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Efficient synthesis and characterization of novel indolizines: exploration of *in vitro* COX-2 inhibitory activity and molecular modelling studies

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Abstract

A series of novel indolizine scaffold incorporating cyano group at 7 position as potential cyclooxygenase (COX)-2 inhibitors has been synthesized and characterized by FT-IR, NMR, LC-MS, and elemental analysis. Molecular modelling study was carried out to explore the COX-2 binding properties of the synthesized compounds, in an endeavour to provide additional insights on inhibitory potential to treat and/or manage inflammation and pain. All compounds demonstrated comparable docking scores with that of Indomethacin, a potent nonsteroidal anti-inflammatory drug. Additional empirical scoring functions were also investigated to estimate the best ligand orientation into the binding site. Halogen atoms at para position of the benzoyl ring at third position of indolizine nucleus showed promising COX-2 inhibitory activity. The observed promising activity was favoured by ethyl moiety at second position of the indolizine nucleus. These findings will provide insights into structural requirement for designing novel indolizine scaffolds as potent COX-2 inhibitors.

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used therapeutic agents for the management and treatment of chronic musculoskeletal pathologies namely rheumatoid arthritis and osteoarthritis [1]. They are known to act by inhibiting the biosynthesis of pain and inflammation mediating prostaglandins. Opioid receptors are known for controlling severe to chronic pain and various agonists [2-7] and antagonists [8] have been reported. NSAIDs exert their molecular mechanism of action as cyclooxygenase inhibitors (COX), which converts arachidonic acid into prostanoids which appear as physiopathological effectors. COX-1 and 2 are the two isoforms of COX enzyme. NSAIDs are classified into several chemical classes, generally selective and non-selective for COX-2 enzyme inhibition. Some of the nonselective and selective COX-2 enzyme inhibitors are shown in Fig. 1. Inhibition of COX-2 activity for the production of prostaglandins produces good anti-inflammatory and anti-nociceptive activities. Coincidently COX-1 enzyme is also inhibited in this process, resulting in unwanted side effects such as gastrointestinal tract disturbances and adverse cardiovascular effects. This non selective nature of NSAIDs towards COX inhibition led to the development of selective COX-2 enzyme inhibitors. Indolizine represents an important class of nitrogen-fused heterocycles that are ubiquitous in many natural products and biologically active compounds [9-11].

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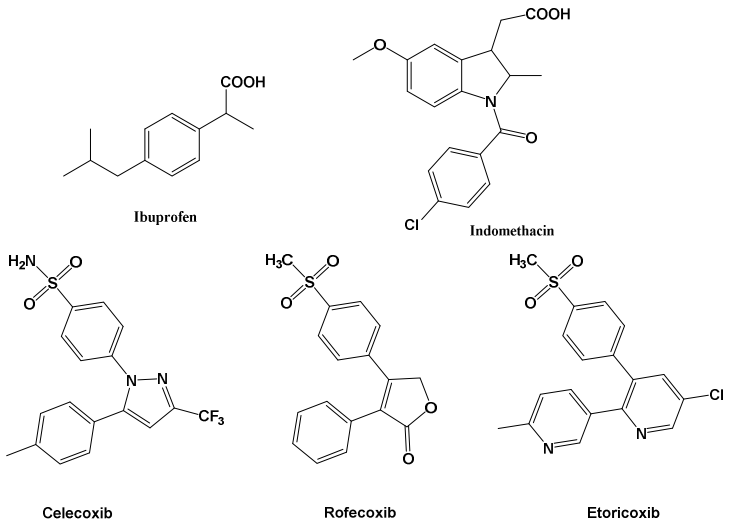

Fig. 1 Selective COX-2 enzyme inhibitors (Ibuprofen, Indomethacin, Celecoxib, Rofecoxib, and Etoricoxib).

Many synthetic indolizine derivatives have found wide applications in the pharmaceutical field as analgesic [12], anti-inflammatory inhibitors [13], anticancer [14], antidiabetic [15, 16], antihistaminic [17], COX-2 inhibition [18, 19], antileishmanic [20], antimicrobial [21], antimutagenic [22], antioxidant [23], antitubercular [24, 25], antiviral [26], larvicidal [27, 28], and herbicidal activities [29]. They are also useful in material science due to their distinctive photophysical properties [30, 31]. Although several methods are reported for the construction of indolizines [32-39], the development of general and efficient synthesis of functionalized indolizines is still highly attractive [7, 40]. Furthermore substituted indolizines and their derivatives have also been extensively used as versatile building blocks for the synthesis of dyes [41], biological markers [42, 43], and electroluminescent materials with enhanced electronic and photonic properties [44, 45].

Computational methods have been successfully applied for the development of novel chemical entities with bioactivity expectations. It facilitates the prediction of the mechanism of action of molecule through the investigation of the binding property within the cavity site of the enzyme receptor [46, 47]. Preliminary molecular modelling analysis of the indolizine scaffold reveals the potential of incorporating the cyano group at 7 position of indolizine ring intending to interact with the lipophilic cavity site of COX-2 receptor, normally referred as selective COX-2 inhibitors. In continuation of our efforts in search of novel heterocyclic scaffolds for promising pharmacological properties [48, 49] and polymorphism behavior [50, 51], we wish to report our preliminary structure activity relationships of the new design incorporated in the cyano group into the indolizine scaffold against COX-2 inhibitory activity (Fig. 2).

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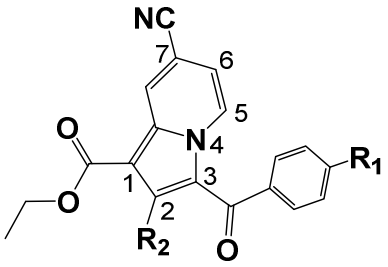

Fig. 2 Design of novel indolizine scaffold incorporating cyano group at 7 position as potential COX-2 inhibitors.

Indomethacin and indolizine compounds were overlaid in the active site, demonstrating the similarities of structures (Fig. 3). The requirement of this scaffold has been evaluated for the inhibition of COX-2 activity.

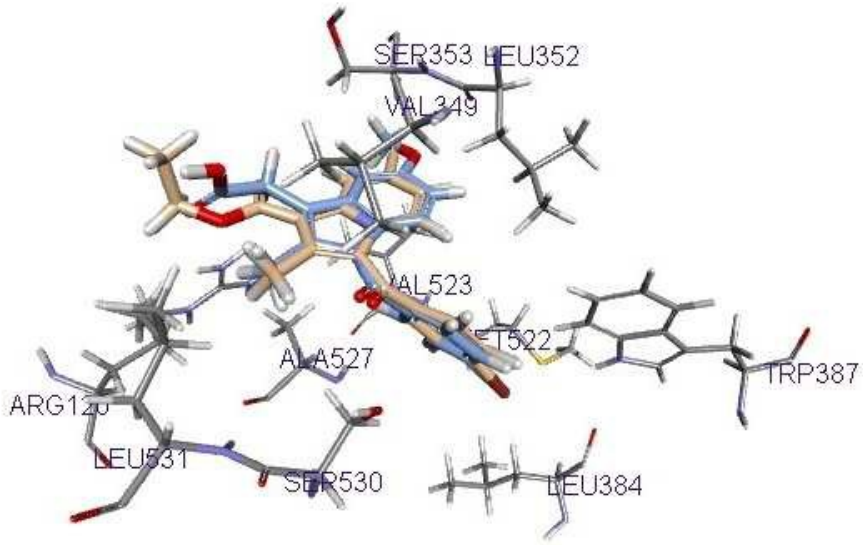

Fig. 3 3D overlay of Indomethacin (bleu) with Indolizine (salmon) in the active site of COX-2 receptor shown as grey (PDB code: 4COX).

The synthetic scheme for the construction of novel indolizine scaffold incorporating cyano group at seventh position is described in Scheme 1.

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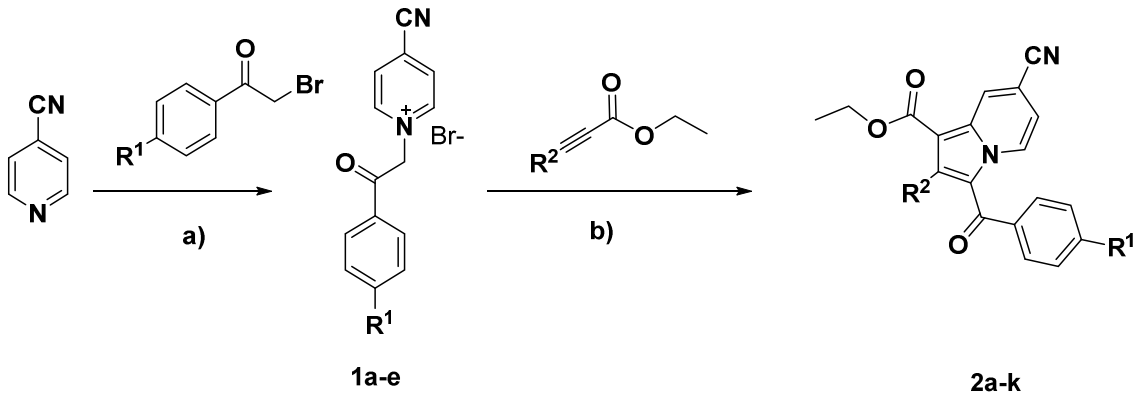
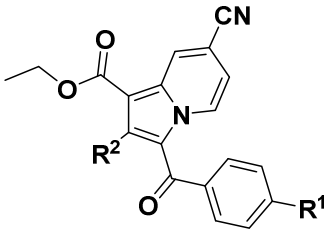


Scheme 1 Synthetic scheme for the construction of novel indolizine scaffolds (**2a-k**): (a) acetone, r.t., 5h; (b) DMF, K_2CO_3, 30 min.

Table 1 Physicochemical constants of 1,2,3 trisubstituted 7-cyano indolizine scaffolds **2a-k**



Comp	Mol formulae (Mol weight)	R^1	R^2	Yield[a,b] (%)	m.p (°C)	ClogP[c]
2a	$C_{21}H_{17}FN_2O_3$ (364)	F	C_2H_5	87	133-134	4.9790
2b	$C_{21}H_{17}BrN_2O_3$ (424)	Br	C_2H_5	91	168-169	5.6990
2c	$C_{22}H_{17}ClN_2O_5$ (424)	Cl	$COOC_2H_5$	89	179-180	4.0037
2d	$C_{21}H_{15}N_3O_3$ (357)	CN	CH_3	88	182-183	3.7863
2e	$C_{19}H_{13}BrN_2O_3$ (396)	Br	H	92	174-175	4.6710
2f	$C_{21}H_{18}N_2O_4$ (362)	OCH_3	CH_3	86	136-137	4.4701
2g	$C_{20}H_{15}BrN_2O_3$ (410)	Br	CH_3	89	123-124	5.1700
2h	$C_{22}H_{20}N_2O_4$ (376)	OCH_3	C_2H_5	87	110-111	4.9991
2i	$C_{21}H_{17}ClN_2O_3$ (380)	Cl	C_2H_5	85	128-129	5.5490
2j	$C_{23}H_{17}N_3O_5$ (415)	CN	$COOC_2H_5$	91	184-185	2.7645
2k	$C_{20}H_{13}N_3O_3$ (343)	CN	H	90	189-190	3.2873

[a] All of the products were characterized by spectral and physical data.
[b] Yields after purification by column chromatography method.
[c] *c*Log*P* was calculated using ChemDraw Professional 16.

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Experimental

Chemistry

All the commercially available chemicals were procured from Sigma-Aldrich, India. All the chemical reactions of Scheme-1 were carried out under nitrogen atmosphere using dry solvents. Progress of the chemical reactions was monitored on thin layer chromatography (TLC). TLC was performed on Sigma-Aldrich Silica gel on TLC aluminium foils with n-hexane and ethyl acetate (4:6) as solvent system and visualization in iodine chamber. Melting points were determined on a Büchi melting point B-545 apparatus. The FT-IR spectra were recorded on a Shimadzu FT-IR spectrometry. NMR (400 MHz) spectra were recorded at ambient temperature using $CDCl_3$ as a solvent using Bruker-400 spectrometer. Chemical shift values are measured in δ ppm and were referenced with TMS. The peak multiplicities were given as follows; s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. LC-MS analysis was performed on Agilent LC-1200 series coupled with 6140 single quad mass spectrometer with ESI +ve mode, MS range 100-800. Elemental analysis was performed on Thermo Finnigan FLASH EA 1112 CHN analyzer.

General procedure for the preparation of 4-cyano-1-(2-(4-fluorophenyl)-2-oxoethyl)pyridin-1-ium bromide (1a-e)

To a stirred solution of isonicotinonitrile (1g, 9.60 mmol) in dry acetone (10 mL), 4-fluoro phenacylbromide (2.97g, 9.6 mmol) was added and stirred at room temperature for 5h. Progress of the chemical reaction was monitored on TLC. The solid formed was separated by filtration and dried under vacuum to afford 99% yield of 4-cyano-1-(2-(4-fluorophenyl)-2-oxoethyl)pyridin-1-ium bromide. Remaining compounds **1b-e** were prepared by following similar protocol.

General procedure for the synthesis of ethyl 7-cyano-2-ethyl-3-(4-fluorobenzoyl)indolizine-1-carboxylate (2a)

To a stirred solution of 4-cyano-1-(2-(4-fluorophenyl)-2-oxoethyl)pyridin-1-ium bromide (0.5g 1.5 mmol) in DMF, ethylpentynoate (0.197g 1.5 mmol), and K_2CO_3 (0.431g 3.1 mmol) were added and stirred at room temperature for 30 min. Progress of the chemical reaction was monitored by TLC. The reaction medium was evaporated under reduced pressure, diluted with brine solution and the aqueous layer was extracted two times with ethyl acetate. The

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organic layer was collected and evaporated to obtain crude product and purified by column chromatography using 60-120 mesh silica gel with hexane ethylacetate solvent mixture to afford 0.48g (87%) of ethyl 7-cyano-2-ethyl-3-(4-fluorobenzoyl)indolizine-1-carboxylate (**2a**) Remaining compounds from the series **2b-k** were prepared by following the similar procedure and physicochemical constants of 1,2,3 trisubstituted 7-cyano indolizine scaffolds **2a-k** are tabulated in Table 1.

Ethyl 7-cyano-2-ethyl-3-(4-fluorobenzoyl)indolizine-1-carboxylate (2a)

Yellow crystalline compound; IR (neat cm^{-1}): 2974, 2227, 1689, 1608, 1379, 1224; ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 9.29-9.27 (m, 1H), 9.10 (s, 1H), 7.81-7.74 (m, 2H), 7.24-7.16 (m, 2H), 6.98-6.96 (m, 1H), 4.49-4.44 (q, J = 7.2Hz, 2H), 2.83-2.77 (q, J = 7.2Hz, 2H), 1.49-1.43 (t, J = 7.2Hz, 3H), 1.05-1.01 (t, J = 7.2Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 186.90, 166.94, 164.40, 163.73, 143.50, 136.48, 135.87, 135.84, 131.66, 131.56, 127.68, 125.90, 123.72, 117.63, 116.12, 115.90, 113.58, 108.43, 107.32, 60.51, 19.90, 15.85, 14.43. LC-MS (ESI, Positive): *m/z* $(M + H)^+$ 365.2; Anal calculated for: $C_{21}H_{17}FN_2O_3$: C, 69.22, H, 4.70, N, 7.69: Found: C, 69.20, H, 4.72, N, 7.72.

Ethyl 3-(4-bromobenzoyl)-7-cyano-2-ethylindolizine-1-carboxylate (2b)

Brown crystalline compound; IR (neat cm^{-1}): 2981, 2227, 1697, 1627, 1585, 1571, 1423, 1217; ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.15-9.13 (m, 1H), 8.75 (s, 1H), 7.67-7.60 (m, 4H), 6.99-6.98 (m, 1H), 4.48-4.42 (q, *J* = 7.2Hz, 2H), 2.80-2.75 (q, *J* = 7.2Hz, 2H), 1.47-1.43 (t, *J* = 7.2Hz, 3H), 1.05-1.01 (t, *J* = 7.2Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 186.86, 163.95, 138.60, 137.72, 136.49, 132.14, 130.61, 127.92, 127.87, 125.52, 124.12, 117.54, 113.90, 108.95, 108.40, 60.54, 14.92, 14.50. LC-MS (ESI, Positive): *m/z* $(M + H)^+$ 425.1; Anal calculated for: $C_{21}H_{17}BrN_2O_3$: C, 59.31, H, 4.03, N, 6.59: Found: C, 59.36, H, 3.59, N, 7.02.

Diethyl 3-(4-chlorobenzoyl)-7-cyanoindolizine-1,2-dicarboxylate (2c)

Brown crystalline compound; IR (neat cm^{-1}): 2948, 2231, 1739, 1706, 1629, 1583, 1517, 1446, 1228; ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.53-9.51 (m, 1H), 8.77 (s, 1H), 7.84-7.81 (m, 2H), 7.68-7.65 (m, 2H), 7.18-7.16 (m, 1H), 4.48-4.42 (q, *J* = 7.2Hz, 2H), 2.80-2.75 (q, *J* = 7.2Hz, 2H), 1.47-1.43 (t, *J* = 7.2Hz, 3H), 1.05-1.01 (t, *J* = 7.2Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 185.53, 164.14, 162.37, 139.23, 136.91, 135.42, 131.69, 130.18, 128.98, 128.71, 126.30, 122.31, 116.85, 110.40, 52.63, 52.25, 24.99, 22.46. LC-MS (ESI, Positive): *m/z* (M +

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H)$^+$ 425; Anal calculated for: $C_{22}H_{17}ClN_2O_5$: C, 62.20, H, 4.03, N, 6.59: Found: C, 62.16, H, 3.58, N, 6.49.

Ethyl 7-cyano-3-(4-cyanobenzoyl)-2-methylindolizine-1-carboxylate (2d)

Yellow crystalline compound; IR (neat cm^{-1}): 2977, 2227, 1697, 1618, 1593, 1515, 1217, 1112; ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.34-9.32 (m, 1H), 8.73 (s, 1H), 7.69-7.66 (m, 2H), 7.51-7.48 (m, 2H), 7.03-7.01 (m, 1H), 4.47-4.41 (q, J = 7.2Hz, 2H), 2.29 (s 3H), 1.47-1.43 (t, J = 7.2Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 186.73, 163.96, 139.36, 138.15, 137.67, 136.47, 130.52, 129.16, 127.86, 125.53, 124.16, 117.55, 113.87, 108.91, 108.38, 60.54, 14.90, 14.50. LC-MS (ESI, Positive): *m/z* (M)$^+$ 357.2; Anal calculated for: $C_{21}H_{15}N_3O_3$: C, 70.58, H, 4.23, N, 11.76: Found: C, 70.54, H, 4.19, N, 11.72.

Ethyl 3-(4-bromobenzoyl)-7-cyanoindolizine-1-carboxylate (2e)

Brown crystalline compound; IR (neat cm^{-1}): 2989, 2231, 1703, 1622, 1583, 1525, 1465, 1346, 1222; ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.94-9.92 (m, 1H), 8.79 (s, 1H), 7.87 (s, 1H), 7.70-7.69 (m, 4H), 7.19-7.17 (m, 1H), 4.45-4.40 (q, J = 7.2Hz, 2H), 1.45-1.40 (t, J = 7.2Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 184.78, 163.03, 137.63, 137.03, 131.97, 131.69, 130.58, 129.29, 128.73, 127.27, 125.64, 123.77, 117.18, 114.99, 109.99, 109.57, 60.89, 14.50. LC-MS (ESI, Positive): *m/z* (M + H)$^+$ 397.1; Anal calculated for: $C_{19}H_{13}BrN_2O_3$: C, 57.45, H, 3.30, N, 7.05: Found: C, 57.39, H, 3.26, N, 7.12.

Ethyl 7-cyano-3-(4-methoxybenzoyl)-2-methylindolizine-1-carboxylate (2f)

Yellow crystalline compound; IR (neat cm^{-1}): 2979, 2223, 1691, 1627, 1600, 1514, 1257, 1224; ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.14-9.12 (m, 1H), 8.71 (s, 1H), 7.76-7.73 (m, 2H), 7.00-6.93 (m, 3H), 4.47-4.41 (q, J = 7.2Hz, 2H), 3.91 (s, 3H), 2.34 (s 3H), 1.47-1.44 (t, J = 7.2Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 186.78, 164.17, 163.81, 139.2, 136.45, 135.90, 132.07, 131.80, 127.56, 125.60, 124.78, 117.78, 114.07, 113.29, 107.99, 107.09, 60.39, 55.58, 14.69. LC-MS (ESI, Positive): *m/z* (M + H)$^+$ 363.1; Anal calculated for: $C_{21}H_{18}N_2O_4$: C, 69.60, H, 5.01, N, 7.73: Found: C, 69.58, H, 4.99, N, 7.72.

Ethyl 3-(4-bromobenzoyl)-7-cyano-2-methylindolizine-1-carboxylate (2g)

Yellow crystalline compound; IR (neat cm^{-1}): 2989, 2225, 1687, 1618, 1581, 1512, 1440, 1288, 1213; ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.35-9.33 (m, 1H), 8.74 (s, 1H), 7.68-7.65 (m, 2H), 7.61-6.58 (m, 2H), 7.04-7.01 (m, 1H), 4.47-4.41 (q, J = 7.2Hz, 2H), 2.29 (s 3H), 1.47-1.43 (t, J = 7.2Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 187.18, 163.68, 143.87, 138.47,

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136.65, 132.07, 130.44, 127.93, 127.80, 125.85, 123.57, 117.58, 113.72, 108.67, 107.43, 60.53, 19.93, 15.93, 14.92. LC-MS (ESI, Positive): *m/z* $(M + H)^+$ 411.2; Anal calculated for: $C_{20}H_{15}BrN_2O_3$: C, 58.41, H, 3.68, N, 6.81: Found: C, 58.42, H, 3.69, N, 6.82.

Ethyl 7-cyano-2-ethyl-3-(4-methoxybenzoyl)indolizine-1-carboxylate (2h)

Yellow crystalline compound; IR (neat cm^{-1}): 2979, 2225, 1687, 1614, 1595, 1515, 1438, 1377, 1271; ^1H NMR (400 MHz, $CDCl_3$) δ 8.88-8.86 (m, 1H), 8.72 (s, 1H), 7.78-7.74 (m, 2H), 7.01-6.98 (m, 2H), 6.92-6.90 (m, 1H), 4.48-4.42 (q, J = 7.2Hz, 2H), 3.91 (s, 3H), 2.88-2.82 (q, J = 7.2Hz, 2H), 1.47-1.44 (t, J = 7.2Hz, 3H), 1.08-1.04 (t, J = 7.2Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 187.06, 163.89, 142.38, 135.93, 131.80, 131.72, 127.36, 125.97, 124.30, 117.83, 114.07, 113.10, 107.59, 106.99, 60.39, 55.59, 19.89, 15.84, 14.45. LC-MS (ESI, Positive): *m/z* $(M + H)^+$ 377.1; Anal calculated for: $C_{22}H_{20}N_2O_4$: C, 70.20, H, 5.36, N, 7.44: Found: C, 70.16, H, 5.38, N, 7.42.

Ethyl 3-(4-chlorobenzoyl)-7-cyano-2-ethylindolizine-1-carboxylate (2i)

Yellow crystalline compound; IR (neat cm^{-1}): 2968, 2229, 1685, 1602, 1585, 1521, 1379, 1215; ^1H NMR (400 MHz, $CDCl_3$) δ 9.30-9.28 (m, 1H), 8.64 (s, 1H), 7.77-7.74 (m, 2H), 7.53-7.50 (m, 2H), 7.16-7.13 (m, 1H), 4.46-4.41 (q, J = 7.2Hz, 2H), 2.76-2.72 (q, J = 7.2Hz, 2H), 1.47-1.44 (t, J = 7.2Hz, 3H), 1.06-1.02 (t, J = 7.2Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 186.76, 166.39, 164.66, 164.54, 163.87, 162.09, 144.21, 140.61, 140.53, 139.91, 137.93, 137.91, 137.07, 137.04, 131.26, 131.17, 130.72, 130.64, 128.08, 122.54, 122.51, 121.91, 116.72, 115.81, 115.61, 115.59, 115.41, 113.92, 113.70, 104.95, 59.93, 20.04, 16.01, 14.46. LC-MS (ESI, Positive): *m/z* $(M)^+$ 381.1; Anal calculated for: $C_{21}H_{17}ClN_2O_3$: C, 66.23, H, 4.50, N, 7.36: Found: C, 66.26, H, 4.49, N, 7.32.

Diethyl 7-cyano-3-(4-cyanobenzoyl)indolizine-1,2-dicarboxylate (2j)

Brown crystalline compound; IR (neat cm^{-1}): 2983, 2229, 1733, 1697, 1627, 1512, 1436, 1380, 1224; ^1H NMR (400 MHz, $CDCl_3$) δ 9.67-9.65 (m, 1H), 8.82 (s, 1H), 7.81-7.75 (m, 4H), 7.23-7.22 (m, 1H), 4.43-4.39 (q, J = 7.2Hz, 2H), 3.74-3.71 (q, J = 7.2Hz, 2H), 1.39-1.35 (t, J = 7.2Hz, 3H), 1.13-1.09 (t, J = 7.2Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 185.01, 164.70, 163.67, 161.78, 143.37, 142.24, 138.86, 135.97, 132.52, 132.17, 132.01, 131.81, 129.51, 129.24, 129.17, 128.89, 128.81, 126.22, 121.34, 120.14, 117.74, 116.61, 115.59, 114.96, 110.96, 107.81, 62.27, 61.37, 14.24, 13.59. LC-MS (ESI, Positive): *m/z* $(M + H)^+$ 416.1; Anal calculated for: $C_{23}H_{17}N_3O_5$: C, 66.50, H, 4.13, N, 10.12: Found: C, 66.49, H, 4.18, N, 10.13.

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Ethyl 7-cyano-3-(4-cyanobenzoyl)indolizine-1-carboxylate (2k)

Yellow crystalline compound; IR (neat cm^{-1}): 2979, 2231, 1703, 1625, 1529, 1469, 1346, 1224; ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.99-9.97 (m, 1H), 8.82 (s, 1H), 7.93-7.83 (m, 5H), 7.24-7.22 (m, 1H), 4.46-4.41 (q, J = 7.2Hz, 2H), 1.45-1.41 (t, J = 7.2Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 183.98, 162.83, 142.53, 137.41, 132.50, 132.30, 129.42, 129.39, 129.31, 128.51, 125.65, 123.37, 119.70, 117.87, 117.70, 115.90, 115.67, 110.58, 109.97, 61.09, 14.48. LC-MS (ESI, Positive): m/z $(M)^+$ 343.1; Anal calculated for: $C_{20}H_{13}N_3O_3$: C, 69.97, H, 3.82, N, 12.24: Found: C, 69.99, H, 3.86, N, 12.22.

***In vitro* cyclooxygenase (COX-2) inhibition study**

The designed test compounds **2a-k** were screened for *in vitro* human recombinant COX-2 enzyme inhibitory activity as described previously [52].

Statistical analysis

The one-way investigation of variance (ANOVA) was used to compare the *in vitro* COX 2 inhibitory activity of ethyl 7-cyano-2-subsituted-3-(4-substituedbenzoyl)indolizine-1-carboxylates **2a-k** with nonselective (Indomethacin) and selective (Celecoxib) standard compounds. Bonferroni test was used for post hoc analysis to account for the increased possibility of type I error [53]. Before ANOVA testing, data were transformed to ranks [54] to fit better the assumptions of the test. In all cases, a value of $p < 0.05$ was considered statistically significant.

Molecular docking

Docking studies of the studied compounds were carried out using Accelry's Discovery Studio 4.0 software employing CHARMm force fields algorithm. The X-ray co-crystal structure of Indomathecin and COX-2 enzyme was retrieved from the protein databank (PDB code: 4COX). All ligands and water molecules were extracted from the protein. The latter was subjected to cleaning and protein preparation protocol for repairing protein by adding missing amino acids and for removing any internal unfavorable interactions. The ligand structure of indomethacin was then inserted into the prepared protein for defining the receptor's binding

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site. Molecular Docking was performed by using CDocker protocol, where the flexible ligand was docked into the rigid receptor. Docking scores of the ten best conformations were reported as CDocker energy and CDocker interaction energy and were ranked according to CDocker energy. The stronger negative CDocker scores indicate a more favorable binding interaction. To ensure the optimal ligand orientation into the active binding site of the receptor, the following scoring functions PLP1, PLP2, Jain, and PMF were further examined to each ten binding complexes. The highest negative scores of PLP1, PLP2, Jain, and PMF, indicating the strongest receptor-ligand binding affinities, were considered.

Results and discussion

Synthesis of designed compounds

The novel indolizine scaffolds **2a-k** incorporating cyano group at 7 position were obtained according to the synthetic strategy described in Scheme 1. Substituted pyridin-1-ium bromides **1a-e** as intermediates were prepared by reacting 4-cyanopyridine with substituted phenacylbromides at room temperature in acetone medium as shown in step one of Scheme 1. The substituted indolizine derivatives **2a-k** were prepared by stirring a solution of 4-cyano-1-(2-(4-fluorophenyl)-2-oxoethyl)pyridin-1-ium bromides in dimethyl formamide, ethylpentynoate, and K_2CO_3 room temperature for 30 min and the yield obtained were in the range of 85-92%. All the synthesized compounds were characterized by spectroscopic techniques. FT-IR spectra of indolizine scaffolds **2a-k** revealed nitrile stretching in the range of 2223-2231 cm^{-1}. In ^{13}C-NMR carbonyl carbon of benzoyl and ester group is observed in the range of 183.98-187.18 and 162.83-166.94, respectively. In LC-MS, molecular ion peak of the title compounds was in compliance with molecular weight of the compounds. ChemDraw Professional 16 was used to calculate cLogP of the title compounds and the values were in the range of 2.7645-5.6990 (Table 1).

In vitro COX-2 inhibitory activity

The designed test compounds (**2a-k)** were screened for human recombinant COX-2 inhibitory activity using an enzyme immune assay kit and the IC_{50} of the test and standard compounds were evaluated and tabulated in Table 2. Compound **2a** with fluorine atom at para positon of benzoyl group appeared as the most promising candidate, whereas compounds **2b** and **2i** with bromine and chlorine at para position of benzoyl group exhibited

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IC_{50} values at 7.24 and 8.38 μM, respectively. In all the three promising compounds, **2a**, **2b** and **2i**, the ideal substituent at second position of indolizine nucleus was ethyl group.

Table 2 *In vitro* COX-2 inhibitory activity of ethyl 7-cyano-2-subsituted-3-(4-substituedbenzoyl) indolizine-1-carboxylate scaffolds **2a-k**.

Compound	Substituents		IC_{50} (μM)[a]
	R^1	R^2	
2a	F	C_2H_5	$6.63+0.03^{c}$
2b	Br	C_2H_5	7.24 ± 0.03^{a}
2c	Cl	$COOC_2H_5$	11.81 ± 0.03^{bd}
2d	CN	CH_3	8.36 ± 0.03^{cd}
2e	Br	H	14.21 ± 0.03^{b}
2f	OCH_3	CH_3	36.07 ± 0.03^{c}
2g	Br	CH_3	10.33 ± 0.03^{cd}
2h	OCH_3	C_2H_5	39.24 ± 0.03^{ab}
2i	Cl	C_2H_5	8.38 ± 0.03^{c}
2j	CN	$COOC_2H_5$	21.02 ± 0.03^{e}
2k	CN	H	9.51 ± 0.03^{d}
Celecoxib	-	-	0.05 ± 0.03^{bd}
Indomethacin	-	-	$6.84+0.03^{a}$

[a] IC_{50} value is the concentration of test and standard compounds required to produce 50% inhibition of human recombinant COX-2 by means of three determinations using the enzyme linked immuno sorbent assay kit. IC_{50} value not sharing the same superscript letter differ significantly (p<0.05).

Molecular docking

Molecular modelling is an important tool at early stage of drug discovery process. It has been accelerated by the increasing availability of X-ray structures of inhibitors complexes. The

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bioactivity of the molecule can be accurately predicted by analyzing the nature of ligand-receptor interactions through docking studies. To gain insights into the potency of the newly synthesized compounds, the binding modes between the ligand and COX-2 receptor were examined to identify the interactions with key amino acid residues. Docking studies were conducted through CDocker and scoring function modules in Accelry's Discovery Studio 4.0 software. For structural similarities of the studied compounds with Indomethacin, the X-ray structure of COX-2 crystallized with the Indomethacin inhibitor (PDB: 4COX) was chosen. Docking scores were reported as CDocker energy and CDocker interaction energy. CDocker Energy represents the ligand-receptor interaction energy and the internal ligand strain energy. The strongest negative CDocker Energy and CDocker interaction Energy scores indicate the most favorable binding. To predict the most plausible ligand orientation within the binding active site, the scoring functions as Piecewise Linear Potential 1 (PLP1) [55], PLP2 [56], Jain [57] and Potential of Mean Force (PMF) [58] for each pose were taken into account. Higher negative scores of PLP1, PLP2, Jain, and PMF indicate stronger receptor-ligand binding affinities. The docking results showed that all studied compounds have favorable binding interaction with the receptor similar with that observed for Indomethacin (Table 3). Two distinct binding modes were observed among the docking conformation pose, resulting from the projection of the cyano group of the indolizine ring towards HIS 90 participating in hydrogen bonding interaction or towards ARG 120 exhibiting π-cation interaction with indolizine ring and hydrogen bonding interaction with TYR355 from the oxygen of carbonyl ester. In addition, the insertion of benzoyl ring occurred in the same region as *p*-chloro benzoyl moiety of Indomethacin, interacting mainly with the amino acid residues TRP 387, TYR 385, PHE 381, LEU 384, and MET 522 through hydrophobic interactions and van der Waals interactions (Scheme 2). Most of compounds exhibited hydrogen bonding with one of the following amino acid residues HIS 90, TYR 355 or ARG 120. With the exception of compound **2d**, the cyano group on benzoyl ring participates in hydrogen bonding with NH of TRP 387 showing its good inhibitory activity. Preliminary structure activity relationships demonstrates that the presence of the electron withdrawing group at para position of the benzoyl ring i.e. –F (**2a**), -Br (**2b**) and –Cl (**2i**) is more favorable to the bioactivity compared to the electron donating group i.e. $–OCH_3$ (**2h**). For instance, compounds **2h** and **2f**, having $-OCH_3$ group, exhibited weak activity and lack of critical hydrophobic interactions with the residues TRP 387, TYR 385, PHE 381, LEU 384, and MET 522. Another interesting binding feature is the hydrophobic interaction of alkyl group at 2-position of indolizine ring with the residues LEU 531, VAL 349, and ALA 527 in comparison with compound **2b** (ethyl group), **2g** (methyl

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group), and **2e** (hydrogen group). The latter did not exhibit any interactions explaining the diminishment of inhibitory activity. The replacement of alkyl group by ester at 2 position led to compounds **2c** and **2j**. Interestingly, both showed positive CDocker scores and negative CDocker interaction energy scores comparable to Indomethacin. Considering their high internal ligand strain energies, they exerted significant activities. Compound **2c** displayed four hydrogen bonding interactions with the residues ARG120 and HIS90 conferring greater activity over compound **2j**, showing only one hydrogen bonding interaction with the amino acid ARG120. Moving to compound **2g**, the docking study revealed that the hydrophobic interactions were the main interactions contributing to its good inhibitory activity (Fig. 4). To ensure that, the predicted binding mode of the indolizine analogues is accurate, the docking of Celecoxib, into the COX-2 crystal structure's active site containing Indomethacin, was performed. Celecoxib was found to adopt identical orientation into the binding site as reported in protein databank (PDB code: 3LN1), involving strong hydrogen bonding interactions with the residues HIS90, PHE518, SER353, LEU 352 and GLN192. From the molecular study, the nature of substituent on the position of indolizine ring and substituent at para position of the benzoyl ring play a critical role in binding interaction with COX-2 receptor. The introduction of cyano group at 7 position of indolizine ring, have a positive impact on the bioactivity of compounds by generating molecular interaction within the binding site. Some of the designed indolizines may possess some selectivity towards COX-2 over COX-1 inhibition. COX-2 selectivity of inhibitors is attributed by the absence of ion-pair and/or hydrogen bonding interaction with the residue ARG 120 [59-61]. For instance, compounds **2b** and **2d** do not involve such interaction and therefore they can be regarded as potential selective inhibitors.

Table 3 COX-2 inhibitory activity, docking scores of the best pose of the ligand complex into the active binding site of COX-2 receptor (PDB code: 4COX). The most favorable binding indicated by the strongest negative CDocker Energy and CDocker interaction Energy. Higher negative scores of PLP1, PLP2, Jain and PMF indicate stronger receptor-ligand binding affinities.

Comp code	COX-2 Inhibitory (μM)	CDocker Energy (kcal/mol)	CDocker Interaction Energy (kcal/mol)	-PLP1	-PLP2	Jain	-PMF	Nb of H-Bonding	Residues (interacting ligand atom, H-bond length A^0)
2a	6.6	-16	-41	112	103	5.8	98	1	TYR355 (C=O ester, 2.34)
2b	7.2	-17	-40	118	115	5.7	95	1	HIS90 (CN, 3.09)
2h	39	-8	-43	130	126	6.6	106	1	HIS90 (CN, 2.46)
2i	8	-17	-45	112	111	6.0	94	1	TYR355 (C=O ester, 2.36)
2d	8	-22	-45	121	110	5.8	90	1	TRP387 (CN benzoyl, 3.03)
2f	36	-27	-49	118	112	6.1	107	1	TYR355 (C=O ester, 2.30)
2g	10	-25	-46	112	104	5.1	91	0	
2e	14	-28	-48	112	108	5.2	89	1	ARG120 (O ester, 2.57)
2k	9	-23	-44	118	109	5.0	89	1	ARG120 (O ester, 2.73)
2c	12	5	-40	120	119	7.9	87	4	ARG120 (C=O ester, 2.09, 3.01; C=O ester, 2.99) HIS90 (CN, 2.49)
2j	21	6	-43	131	128	7.0	85	1	ARG120 (C=O ester, 2.75)
Indomethacin	6.8	-13	-49	110	106	5.0	94	1	ARG120 (C=O ester, 2.27)
Celecoxib	0.02	-9	-44	111	99	5.5	85	6	HIS90 (SO_2, 2.74) PHE518 (SO_2, 2.86) SER353 (NH_2, 2.17) LEU352 (NH_2, 1.91) GLN192 (NH_2, 2.75)

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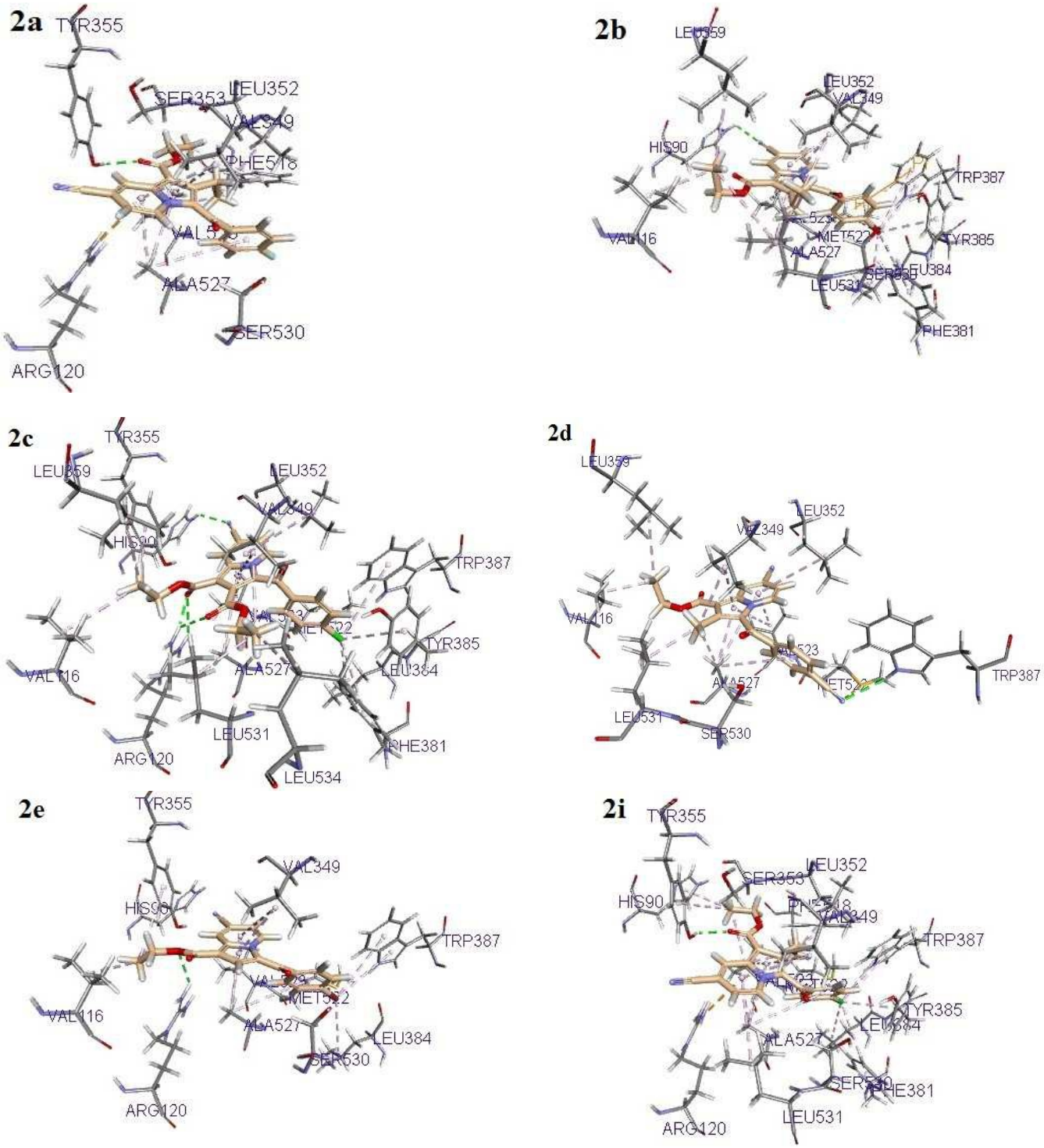

Fig. 4 Predicted binding modes of selected compounds (salmon-filled spheres) with COX-2 receptor (PDB code: 4COX), Dotted green line represents H-bonding interaction, violet dotted line represents hydrophobic interaction and amino acid residues are represented in grey.

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Conclusion

In the present study, we have described the chemistry and the *in vitro* COX-2 enzyme inhibitory activity of 1,2,3 trisubstituted 7-cyano indolizine scaffolds **2a-k**. The reactions performed to obtain indolizine derivatives **2a-k** were eco-friendly and carried out at room temperature with good yield. The test compounds **2a**, **2b,** and **2d** exhibited promising COX 2 inhibition activity from the series when compared to standard Indomethacin and Celecoxib. Halogen atoms at para position of the benzoyl ring at third position of indolizine nucleus possess promising COX-2 enzyme inhibitory activity. This promising activity was favoured by ethyl moiety at second position of the indolizine nucleus. However, lowest homologous of ester functional group at second position of indolizine nucleus didn't favour the good activity. Further biological evaluation must be conducted to confirm our molecular studies. Data amassed in the present study could be taken into account to develop novel indolizine scaffolds for improving the potency of this class of compound as potential selective COX-2 inhibitors.

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Supplementary data

Electronic supplementary information (ESI) available: FT-IR, ^{1}H & ^{13}C NMR spectra.

Competing interests

The authors declare that they have no competing interests.

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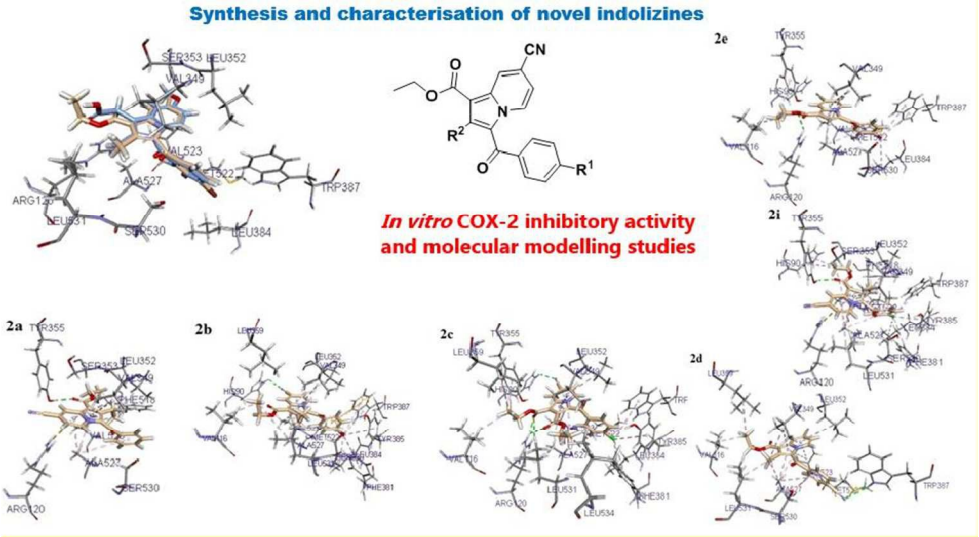

Efficient synthesis and characterization of novel indolizine scaffolds as COX-2 inhibiting agents including molecular modelling studies