13.7 Hz ,1H), 3.13 (s, 3H), 3.07-2.90 (m, 2H), 2.56-2.44 (m, 1H), 2.41-2.31 (m, 2H), 2.29(s, 3H), 1.88(d, 2H, *J* = 12.4Hz 1.14 (t, 3H, *J* = 7.36

Hz); MS (ESI): m/z 367 $[M+1]^+$; HRMS (ESI *m/z*) Calcd for $C_{21}H_{26}N_4O_2$: 367.16937 and found: 367.16894; Purity: 97.44% (R_t = 10.043).

(*E*)-N-ethyl-4-((9-oxo-1,2-dihydropyrrolo[2,1-b]quinazolin-3(9H)-ylidene)methyl)piperidine-1-carboxamide (10g). The titled compound was prepared in 95% yield as a white solid by treating compound **8** with ethyl isocyanate using the procedure detailed above; mp 138 -139 ^oC; TLC: EtoAc/ Hexanes (9:1), R_f ~ 0.5; ^1H NMR (DMSO-d_6, 300 MHz): δ 8.28 (d, 1H, J = 7.5 Hz), 7.86-7.82 (m, 1H), 7.74 (d, 2H, *J* = 3.0 Hz), 7.57 (d, 2H, *J* = 8.3 Hz), 7.47-7.41 (m, 1H), 7.17 (d, 2H, *J* = 8.3 Hz), 4.3 (t, 2H, *J* = 7.5 Hz), 3.72-3.46 (m, 1H), 3.02-2.90 (m, 4H), 2.42-2.36 (m, 3H), 1.80-1.64 (m, 2H),1.57-1.48(m, 2H); MS (ESI): m/z 353 $[M+1]^+$; HRMS (ESI *m/z*) Calcd for $C_{20}H_{24}N_4O_2$: 353.1644 and found: 353.16419; Purity: 96.30 % (R_t = 5.413).

sEH IC_{50} assay procedure

For the recombinant affinity purified sEHs (human), we used a fluorescent-based assay to determine IC_{50} [5]. Enzymes (~1 nM human sEH) were incubated with inhibitors for 5 min in 25 mM Bis-Tris/HCl buffer (200 µL; pH 7.0) at 30 ^oC before substrate (cyano(2-methoxynaphthalen-6-yl)methyl trans-(3-phenyl-oxyran-2-yl)methyl carbonate (CMNPC) was added ([S] =5µM). Activity was assessed by measuring the appearance of the fluorescent 6-methoxynaphthaldehyde product (λ_{em} = 330 nm, λ ex = 465 nm) at 30 ^oC during a 10 min incubation (Spectramax M2; Molecular Device, Inc., Sunnyvale, CA). The IC_{50}s were calculated from at least three separate runs, each in triplicate, to obtain the standard deviation given in the Results section. The IC_{50} was determined from at least four points in the linear region of the inhibition curve with at least one point above and one below the IC_{50}.

COX-2 Inhibitory assay:

The ability of the test compounds listed in the Table 1 to inhibit COX-2 (% Inhibition at 1 µM concentration) was determined using an enzyme immunoassay (EIA) kit (Catalog number 560101, Cayman Chemical, Ann Arbor, MI, USA). Briefly, 20 µl of compounds (final concentration of 1µM) was added to the reaction mixture contains 960 µl of buffer solutions (0.1 M Tris HCl pH 8.0 containing 5 mM EDTA and 2 mM phenol) with either COX-2 (10 µL) enzyme in the presence of heme (10 µL). These solutions were incubated for a period of 5 min at 37 ^oC after which 10 µL of arachidonic aid (100 mM) solution was added and the COX reaction was stopped by the addition of 50 µL of 1M HCl after 2 min. The concentration of PGF2α, produced from PGH2 by reduction with stannous chloride was measured by enzyme immunoassay (Acetylcholine esterase competitive EIA). This assay is based on the competition between PGs and a PG-acetyl cholinesterase 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 colour that absorbs at 406 nm. The intensity of this colour, determined spectrophotometrically (Spectramax M4, Molecular devices, USA) 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. Percentage inhibition was calculated by the comparison of compounds treated to various control incubations.

RESULTS AND DISCUSSION

Chemistry:

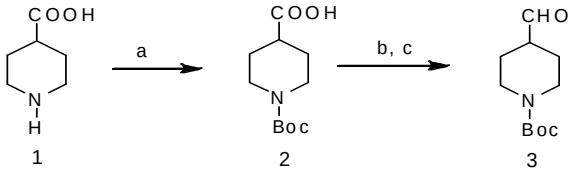
The novel piperidine amide derivatives of substituted Isaindigotone were synthesized starting from isonipecotic acid **1**. The NH group of the isonipecotic acid **1** was protected [25] using $(Boc)_2O$ and NaOH as base to give Boc protected acid **2** in 97% yield. The acid **2** was reduced to its aldehyde **3** in two steps [26] using *N, N*-Dimethylchloromethylenammonium chloride **(Scheme 1)**. The condensation of pyrrolidin-2-one and 2-ami no benzoic acid in presence of anhydrous toluene and $POCl_3$ was carried out and to the condensed intermediate (**6**) the coupling of Boc protected aldehyde **3** was carried out in presence of acetic anhydride under refluxed condition in **Scheme 2**. The compounds **7** upon Boc deprotection [27] using HCl gave key substituted Isaindigotone intermediates **8** in 85% yield **(Scheme 3)**. The intermediates **8** was treated with various isocyanate in the presence of triethylamine as base and solvent THF afforded corresponding novel piperidine urea derivatives of substituted Isaindigotone **(10a–l)** respectively in approximately 85 – 90% yield (**Scheme 3**).

sEH Inhibitory assay

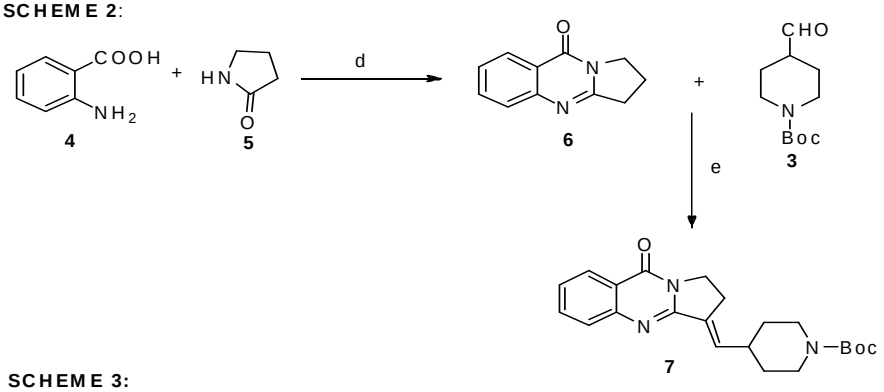
The ability of the synthesized compounds to inhibit sEH enzyme was evaluated by *In-vitro* biological evaluation of all compounds for sEH activity is planned to be done using Soluble Epoxide Hydrolase Inhibitor Screening Assay kit (10011671). The assay utilizes (3-phenyl-oxiranyl)-acetic acid cyano-(6-methoxy-naphtalen-2-yl)-methyl ester (PHOME) as substrate. When the epoxide moiety of PHOME is hydrolyzed by epoxide hydrolase, an intramolecular

cyclization occurs which results in the release of cyanohydrins under basic conditions. The cyanohydrin quickly decomposes into cyanide ion and highly fluorescent 6-methoxy-2-methoxy-2-naphthaldehyde which can be analyzed using an excitation wavelength of 330 nm and an emission wavelength of 465nm [28].

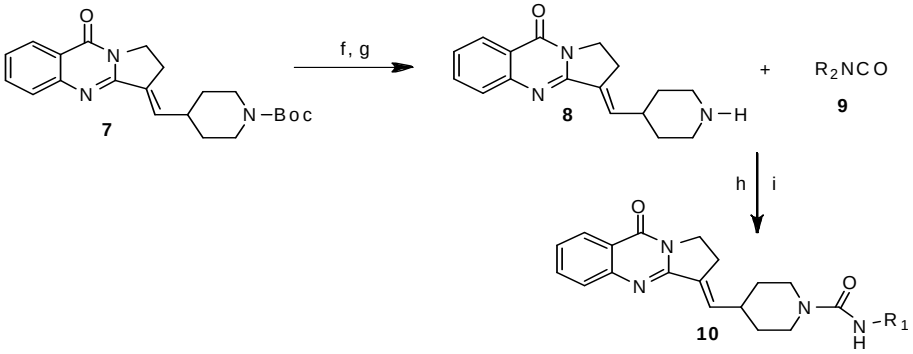
SCHEME 1:



SCHEME 2:



SCHEME 3:



Scheme 1: Reagents and conditions: Synthesis of amide derivatives of substituted Isaindigotone. (a) NaOH, $(Boc)_2O$, THF, 0 °C to rt, 24 h, 97%; (b) *N, N*-Dimethylchloromethylenammonium chloride, DCM, Oxalyl chloride, 0 °C, 1h, (c) *N, N*-Dimethylchloromethyl enammonium chloride, THF, Pyridine, acetonitrile , $LiAlH_4$ -30 °C- 0 °C, 30min, Scheme 2: d) $POCl_3$, toluene, reflux, 4h (e) acetic anhydride, reflux Scheme 3: (f) EtOH, rt, 20 h, 83%; (g) HCl, EtOH, rt, 4 h; (h) THF (i) Et_3N, 0°C to rt, 3 h (85-90%

Table 1: Inhibition of Human recombinant sEH by compound 10a-g

Compound Code.	R	% sEH Inhibition(100 µM)[a]	IC $_{50}$ Value (µM)[b]
10a	R=Cyclohexyl	102.86	22.256±1.32
10b	R= p-tolyl	81.95	10.082±0.84
10c	**R= *p*-Cl-Ph**	**98.95**	**0.275±0.027**
10d	**R= *p*-OMe-Ph**	102.70	**0.699±2.11**
10e	**R= Butyl**	103.28	**0.124±0.011µM**
10f	R= Pr	99.00	12.607±0.68
10g	**R= Ethyl**	116.97	**0.110 ±0.01µM**

**Values are the means (SD of three independent experiments with sEH Fluorescent Inhibitor Screening Assay Kit (catalogue no. 10011671, Cayman Chemicals Inc., Ann Arbor, MI).[a] Determined via a kinetic fluorescent assay, results are means (SD of three separate experiments. Percent inhibition at 100 µM concentration.[b] Data points are triplicate average. We observed coefficient variation between 5 and 10%*

The preliminary *in vitro* findings of sEH enzyme inhibition studies showed that, all the compounds 10a-g series were shown 54-100% inhibition of the sEH enzyme at 100 µM concentration & compounds 10e and 10g emerged as the most potent sEH inhibitors, displaying IC_{50} values of **0.124±0.011µM** and **0.110 ±0.01µM** for in-vitro sEH inhibition respectively which is comparable to that of standard AUDA **0.0065±0.002** (**Table 1**).

These results clearly indicate that all the compounds are showing potent sEH inhibition. This activity may be due to the additional interactions of urea linked functionalities.

Table 2: List of compounds with their COX-2/COX-1inhibition potencies

S. No.	*Compound*	*% inhibition of COX-2[a]*	*% inhibition of COX-1[b]*
1	**10a**	91.4	0
2	**10b**	53.6	-
3	**10c**	86.1	0.9
4	**10d**	81.8	0
5	**10e**	91.8	-
6	**10f**	100	-
7	**10g**	69.9	-
8	Celecoxib	39.2	-
9	Ibuprofen	-	80.4

[a] *Results obtained at the concentration of 1 µM*
[b] *Results obtained at the concentration 10 µM*

COX-2 inhibitory assay: The ability of the synthesized compounds to inhibit COX-2 enzyme was evaluated by *in vitro* assay technique using COX-2 inhibitory assay kit (Cayman chemical catalogue no. 560131) by following the given standard protocol [29, 30]. The preliminary *in vitro* findings of COX-2 enzyme inhibition studies showed that, all the compounds (**10a-g**) were shown 54-100% inhibition of the COX-2 enzyme at 1 µM concentration, where as reference drug celecoxib showed 39% inhibition (Table 1). These results clearly indicate that all the compounds are showing more potency with respect to COX-2 inhibition than the lead compound celecoxib. This increased activity may be due to the additional hydrogen bindings. On the other hand COX-2 inhibitory activity of all the compounds reveals that introduction of piperidine ring substituted urea and tolerates the pharmacophore model for COX-2 inhibition. Compounds **10f**, **10h** and **10g** were evaluated for their COX-1 inhibitory activity by taking ibuprofen as a reference compound (**Table 2**). All these tested compounds shown no COX-1 inhibitory activity even at 10 mM concentration. These results evidence the high potency of the pyrazole nitrate esters and their corresponding alcohol metabolites for selective inhibition of COX-2 enzyme.

CONCLUSION

A series of novel dual COX-2 and sEH inhibitors were synthesized. The compounds synthesized to demonstrate significant inhibition sEH. This study has demonstrated that a grouping of Isaindigotone and piperidine mediated urea (sEH pharmacophore) side chain can improve the inhibitory activity of the resulting molecule on sEH in vitro. This study has demonstrated that the sEH inhibitor **10e** & **10g** displays improved in vitro efficacy in fluorescence based sEH assay.

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