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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Identification of 3-hydroxy-4[3,4-dihydro-3-oxo-2*H*-1,4-benzoxazin-4-yl]-2,2-dimethyldihydro-2*H*-benzopyran derivatives as potassium channel activators and anti-inflammatory agents

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Abstract The present study described the design, synthesis and identification of 3-hydroxy-4[3,4-dihydro-3-oxo-2*H*-1,4-benzoxazin-4-yl]-2,2-dimethyldihydro-2*H*-benzopyran derivatives. Their biological activity was tested for K_{ATP} channel opener as antihypertensives, COX-1 and COX-2 activity. The results were compared with the activity of cromakalim, ibuprofen and celecoxib. The study aimed at exploring the influence of introduction of a benzoxazine substituent at position 6 of various derivatives of benzopyrans in order to improve biological activity. Several compounds were found to be equipotent or even more potent than cromakalim. Out of these nitro-substituted benzopyrans, nitro substitution at benzoxazino group possessed potent antihypertensive activity in the *R/S* isomers. With amino derivatives, activity remains constant when compared with standard cromakalim. Similarly, compounds **17b**, **17c**, **17e** and **17h** have exhibited around 40 % inhibition of COX-1 as compared to the inhibition of COX-2. Only two compounds **17g** and **17i** exhibited effective inhibition more than 50 % of COX-2 compared with the inhibition of COX-1 at a concentration of 0.3 mg/ml.

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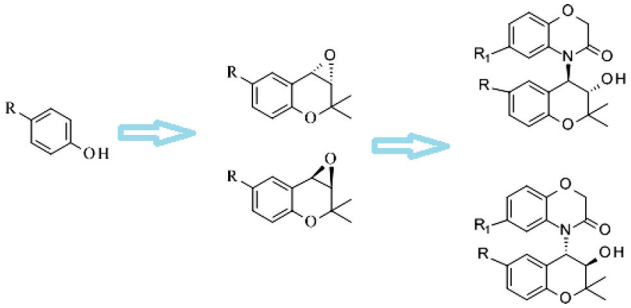
Graphical Abstract



Keywords Benzopyran · Benzoxazine · K channel opener · COX-1 · COX-2 · Anti-inflammatory

Introduction

Benzopyran-based cromakalim is K_{ATP} channel opener, which is reported as potential class of therapeutic agents possessing wide variety of cardioprotective activities such as antihypertensive, antiischaemic, myorelaxant, antiarrhythmic, skeletal muscle relaxants, COX-2 inhibitors, antimicrobials, anti-inflammatory, urinary incontinence, antiasthmatics, anticonvulsants and antiangiogenic (Florence *et al.*, 2014). Out of all the numerous activities related to K_{ATP} channels, antihypertensive activity is the most widely studied, used and well established (Sanfilippo *et al.*, 1993). These K_{ATP} channel openers acts mainly by the opening of potassium channels. Mechanism of action of cromakalim suggests that its antihypertensive effect is associated with cell membrane hyperpolarization and their affinity towards potassium channels. They express pre- and

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post-synaptically in many brain regions; they open and close in response to changes in intra-cellular ATP ratios. Low ATP levels open these channels, allowing K^+ efflux and cell hyperpolarization (Burrell *et al.*, 1990). K_{ATP} channel openers such as benzopyrans have been intensely investigated because of their wide distribution, key role in linking cellular metabolism to membrane potential and therapeutic values. They have attracted considerable attention because of the evidence of their potential value in the treatment of those disorders in which smooth muscle contraction is involved (Caliendo *et al.*, 2002).

Benzopyran nucleus has been modified in both the aromatic ring and in the pyran moiety. Structure–activity studies have shown that the different positions of the benzopyran ring have been variously substituted permitting the optimal activity to be correlated with a specific set of structural characteristics and stereo chemical features in the molecule (Lang and Wenk, 1988). When cromakalim aromatic ring was replaced by pyridine and thiophene systems, potent pyran[3,2-*c*]pyridines and thieno[3,2-*b*]pyrans were identified (Sanfilippo *et al.*, 1992; Press *et al.*, 1993). The replacement of the pyran nucleus with different heterocyclic and non-heterocyclic systems often produced low or moderately active compounds when compared with the benzopyran series (Ashwood *et al.*, 1991). Similarly, there are several benzoxazine derivatives depending on the position of the oxygen and nitrogen in the ring, possessing large number of pharmacologically active molecules such as calcium channel antagonists, central nervous system drugs, analgesic and others (Verma *et al.*, 2011). Therefore, benzoxazine derivatives have been reported to show considerable pharmacological actions such as antimicrobial, antimycobacterial, antidiabetic, antihypolipidemic, and antidepressant (Macías *et al.*, 2005). The literature review showed that Hiroshi K and co-workers have synthesized novel benzoxazine derivatives (**1**) and claimed that these compounds possess K^+ channel opening activity and can be used as antiasthmatic, antiepileptic and antihypertensive agent (Koga *et al.*, 1999). Russell et al. (Russell *et al.*, 1993) have synthesized highly potent fluoroalkyl benzoxazine pyridine-*N*-oxide derivatives (**2**) and (**3**) as potassium channel openers. Kusumoto *et al.* (1994) have studied the antihypertensive and cardiovascular properties of a new potassium channel opener, TCV-295 (**4**) in rats and dogs. Cecchetti *et al.* (2003) have synthesized highly potent 1,4-benzothiazine derivatives (**5**) as K_{ATP} openers. Koini *et al.* (2009) have synthesized and studied 5,7,8-trimethyl-benzopyran (**6**) and 5,7,8-trimethyl-1,4-benzoxazine amino amide derivatives (**7**) as novel antiarrhythmic agents against ischaemia–reperfusion injury. Structure–activity studies indicated that the replacement of the pyran oxygen with NH or CH_2 to produce tetrahydroquinolines and tetrahydronaphthalenes, as well as the ring expansion to benzoxazepines, had detrimental effect on the potency (Buckle *et al.*, 1991a, b). Also slight reduction in potency was also observed with the elimination of the pyran oxygen as in the case of indanes (Buckle *et al.*, 1991a, b). However, replacements of the pyran ring with oxazine systems have shown potent vasodilators (Yamada *et al.*, 1993).

Hence, we planned the synthesis of the benzoxazine benzopyrans without altering the nucleus of the basic cromakalim. We have attempted to synthesize various substituted benzopyrans and carry out the biological studies. To test whether the synthesized molecules are biologically active and do they possess activity, they were tested for antihypertensive and COX-2 activity, and the results were compared with the activity of existing cromakalim. Antihypertensive activity of the synthesized compounds was carried out by direct method in the anaesthetized rats (Sanfilippo *et al.*, 1993). Benzopyran-based cromakalim, pinacidil, aprikalim and diazoxide are ATP-sensitive potassium channel openers which are potent antihypertensive agents acting via peripheral vasodilation; because of the ability of these agents to open potassium channels in several tissue types, their usage is somewhat limited over the existing antihypertensive agents. Tissue-selective K_{ATP} openers are clearly required to explore the potential of these agents in disease states. Hence, various derivatives have been synthesized in correlation with these benzopyrans with tissue selectivity. The objective was achieved, and antihypertensive activity was carried out by the direct method using anaesthetized albino rats.

Number of uses of benzopyran-based cromakalim are as follows: widely used as non-steroidal anti-inflammatory drugs by the inhibition of COX-2 inhibitory activity without influencing much on the normal physiological functions of COX-1, expressed virtually in all tissues and involved in the regulation of physiological functions and in maintaining platelet aggregation and homeostasis of the GI tract and the kidney; mainly, 2,3-diarylbenzopyran derivatives have been extensively used as COX-2 inhibitors (Matralis *et al.*, 2011). Based on the above information, newly synthesized molecules were evaluated for their ability to inhibit COX-2 and COX-1 enzymes by in vitro colorimetric COX (ovine) inhibitor assay method which utilizes the peroxidase component of the enzyme cyclooxygenase. Docking studies were also performed to find out in silicon interaction of benzoxazine benzopyrans derivatives with COX-1 and COX-2 target protein (Zhang *et al.*, 2002).

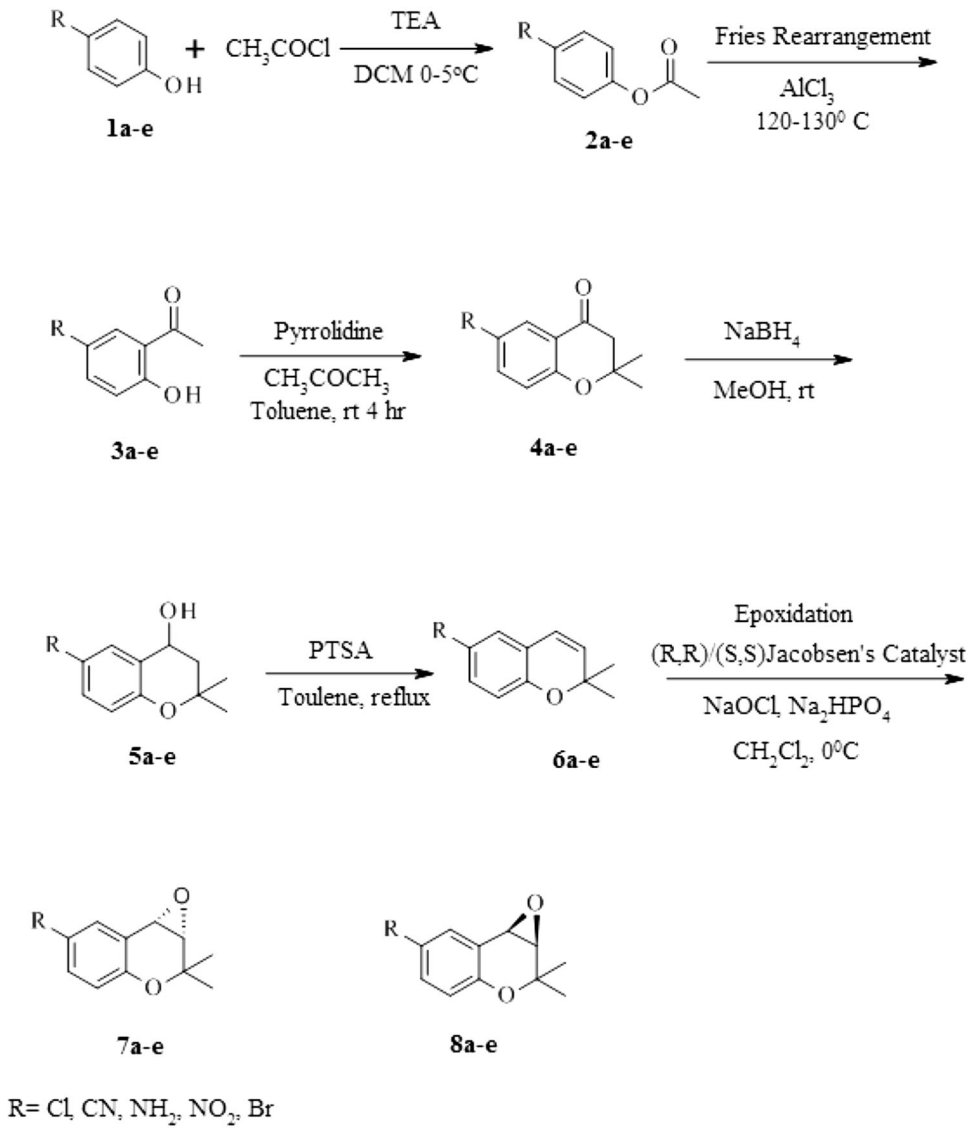
Results and discussion

Chemistry

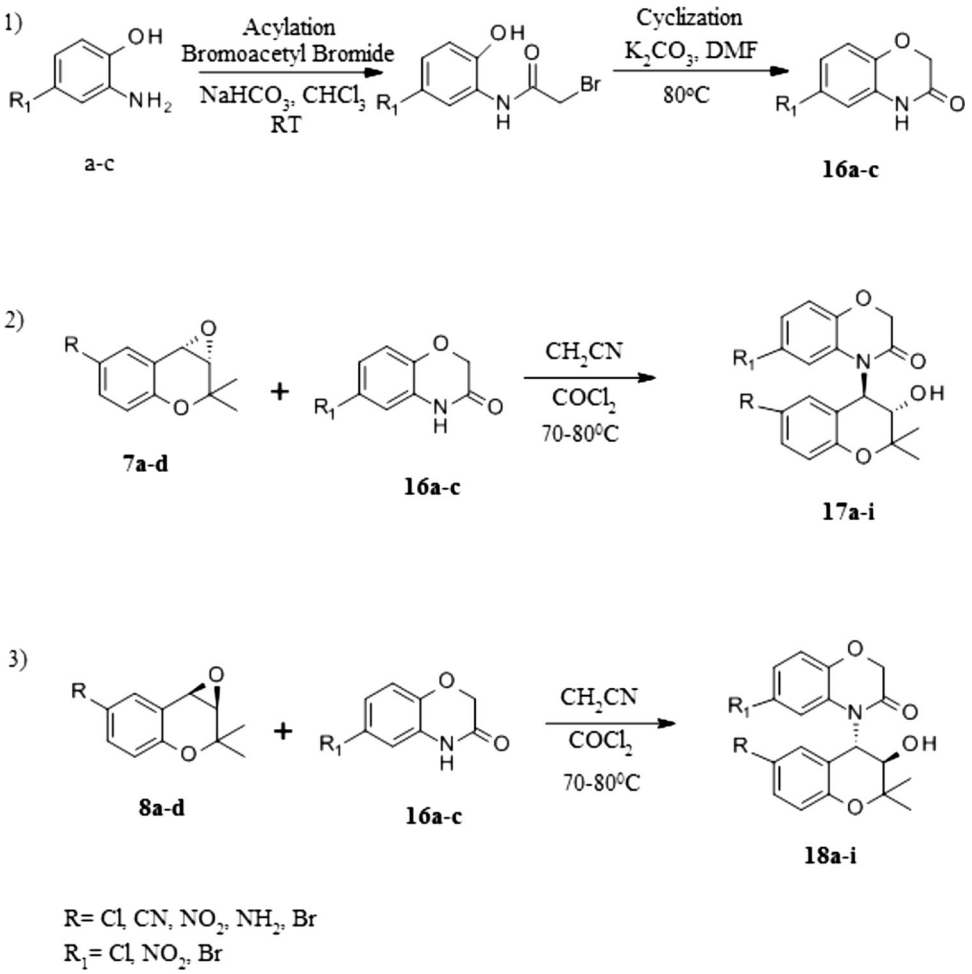
6-Substituted-2,2-dimethyl-2,7*b*-dihydro-1*aH*-oxireno[2,3-*c*]chromene (epoxide) derivatives were synthesized using *R*/*R*- and *S*/*S* Jacobsen's catalyst for the synthesis of targeted

3-hydroxy-4[3,4-dihydro-3-oxo-2*H*-1,4-benzoxazin-4-yl]-2, 2-dimethyldihydro-2*H*-benzopyrans. The synthesis of 6-substituted-2,2-dimethyl-2,7*b*-dihydro-1*aH*-oxireno[2,3-*c*]chromene (epoxide) using *R*/*R*- and *S*/*S* Jacobsen's catalyst provided optimum yield. Compounds **7a–e** and **8a–e** were confirmed by spectral and elemental methods of analysis. Synthetic route for the targeted compounds are summarized in Scheme 1.

Synthesis of substituted benzoxazino benzopyrans was achieved in several steps involving the synthesis of intermediate benzoxazino moiety and synthesis of the above epoxides using various derivatives of benzopyrans. 6-Substituted 3,4-dihydro-3-oxo-2*H*-1,4-benzoxazine **16a–c** were synthesized using bromoacetyl bromide in ice-cold bath solution of 4-substituted 2-amino phenols in saturated solution of sodium carbonate in $CHCl_3$ at room temperature for 3 h to obtain crude oil. After washing several times with water and drying over anhydrous sodium sulphate, anhydrous K_2CO_3 was added and heated at 80 °C with stirring for 3 h. After washing with water and extracted several times with $CHCl_3$, combined extracts were dried over anhydrous Na_2SO_4 and evaporated in vacuum to get white solid product **16a–c**. 3470.25 cm^{-1} (N–H), 1214.56 cm^{-1} (C–O–C) and 2964.42 cm^{-1} (CH–Ali) indicated the synthesis of above intermediates. The target molecule 3-hydroxy-4[3,4-dihydro-3-oxo-2*H*-1,4-benzoxazin-4-yl]-2,2-dimethyldihydro-2*H*-benzopyrans [**17a–i** and **18a–i**] was synthesized using various *R*/*S* epoxy benzopyran derivatives with $COCl_2$ in acetonitrile solution. After washing with aqueous $NaHCO_3$ and extracting with ethyl acetate, the organic layers are dried over $NaSO_4$; the residue was further recrystallized to get the above product (Scheme 2). Synthesis of targeted molecules was confirmed using elemental analysis; spectrophotometric evaluations and optical rotations were confirmed by



Scheme 1 Scheme for the preparation of 6-substituted-2,2-dimethyl-2,7b-dihydro-1a*H*-oxireno[2,3*c*]chromene (epoxidation using R/R- or S/S Jacobsen's catalyst) [**7a–e**] and [**8a–e**]



Scheme 2 Scheme for the synthesis of 3,4-dihydro-3-hydroxy 2,2-dimethyl-4-[*N*(3,4-dihydro-3-oxo-2*H*-1,4 benzoxazine)]-2*H*-benzopyran

polarimetric studies. FTIR spectra showed that C–N stretch at 1263.56 cm^{-1} and Ar–Cl at 710.72 cm^{-1} confirms the identification of the functional groups. Mass spectrum shows the presence of molecular ion peak at 394.21 *m*/*z*, whereas the calculated mass of the compound was 395.0 *m*/*z*.

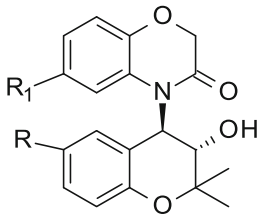
Biological evolution and structure–activity relationship (SAR)

When benzoxazino benzopyrans were subjected to antihypertensive activity, we have observed that the nitro group-substituted benzopyrans with the nitro substitution at benzoxazino group possessed good antihypertensive activity in the *R*/*S* isomers, even so with the amino derivatives the activity remains when compared with the standard cromakalim. The cyano derivative as usual was also active with *R*/*S* isomer; this was not the case with the other derivatives that are summarized in Table 1.

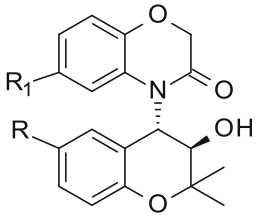
From the above observation, it is clear that when substituted with cyano group at position 6 and R_1 with bromine group, the compound has shown good antihypertensive activity and R isomer of the same substitutions has also shown moderate action. Amino derivative substituted with bromo-oxazino group possess minimal activity. Surprisingly, in the benzoxazino derivative where R = NO_2 and R_1 = NO_2, both the *R*/*S* isomers have shown good activity, whereas the same nitro group with a bromo substituent at benzoxazino position has shown no activity. But when nitro derivative of benzopyran substituted with a bromo benzoxazino compound, there was no activity observed. Even *R* isomer containing cyano substitution at position 6 and bromo group at the benzoxazino group did not show activity. Amino substitution at position 6 in both *R* and *S* has shown moderate activity (Table 2).

When synthesized compounds **17b**, **17c**, **17e**, **17g**, **17h** and **17i** were subjected to in vitro colorimetric COX (ovine) inhibitor assay for evaluation of their ability to inhibit COX-2 and COX-1, the peroxidase component of cyclooxygenase was utilized. The peroxidase activity is assayed colorimetrically by monitoring the appearance of oxidized *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD) during the reduction of PGG_2 to PGH_2, at 590 nm. The results of in vitro COX enzyme inhibition assay studies are summarized in Table 3. The results showed that the

Table 1 Physical and analytical data of compounds **17a–i** and **18a–i**



17a-i



18a-i

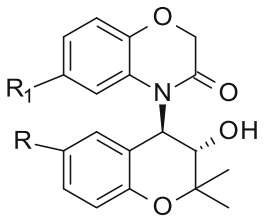
Comp. No.	R	R_1	Mol. formula	Mol. weight	Melting point	% Yield	Rf value
17a	Cl	Cl	$C_{19}H_{17}Cl_2NO_4$	394.25	240	40	0.7
17b	Br	NO_2	$C_{19}H_{17}BrN_2O_6$	449.25	210	44	0.15
17c	NO_2	Br	$C_{19}H_{17}BrN_2O_6$	449.25	184	60	0.11
17d	CN	Br	$C_{20}H_{17}BrN_2O_4$	429.26	189	45	0.2
17e	NH_2	Cl	$C_{19}H_{19}ClN_2O_4$	374.82	213	36	0.7
17f	NH_2	Br	$C_{19}H_{19}BrN_2O_4$	419.27	178	35	0.9
17g	NH_2	NO_2	$C_{19}H_{19}N_3O_6$	385.37	195	49	0.3
17h	NO_2	Cl	$C_{19}H_{17}ClN_2O_6$	404.8	176	37	0.4
17i	NO_2	NO_2	$C_{19}H_{17}N_3O_8$	415.35	149	67	0.12
18a	Cl	Cl	$C_{19}H_{17}Cl_2NO_4$	394.25	231	36	0.1
18b	Br	NO_2	$C_{19}H_{17}BrN_2O_6$	449.25	201	41	0.2
18c	NO_2	Br	$C_{19}H_{17}BrN_2O_6$	449.25	182	53	0.12
18d	CN	Br	$C_{20}H_{17}BrN_2O_4$	429.26	176	42	0.2
18e	NH_2	Cl	$C_{19}H_{19}ClN_2O_4$	374.82	208	38	0.7
18f	NH_2	Br	$C_{19}H_{19}BrN_2O_4$	419.27	172	35	0.7
18g	NH_2	NO_2	$C_{19}H_{19}N_3O_6$	385.37	179	45	0.3
18h	NO_2	Cl	$C_{19}H_{17}ClN_2O_6$	404.8	182	40	0.5
18i	NO_2	NO_2	$C_{19}H_{17}N_3O_8$	415.35	153	43	0.4

compounds **17b**, **17c**, **17e** and **17h** have exhibited around 40 % inhibition of COX-1 (39.07, 42.89, 39.21 and 51.68 %), as compared to the inhibition of COX-2 (9.09, 21.08, 14.06 and 16.93 %) at a concentration of 0.3 mg/ml. Only two compounds **17g** and **17i** exhibited effective inhibition of COX-2 (51.04 and 54.88 %), compared with the inhibition of COX-1 (17.63 and 26.7 %) at a concentration of 0.3 mg/ml. The blank has 38.09 % COX-1 inhibition and 8.69 % COX-2 inhibition activity. The standard celecoxib has 86.95 % inhibition of COX-2 and 33.33 % COX-1 inhibition, whereas ibuprofen has 80.95 % inhibition of COX-1 and 13.04 % COX-2 inhibition (Fig. 1).

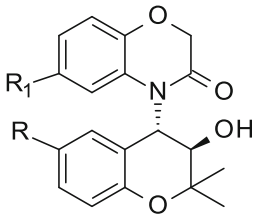
Docking studies

All molecules have shown similar docking mode in the active site of the enzyme. The active site is separated from the initial substrate binding site by a constriction comprised of three residues: Arg-120, Tyr-355 and Glu-524. This constriction must expand for any substrates, inhibitors or products to access or depart the active site. The catalytically essential Tyr-385 is located approximately 13 Å above Arg-120 and across the active site from Ser-530. Ser-530 is not essential for COX. Most of the residues in the COX active sites are identical between the two isoforms, but there are notable exceptions in a region across the active site from Arg-120 and bordered by hydrophobic residues at position 523 (Val in COX-2, Ile in COX-1) (Marnett, 2002). The difference in steric bulk at position 523 provides differential access to space in a side pocket (accessible in COX-2, not accessible in COX-1). Conserved differences also exist at the base of the side pocket (Arg-513 in COX-2, His-513 in COX-1) (Schneider *et al.*, 2001). Evaluation was done with docking score, and single best pose is generated as the output for particular ligand. The docked conformation of the highest docking score molecule 17i is shown in Fig. 2. 17i compound shows the strong H-bonding interaction with Thr-206, His-207 of

Table 2 Antihypertensive activity of compounds **17a–i** and **18a–i**



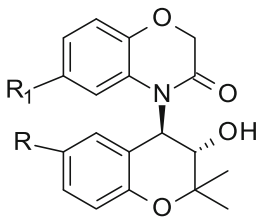
17a-i



18a-i

Comp. No	Parameter	Baseline	With Adrenaline	With test alone	With test +Adrenaline	Inference
17b	SBP	114.8 (±0.256)	159.3 (±1.250)	149.6 (±1.022)	152.1 (±0.228)	Minimal activity
R = Br	DBP	66.0 (±1.250)	92.8 (±2.930)	86.5 (±1.110)	88.2 (±1.027)	
$R_1 = NO_2$	MABP	91.7 (±0.773)	129.7 (±2.145)	119.0 (±2.410)	122.6 (±3.009)	
	HR	316.1 (±4.077)	390.8 (±1.225)	376.0 (±3.008)	380.4 (±3.505)	
17d	SBP	129.7 (±1.980)	188.4 (±2.158)	132.0 (±0.057)	141.8 (±2.880)	Antihypertensive
R = CN	DBP	86.1 (±1.489)	118.4 (±1.012)	85.7 (±1.550)	90.2 (±1.709)	
$R_1 = Br$	MABP	114.8 (±2.058)	136.7 (±2.008)	116.0 (±1.546)	126.9 (±1.148)	
	HR	397.2 (±1.258)	433.8 (±3.113)	409.2 (±0.018)	420.7 (±2.220)	
17f	SBP	124.5 (±3.150)	167.9 (±0.225)	156.0 (±1.087)	159.7 (±1.009)	Moderately active
$R = NH_2$	DBP	90.7 (±1.054)	123.0 (±0.704)	112.8 (±1.883)	115.4 (±1.007)	
$R_1 = Br$	MABP	116.7 (±0.117)	154.7 (±0.140)	138.4 (±1.478)	142.0 (±1.888)	
	HR	308.7 (±0.114)	359.6 (±0.488)	327.9 (±1.048)	322.0 (±4.258)	
17g $R = NH_2$	SBP	130.8 (±2.112)	172.9 (±1.088)	139.6 (±1.159)	144.0 (±1.029)	Antihypertensive
$R_1 = Br$	DBP	86.7 (±1.220)	120.9 (±3.500)	89.8 (±2.004)	92.0 (±1.780)	
	MABP	109.6 (±2.365)	148.2 (±2.238)	119.0 (±2.112)	126.8 (±1.155)	
	HR	311.1 (±5.708)	342.8 (±0.117)	328.9 (±2.007)	331.0 (±0.770)	
17i	SBP	112.7 (±0.228)	168.9 (±1.580)	126.0 (±1.050)	129.7 (±2.550)	Antihypertensive
$R = NO_2$	DBP	72.7 (±1.007)	100.1 (±2.080)	79.8 (±0.502)	82.0 (±3.008)	
$R_1 = NO_2$	MABP	95.7 (±4.228)	126.0 (±2.710)	102.7 (±1.007)	108.1 (±0.013)	
	HR	326.1 (±1.056)	364.7 (±2.117)	334.0 (±1.443)	339.7 (±0.227)	
18b	SBP	117.9 (±3.146)	159.8 (±2.118)	156.7 (±1.084)	157.6 (±1.228)	No Activity
R = Br	DBP	67.8 (±1.057)	98.7 (±1.228)	97.3 (±1.008)	100.2 (±3.096)	
$R_1 = NO_2$	MABP	90.2 (±1.007)	119.7 (±3.189)	120.6 (±2.107)	120.8 (±1.528)	
	HR	304.0 (±4.127)	346.8 (±0.221)	349.7 (±2.397)	350.4 (±1.886)	
18d	SBP	105.1 (±0.404)	144.7 (±0.786)	116.9 (±1.228)	120.0 (±2.505)	Antihypertensive
R = CN	DBP	60.7 (±1.056)	91.5 (±0.258)	68.7 (±2.115)	76.1 (±0.156)	
$R_1 = Br$	MABP	78.9 (±1.008)	115.0 (±3.489)	80.8 (±2.007)	91.5 (±0.117)	
	HR	268.1 (±1.470)	317.9 (±3.118)	278.0 (±0.220)	289.7 (±1.551)	
18g	SBP	122.7 (±2.022)	168.0 (±1.058)	159.7 (±2.580)	160.8 (±2.008)	Moderately active
$R = NH_2$	DBP	74.8 (±2.228)	102.6 (±0.019)	89.7 (±3.007)	93.9 (±1.739)	
$R_1 = Br$	MABP	100.4 (±2.085)	141.8 (±1.007)	126.7 (±0.227)	129.8 (±0.728)	
	HR	309.7 (±1.228)	341.0 (±4.057)	322.7 (±5.227)	336.0 (±1.270)	
18i	SBP	123.6 (±2.050)	168.2 (±1.204)	132.0 (±0.229)	149.2 (±3.237)	Antihypertensive
$R = NO_2$	DBP	83.9 (±3.691)	120.6 (±1.227)	89.6 (±0.580)	100.9 (±4.336)	
$R_1 = NO_2$	MABP	107.9 (±5.180)	140.8 (±1.239)	118.0 (±1.500)	122.9 (±4.179)	
	HR	290.7 (±0.170)	326.9 (±3.229)	302.7 (±2.007)	315.8 (±2.775)	

Table 3 In vitro COX-1 and COX-2 enzyme inhibition activity data for standard drugs' blank and synthesized compounds **17a–i**



17a-i

Compounds	% Inhibition[a,b]	
	COX-1 (0.3 mg/ml)	COX-2 (0.3 mg/ml)
Celecoxib	33.33	86.95
Ibuprofen	80.95	13.04
Blank	38.09	8.69
17b	39.07	9.09
R = Br, $R_1 = NO_2$		
17c	42.89	21.08
$R = NO_2$, R_1 = Br		
17e	39.21	14.06
$R = NH_2$, R_1 = Cl		
17g	17.63	51.07
$R = NH_2$, $R_1 = NO_2$		
17h	51.68	16.93
$R = NO_2$, R_1 = Cl		
17i	26.70	54.88
$R = NO_2$, $R_1 = NO_2$		

Test concentration used for evaluation is 0.3 mg/ml, dissolved in Methanol

[a] Values are acquired using in vitro ovine COX-1/COX-2 assay kit (Catalog No. 760 111, Cayman Chemicals Inc., Ann Arbor, MI)

[b] Experiments were carried out in duplicate and have less than 10 % error

Cox-1 target protein (Fig. 2a) and Thr-221, Asp-294, Phe-3925 of Cox-2 target protein (Fig. 2c). Aromatic rings provide necessary lipophilic characters to allocate in lipophilic cleft of protein (Fig. 2b, d). All other molecules showed similar docking mode in the active site of the enzyme (Table 4).

Experimental work

Chemistry

Progress of all the reactions were monitored by TLC studies using pre-coated alumina plate using various solvent systems for elution. Formation of the products was carried by the determination of their melting points. Structure of all the compounds was authenticated by the detailed spectral analysis using FTIR and ^{1}HNMR.

Synthesis of 6-substituted-2,2-dimethyl-2,7b-dihydro-1aH-oxireno[2,3-c]chromene (epoxide) using R/R- and S/S Jacobsen's catalyst [7a–d and 8a–d]

Substituted benzopyran epoxide was synthesized by several steps utilizing trial-and-error method starting from phenols. Various substituted phenols were successfully acetylated using acetyl chloride and triethylamine under dichloromethane. These acetylated phenols underwent Fries rearrangement in the presence of aluminium trichloride under controlled temperature of 120–130 °C. The compounds were further cyclized using pyrrolidine under Dean Stack set-up using acetone to get cyclized nucleus, which were further treated with *p*-toluenesulphonic acid (PTSA) to get the nucleus of various substituted benzopyrans. The double-bonded compound was further converted to chiral derivative by treating with a chiral catalyst, Jacobsen's catalyst; the respective *R*/*S* catalyst resulted in the chiral epoxides which are highly reactive.

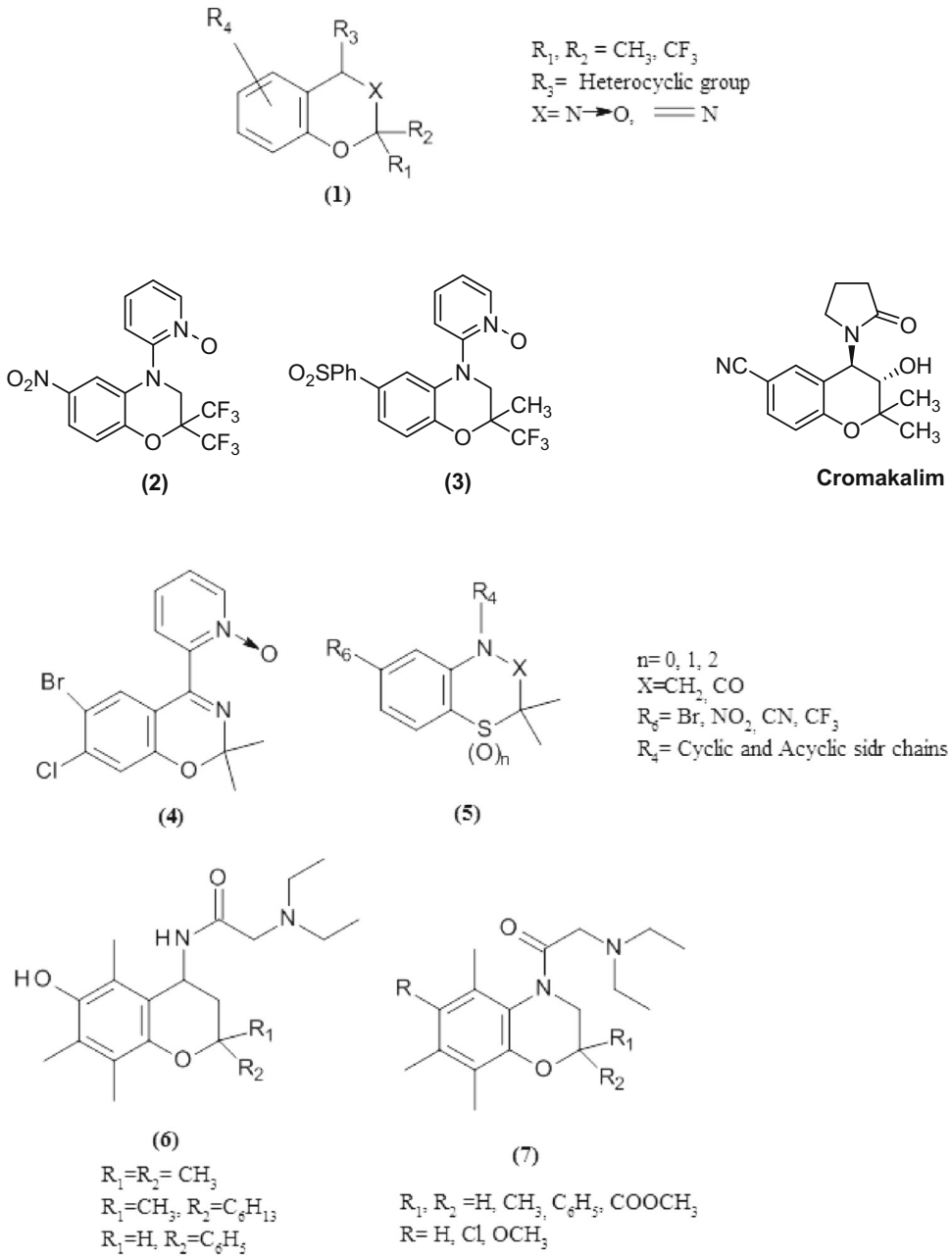


Fig. 1 First-generation K_{ATP} channels openers as potent antihypertensive agents

Synthesis of 6-substituted 3,4-dihydro-3-oxo-2H-1,4-benzoxazine intermediate [16a–c]

Bromoacetyl bromide (3.5 ml, 0.15 mol) was added dropwise to an ice-cold bath solution of 4-substituted 2-amino phenol. To this solution, 0.1 mmol saturated solution of sodium carbonate in approximately 600 ml of $CHCl_3$ was added. The reaction mixture was stirred at room temperature for 3 h and monitored by TLC continuously; layers were separated; and the organic phase was washed several times with water and dried over anhydrous sodium sulphate and concentrated in vacuum to provide a crude oil. To the solution of crude oil product, anhydrous K_2CO_3 (0.1 mol) in 250 ml of DMF was added and heated at 80 °C and stirred for 3 h. After cooling, the reaction mixture was poured into water and extracted several times with $CHCl_3$. Combined organic extracts were dried over anhydrous Na_2SO_4 and evaporated in vacuum to get white solid product **16a–c**. Completion of the reaction was confirmed by the help of TLC, using diethyl ether: *n*-hexane 1:1 as solvent system; percentage yield of the compound was found to 68 %.

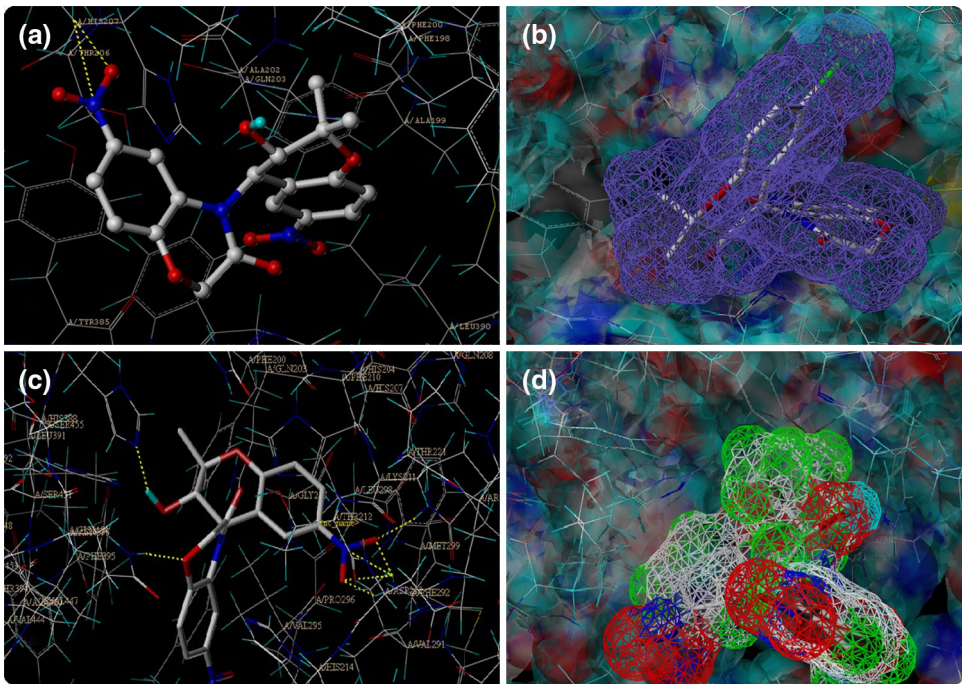


Fig. 2 Docking poses of synthesized compound. **a** Binding interaction of **17i** compound in the binding pocket of Cox-1 target protein (PDB: 3N8X). **b** Hydrophobic fitting of **17i** compound in binding pocket of COX-1 target protein. **c** Binding interaction of **17i** compound in the binding pocket of Cox-2 target protein (PDB: 4COX). **d** Hydrophobic fitting of 17i compound in binding pocket of COX-2 target protein

Table 4 Docking result of synthesized molecules in Cox-1 and Cox-2 target

Molecule	Cox-1		Cox-2	
	Docking score	Crash	Docking score	Crash
17a	4.2361	−2.9798	3.8123	−0.9143
17b	4.6229	−0.6516	3.0801	−1.7626
17c	3.1293	−1.1717	1.2349	−1.7626
17d	2.676	−1.0507	3.5041	−1.7626
17e	2.8071	−1.0989	2.2297	−1.7626
17f	2.6483	−2.9823	2.685	−1.7626
17g	3.7211	−1.0446	5.244	−1.7626
17h	3.3094	−1.4756	3.9415	−1.7626
17i	4.8597	−1.2149	5.9193	−1.7626
18a	2.9451	−1.3396	4.4946	−1.7626
18b	2.7012	−1.3362	5.0748	−1.7626
18c	4.2698	−0.2928	5.366	−1.7626
18d	3.9045	−1.4703	3.1873	−1.7626
18e	3.766	−1.0013	3.4567	−1.7626
18f	3.4929	−1.3137	3.3603	−1.7626
18g	3.4131	−0.9563	5.0594	−1.7626
18h	4.796	−1.6318	4.1827	−1.7626
18i	4.1976	−0.6875	3.8871	−1.7626
Ibuprofen	6.0995	−1.4525	6.2437	−1.7626
Celecoxib	5.3483	−0.5562	6.4778	−1.7626

*Synthesis of 3,4-dihydro-3-hydroxy 2,2-dimethyl-4-[N(3,4-dihydro-3-oxo-2H-1,4-benzoxazine)]-2H-benzopyran [**17a–i** and **18a–i**]*

Various *R*/*S* epoxy benzopyran derivatives (1 g, 4.97 mmol) and 1.03 g of the above intermediate and 0.64 g $COCl_2$ were added to 4 ml of acetonitrile solution and heated to 60 °C, stirring continuously for 50–60 min. After 1 h, TLC was carried out to check the completion of the reaction. The reaction mixture was washed with aqueous $NaHCO_3$ and extracted with ethyl acetate; organic layers are dried over $MgSO_4$; and the residue obtained was further recrystallized by hexane–ethyl acetate and hexane–chloroform to get pure 3,4-dihydro-3-hydroxy 2,2-dimethyl-4-[*N*(3,4-dihydro-3-oxo-2*H*-1,4-benzoxazine)]-2*H*-benzopyran derivatives **17a–i** and **18a–i**. Formation of the above product was confirmed by TLC, using hexane: ethyl acetate 5:1 as solvent system, and further the structure of the synthesized compound was confirmed using FTIR and ^{1}H NMR studies. Optical rotations were confirmed by polarimetric studies.

*6-Chloro-4-((3S,4R)-6-chloro-3-hydroxy-2,2-dimethyl-3,4-dihydro-2H-chromen-4-yl)-2H-benzo[b][1,4] oxazin-3(4H)-one (**17a**)*

Yield 40.38 %, mp 240–242 °C; [α]25D +38.00 (c 0.01, Water) IR (KBr) vmax: 1266, 3459, 2929.08, 2977, 1637, 1589, 1363 710 cm^{-1}. ^{1}H NMR (DMSO, 400 MHz), $\delta = 1.40$ (6H, s, CH_3), 4.58 (1H, s, OH), 7.032 (2H, d, $J = 2.4$, CH), 6.928–6.934 (2H, d, $J = 2.4$, CH), 7.24 (1H, s, CH), 7.173 (1H, s, CH), 4.093–4.146 (2H, t, $J = 8.2$, CH_2), 2.038 (2H, t, $J = 3.2$ CH_2), 5.619 (1H, d, $J = 12$, CH), 6.220 (1H, d, $J = 9.6$, CH). 13C NMR (DMSO, 400 MHz): $\delta = 166.3$ (C=O) 151.1, 150.3 (C–O) 129.3, 127.2 (CH, benzene) 125.4, 126.1 (C–Cl) 92.2 (CH–OH) 70.3 (CH_2) 21.2 (CH_3) MS (ESI, *m*/*z*): 395.2 (M + 1, 98 %), 393.0 (M − 1, 52 %). Anal. Calcd. for C19H17Cl2NO4: C, 57.88; H, 4.35; N, 3.55. Found: C, 57.94; H, 4.41; N, 3.27.

*4-((3S, 4R)-6-Bromo-3-hydroxy-2,2-dimethyl-3,4-dihydro-2H-chromen-4-yl)-6-nitro-2H-benzo[b][1,4] oxazin-3(4H)-one (**17b**)*

Yield 44.25 %, mp 210–212 °C. [α]25D +21.40 (c 0.009, Water) IR (KBr) vmax: 3044, 2927, 2976, 1638, 1597, 1361, 1266, 522, 561, cm^{-1}. ^{1}H NMR (DMSO, 400 MHz), $\delta = 1.407$ (6H, s, CH_3), 5.608 (1H, s, OH), 7.067 (2H, d, $J = 2.4$, CH), 7.149 (1H, d, $J = 2.4$, CH), 7.171 (d, 1H, $J = 2.4$, CH), 7.241 (1H, s, CH), 6.649 (1H, s, CH). 13C NMR (DMSO, 400 MHz): $\delta = 166.1$ (C=O) 151.2, 150.5 (C–O) 142.4(C-NO_2) 129.5, 127.2 (CH, benzene) 116.1 (C–Br) 92.3 (CH–OH) 70.2 (CH_2) 21.2 (CH_3) MS (ESI, *m*/*z*): 450.0 (M + 1, 96 %), 448.2 (M − 1, 65 %). Anal. Calcd. for C19H17BrN2O6: C, 50.80; H, 3.81; N, 6.24. Found: C, 50.84; H, 3.79; N, 6.12.

*6-Bromo-4-((3S, 4R)-3-hydroxy-2,2-dimethyl-6-nitrochroman-4-yl)-2H-benzo[b][1,4]oxazin-3(4H)-one (**17c**)*

Yield 60.53 %, mp 184–186 °C. [α]25D +43.12 (c 0.006, 0.5 N HCl) IR (KBr) vmax: 3648, 3092, 2966, 1651, 1600, 1520, 1340, 1214 545 cm^{-1}. ^{1}H NMR (DMSO 400 MHz), δ ppm 1.577 (s, 6H, CH_3), 4.608 (s, 1H, OH), 8.709–8.716 (d, 2H, CH), 8.343 (d, 1H, $J = 2.8$, CH), 6.935–6.941 (d, 1H, $J = 2.4$ CH), 7.107 (s, 1H, CH), 7.084 (s, 1H, CH), 2.749 (t, 2H, CH_2), 8.366 (d, 1H, $J = 2.8$, CH), 6.847 (d, 1H, CH $\delta = 166.3$ (C=O) 151.4, 150.8 (C–O) 129.3, 127.2 (CH, benzene) 138.5 (C–NO_2) 115.4 (C–Br) 92.8 (CH–OH) 70.2 (CH_2) 21.2 (CH_3) MS (ESI, *m*/*z*): 430.2 (M + 1, 94 %), 428.0 (M − 1, 55 %). Anal. Calcd. for C19H17BrN2O6: C, 50.80; H, 3.81; N, 6.24. Found: C, 50.74; H, 3.85; N, 6.32.

*(3S, 4R)-4-(6-Bromo-3-oxo-2,3-dihydrobenzo[b][1,4]oxazin-4-yl)-3-hydroxy-2,2-dimethylchroman carbonitrile (**17d**)*

Yield 45.27 %, mp 189–191 °C. [α]25D +23.5 (c 0.01, Water) IR (KBr) vmax: 3574, 3045, 2914, 2315, 1661, 1610, 1510, 1218, 545 cm^{-1}. ^{1}H NMR (DMSO, 400 MHz) $\delta = 1.457$ (6H, s, CH_3), 4.609 (1H, s, OH), 7.239 (d, 2H, $J = 2.0$ CH), 7.362–7.367 (1H, d, $J = 2.0$, CH), 7.383 (1H, d, $J = 2.0$, CH), 6.783 (1H, s, CH), 6.295 (1H, s, CH), 6.818 (1H, d, $J = 2.0$, CH), 6.927 (1H, d, $J = 2.4$, CH). 13C NMR (DMSO, 400 MHz $\delta = 166.3$ (C=O) 151.2, 150.8 (C–O) 129.2, 127.1 (CH, benzene) 125.4 (C–Br) 105.4 (C–CN) 92.2 (CH–OH) 70.1 (CH_2) 21.2 (CH_3) MS (ESI, *m*/*z*): 430.0 (M + 1, 97 %), 428.2 (M − 1, 62 %). Anal. Calcd. for C19H17BrN2O6: C, 50.80; H, 3.81; N, 6.24. Found: C, 50.83; H, 3.86; N, 6.22.

*(3S, 4R)-4-(6-Bromo-3-oxo-2,3-dihydrobenzo[b][1,4]oxazin-4-yl)-3-hydroxy-2,2-dimethylchroman-6-carbonitrile (**17e**)*

Yield 66.53 %, mp 213–215 °C. [α]25D +0.12 (c 0.01, Water) IR (KBr) vmax: 3709, 2924, 1649, 1475, 1290, 1203 452 cm^{-1}. ^{1}H NMR (400 MHz, DMSO) δ ppm 1.564 (6H, s, CH_3), 7.210 (1H, s, Ar–H), 7.198 (1H, d, $J = 4.0$ Ar–H), 7.266–7.269 (d, 1H, Ar–H), 7.345–7.348 (1H d, $J = 1.2$, CH), 7.90 (d, 1H, $J = 4.0$, Ar–H), 4.024 (2H, s, NH), 7.90 (2H, d, $J = 4.0$ Ar–H), 7.084 (s, OH, 1H), 7.24 (1H, s CH). 13C NMR (DMSO, 400 MHz) $\delta = 166.3$

(C=O) 151.4, 150.2 (C–O) 129.3, 127.2 (CH, benzene) 141.4, (C–NH_2), 128.4 (C–Cl) 92.2 (CH–OH) 70.3 (CH_2) 21.8 (CH_3). MS (ESI, *m/z*): 375.1 (M + 1, 95 %), 373.0 (M − 1, 69 %). Anal. Calcd. for C19H19ClN2O4: C, 60.88; H, 5.11; N, 7.47. Found: C, 60.81; H, 5.18; N, 7.42.

*4-((3S, 4R)-6-Amino-3-hydroxy-2,2-dimethyl-3,4-dihydro-2H-chromen-4-yl)-6-bromo-2H-benzo[b][1,4] oxazin-3(4H)-one (**17f**)*

Yield 61.27 %, mp 178–180 °C. [α]25D +46.00 (c 0.01, Water) IR (KBr) vmax: 3399, 2980, 1615, 1463, 1295, 1268, 617.83, cm^{-1}. ^1H NMR (400 MHz, DMSO) δ ppm 1.561 (6H, s, CH_3), 7.215 (1H, s, Ar–H), 7.187 (1H, d, $J = 4.0$ Ar–H), 7.266–7.269 (d, 1H, Ar–H), 7.345–7.348 (1H d, $J = 1.2$, CH), 7.92 (d, 1H, $J = 4.0$, Ar–H), 4.021 (2H, s, NH), 7.95 (2H, d, $J = 4.0$ Ar–H), 7.089 (s, OH, 1H), 7.24 (1H, s CH). 13C NMR (DMSO, 400 MHz) $\delta = 166.3$ (C=O) 151.1, 150.3 (C–O) 129.3, 127.2 (CH, benzene) 141.4 (C–NH_2), 116.1 (C–Br) 92.2 (CH–OH) 70.3 (CH_2) 21.3 (CH_3). MS (ESI, *m/z*): 420.3 (M + 1, 93 %), 418.0 (M − 1, 64 %). Anal. Calcd. for C19H19BrN2O4: C, 54.43; H, 4.57; N, 6.68. Found: C, 54.48; H, 4.59; N, 6.74.

*4-((3S, 4R)-6-Amino-3-hydroxy-2,2-dimethyl-3,4-dihydro-2H-chromen-4-yl)-6-nitro-2H-benzo[b][1,4] oxazin-3(4H)-one (**17g**)*

Yield 69.52 %, mp 195–197 °C. [α]25D +25.0 (c 0.01, Water) IR (KBr) vmax: 3449, 2822, 1601, 1297, 1352, 1252, 1214 cm^{-1}. ^1H NMR (DMSO, 400 MHz), $\delta = 1.302$ (6H, s, CH_3), 7.069 (1H, s, Ar–H), 6.800 (1H, d, $J = 2.4$, Ar–H), 7.382 (1H, d, $J = 2.4$ Ar–H), 7.328 (1H, d, $J = 2.4$ CH), 8.028 (1H, d, $J = 2.4$, Ar–H), 7.204 (2H, d, $J = 2.4$, Ar–H), 8.050 (1H, d, $J = 2.4$, Ar–H), 4.599 (s, NH, 2H), 7.310 (2H, d, $J = 2.4$, Ar–H), 7.090 (OH, s, 1H), 5.041 (2H, d, $J = 2.4$, CH), 4.599 (t, CH, 1H). 13C NMR (DMSO, 400 MHz): $\delta = 166.3$ (C=O) 151.1, 150.3 (C–O) 129.3, 127.2 (CH, benzene) 141.3, (C–NO_2) 140.1 (C–NH_2) 92.2 (CH–OH) 70.3 (CH_2) 21.3 (CH_3). MS (ESI, *m/z*): 386.2 (M + 1, 95 %), 384.1 (M − 1, 52 %). Anal. Calcd. for C19H19N3O6: C, 59.22; H, 4.97; N, 10.90. Found: C, 59.30; H, 4.99; N, 10.96.

*4-((3S, 4R)-6-Amino-3-hydroxy-2,2-dimethyl-3,4-dihydro-2H-chromen-4-yl)-6-nitro-2H-benzo[b][1,4] oxazin-3(4H)-one (**17h**)*

Yield 47.26 %, mp 176–178 °C. [α]25D +0.21 (c 0.005, N HCl) IR (KBr) vmax: 3484, 2978, 1651, 1519, 1471, 1297, 440 cm^{-1}. ^1H NMR (DMSO, 400 MHz), $\delta = 1.308$ (6H, s, CH_3), 7.061 (1H, s, Ar–H), 6.801 (1H, d, $J = 2.4$, Ar–H), 7.385 (1H, d, $J = 2.4$ Ar–H), 7.323 (1H, d, $J = 2.4$ CH), 8.024 (1H, d, $J = 2.4$, Ar–H), 7.205 (2H, d, $J = 2.4$, Ar–H), 8.051 (1H, d, $J = 2.4$, Ar–H), 7.310 (2H, d, $J = 2.4$, Ar–H), 7.090 (OH, s, 1H), 5.041 (2H, d, $J = 2.4$, CH), 4.599 (t, CH, 1H). 13C NMR (DMSO, 400 MHz): 13C NMR (DMSO, 400 MHz): $\delta = 166.3$ (C=O) 151.1, 150.3 (C–O) 129.3, 127.2 (CH, benzene) 139.4 (C–NO_2) 126.1 (C–Cl) 92.2 (CH–OH) 70.3 (CH_2) 21.4 (CH_3). MS (ESI, *m/z*): 405.1 (M + 1, 96 %), 403.4 (M − 1, 65 %). Anal. Calcd. for C19H17ClN2O6: C, 56.37; H, 4.23; N, 6.92. Found: C, 56.34; H, 4.29; N, 6.84.

*4-((3S,4R)-3-Hydroxy-2,2-dimethyl-6-nitrochroman-4-yl)-6-nitro-2H-benzo[b][1,4]oxazin-3(4H)-one (**17i**)*

Yield 56.46 %, mp 181–183 °C. [α]25D +0.22 (c 0.005, N HCl) IR (KBr) vmax: 3453, 2934, 1634, 1525, 1434, 12,385, 446 cm^{-1}. ^1H NMR (DMSO, 400 MHz), $\delta = 1.305$ (6H, s, CH_3), 7.066 (1H, s, Ar–H), 6.806 (1H, d, $J = 2.4$, Ar–H), 7.384 (1H, d, $J = 2.4$ Ar–H), 7.325 (1H, d, $J = 2.4$ CH), 8.024 (1H, d, $J = 2.4$, Ar–H), 7.203 (2H, d, $J = 2.4$, Ar–H), 8.050 (1H, d, $J = 2.4$, Ar–H), 7.311 (2H, d, $J = 2.4$, Ar–H), 7.093 (OH, s, 1H), 5.041 (2H, d, $J = 2.4$, CH), 4.599 (t, CH, 1H). 13C NMR (DMSO, 400 MHz): 13C NMR (DMSO, 400 MHz): $\delta = 166.3$ (C=O) 151.1, 150.3 (C–O) 129.3, 127.2 (CH, benzene) 139.6 (C–NO_2) 92.2 (CH–OH) 70.5 (CH_2) 21.5 (CH_3). MS (ESI, *m/z*): 416.1 (M + 1, 95 %), 413.4 (M − 1, 66 %). Anal. Calcd. for C19H17N3O8: C, 54.94; H, 4.13; N, 10.12. Found: C, 54.99; H, 4.19; N, 10.14.

Docking studies

Molecular docking is a key tool in structural molecular biology and computer-assisted drug design. The goal of ligand–protein docking is to predict the predominant binding mode of a ligand with a protein of known 3D structure. Docking can be used to perform virtual screening on large libraries of compounds, rank of results and propose structural hypotheses of how the ligand inhibits the target, which is valuable in lead optimization. To investigate the detailed intermolecular interactions, docking studies were carried out between the synthesized derivatives COX-1 and COX-2 target protein. The 3D structure of COX-1 and COX-2 enzyme is well known. For our studies, X-ray crystal structure of COX-1 (3N8X) and COX-2 (4COX) was taken from PDB having resolution of more than 2.0 Å. The molecular docking of different synthesized benzopyran derivatives into COX-1 and COX-2 target protein was performed using Surflex-Dock 2.1 module of SYBYL X 1.2 software. The protein–ligand interactions responsible for the observed activity to identify the binding orientations and processing of the protein included the

deletion of the ligand and the solvent molecules as well as the addition of hydrogen atoms. After ensuring chemical correctness, water molecules in the crystal structures were deleted and hydrogens were added where hydrogen atoms were missing and bond order for crystal ligand and protein were adjusted and minimized up to 0.30 Å RMSD.

Antihypertensive activity

BIOPAC System, Santa Barbara, with calibration was used to record the BP. Adrenaline was used to induce hypertension, left carotid artery was exposed, and the arterial cannula to a blood-pressure transducer and a venous cannula to a syringe were attached. Arterial cannula was connected via the BSL pressure transducer (SS13L) to the BIOPAC Systems.

Toxicity studies to fix up LD_{50}

In order to fix up the dose to carry out the antihypertensive activity, toxicity studies were carried out according to the OECD Guidelines No. 420 and 421. Wister albino rats weighing 200–250 g were chosen; route of administration of the drug was chosen as oral route. Six groups of animals each containing three animals were initially selected. At first, as per the Guidelines No. 420 and 421, given a dose of 70 mg/kg body weight monitored the animal for the toxic symptoms as well as mortality; the animals showed high toxicity symptoms such as increased intestinal motility, diarrhoea, tail erection and irritation to nose; and after 3 h, all the animals were dead. Hence, we decreased the dose to 50 mg/kg body weight and administered it to the next group of animals and monitored for toxic symptoms and mortality. In this dose, animals were safe but showed fewer toxic symptoms and only few were mortal, and toxicity symptoms were diarrhoea, tail erection and irritation to nose. Once again, we decreased the dose, and it was fixed to a dose of 20 mg/kg body weight to the next set of animals and observed for the toxic symptoms and mortality. At this dose, all the animals were safe and no toxic symptoms were seen. Hence, it was concluded that 20 mg/kg body weight dose was safe and recommended dose for further studies (antihypertensive activity).

Direct antihypertensive activity

Direct antihypertensive activity was carried out using the instrument BIOPAC System (MP-36, Santa Barbara, California) for recording BP response. Male albino rats, weighing 200–250 g were used, the rats were anaesthetized with urethane hydrochloride, 1.25 g/kg i.p, and then, the rats were prepared by shaving the neck and inguinal region with animal hair clippers. The jugular vein for drug administration was surgically cannulated, and left carotid artery by dissection for blood-pressure recording was isolated and exposed, using PE-50 tubing. By means of a three-way plastic stop cock and a stainless steel needle at the end of the PE tubing, the arterial cannula to a blood-pressure transducer and a venous cannula to a syringe were attached, and both cannulae with heparinized saline before cannulation were fluid filled. Afterthought, the arterial cannula via the BSL pressure transducer (SS13L) to the BIOPAC Systems, Inc was connected. Criterion for antihypertensive activity will be reduction in systolic arterial pressure by about 10–20 mmHg. Sympathetic system activation to induce hypertension is done by administering adrenaline, 5 μg/kg i.v., flushing the venous cannula with 0.2 ml of normal saline, and then allowing returning to pre-injection level. Effect of antihypertensive blood-pressure compounds on induced hypertension is measured by injecting the test compound 20 mg/kg solution intravenously and allowing to equilibration in the system, and then, adrenaline, 5 μg/kg i.v., as described previously was repeated and blood-pressure response to each procedure was observed and recorded.

SBP—systolic blood pressure
DBP—diastolic blood pressure
MABP—mean arterial blood pressure
HR—heart rate
Values are expressed in mean ± SEM. No of readings: 03

Colorimetric COX (ovine) inhibitor screening assay

There are several assay kits available for measuring COX inhibition. Cayman's colorimetric COX (ovine) inhibitor screening assay measures the peroxidase component of COXs. The peroxidase activity is assayed colorimetrically by monitoring the appearance of oxidized *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD) at 590 nm. Inhibition of COX activity, by a variety of selective and non-selective inhibitors, showed potencies similar to those observed with in vitro methods. Test concentration used for evaluation is 0.3 mg/ml which is dissolved in Methanol.

X = Values are acquired using in vitro ovine COX-1/COX-2 assay kit (Catalog No. 760 111, Cayman Chemicals Inc., Ann Arbor, MI).
Y = Experiments were carried out in duplicate and have <10 % error.

Conclusion

Benzoxazine scaffold with different substituents have also been used in the development phase as potential new drugs.

The versatility of the benzoxazine skeleton, in addition to its relative chemical simplicity and accessibility, makes these chemicals amongst the most promising sources of bioactive compounds. This has led to the discovery of a wide variety of compounds that are of high interest from the point of view of its antimicrobial, antimycobacterial, antidiabetic and antidepressant effects amongst the others. Hence, an attempt has been made to combine benzopyran nucleus along with various benzoxazines. The combination of these two heteroaromatic nucleus is expected to show a great deal of pharmacologic responses. In this series of benzoxazino benzopyrans, we have observed details and can conclude that the Nitro group-substituted benzopyrans with the a nitro substitution at benzoxazino group possessed good antihypertensive activity in the *R/S* isomers, even so with the Amino derivatives the activity remains when compared with the standard cromakalim.

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