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Original Article

IN SILICO DESIGN, SYNTHESIS AND PHARMACOLOGICAL SCREENING OF NOVEL 2-(6-SUBSTITUTED BENZO [D] THIAZOL-2-YL) ISOINDOLINE-1, 3-DIONES AS POTENTIAL COX-2 INHIBITORS FOR ANTI-INFLAMMATORY ACTIVITY

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ABSTRACT

Objective: Benzothiazole is known to be pharmacologically active moiety with anti-inflammatory potential. Present work describes synthesis of some potentially active derivatives of 2-aminobenzothiazoles incorporated with isoindolinediones. The design of target compounds was based on chemical hybridization of two pharmacophores, benzothiazole and isoindole-1, 3-dione. Molecular docking studies of synthesized compounds with COX-2 using V-life MDS-3.5 software revealed significant docked scores for PH-5 and PH-6.

Methods: A series of 2-(6-substituted benzo[*d*]thiazol-2-yl) isoindoline-1, 3-diones **PH(1-6)** was synthesized by heating 6-substituted-2-amino benzothiazoles with phthalic anhydride. These studies established significant correlation between dock score and *in-vivo* anti-inflammatory activity.

Results: The anti-inflammatory activity of synthesized compounds was carried using carrageenan induced rat paw edema method (a mechanistic model for in vivo COX-2 inhibition). Compounds **PH-5** and **PH-6** showed anti-inflammatory activity comparable to the reference drug, diclofenac at 100 mg/kg dose.

Conclusion: Significant correlation between the dock score and the in-vivo anti-inflammatory activity was observed in this study. In conclusion, compounds, PH-5 and PH-6 hold promise as lead compounds for further development as anti-inflammatory agents.

Keywords: Benzothiazole, Phthalimide, Anti-inflammatory activity.

INTRODUCTION

Design of agent with multiple target sites for higher anti-inflammatory potential is one of the most challenging areas in the area of inflammation. Benzothiazoles are known to exhibit diverse pharmacological properties such as antimicrobial, anti-inflammatory, anticancer, antidiabetic, anticonvulsant etc. Benzothiazole ring is a part of several marine and terrestrial natural compounds having useful biological activities [1].

Further, Cyclic imides, such as succinimide, phthalimide, and maleimide have received attention due to their analgesic activities. [2] A number of 1*H*-isoindole-1, 3(2*H*)-dione derivatives have been reported to posses antimicrobial potential. [3, 4, 5] The literature describes synthesis of series of alkyl-substituted phthalimide 1*H*-1,2,3-triazoles displaying potent anti-inflammatory activity.[6]. Non-steroidal anti-inflammatory drugs (NSAIDs) are most frequently prescribed classes of drugs and their benefits and side effects arise due to inhibition of cyclooxygenase (COX), of which there are two isoenzymes, COX-1 and COX-2. The COX-2 is an inducible isoform that leads to inflammation. Both COX isoenzymes have a hydrophobic tunnel, through which the substrate or the inhibitor accesses the active site. [7]

In view of above, compounds containing both phthalimide and benzothiazole pharmacophores may hold promise as anti-inflammatory agent and thus, an *in silico* molecular docking of the molecules was carried out in order to prioritize the molecules for actual synthesis and pharmacological screening against the COX-2 enzyme as anti-inflammatory agents. In the present study, a molecular docking study of these compounds with the pdb structure of COX-2 was performed to study the possible interaction of compounds with COX -2 using V-life MDS 3.5 software. The series of ligands of 2-(6-substituted benzo[d]thiazol-2-yl) isoindoline-1, 3-diones (**PH-1-6**) were screened *in-silico* by this process. Further these molecules were synthesized and screened for their anti-inflammatory potential against COX-2 specific inhibition mechanistic model in carrageenan induced rat paw edema. This resulted in significantly active molecules PH-5 and PH-6 as compared with the docking score justifying the set hypothesis. Prior to the in vivo evaluation for anti-inflammatory activity the compounds were evaluated by Acute Oral toxicity (AOT) studies as per OECD 425 guidelines to fix the dose for evaluation and to determine the LD_{50} of the compounds.

MATERIALS AND METHODS

All the chemicals used in the synthesis were of laboratory grade. The structures of the intermediates and the target compounds were confirmed by melting points, TLC, IR, proton NMR and mass spectral studies. Melting points were determined in open capillary on Veego (Model: VMP-D) electronic apparatus and are uncorrected.

The IR spectra of the synthesized compounds were recorded on Shimadzu 8400-S FTIR spectrophotometer using potassium bromide. The ^{1}H NMR spectra were recorded in $CDCl_3$ on Varian-Mercury 300MHz spectrometer and chemical shifts are given in units as parts per million, downfield from tetra methyl silane (TMS) as an internal standard.

To monitor the reactions as well as to establish the identity and purity of reactants and products, thin layer chromatography was performed on precoated aluminum sheets using toluene: methanol solvent system.

The spots were visualized under ultra- violet light. In case of AOT studies the healthy swiss albino mice were employed of 3500g for biological evaluation. The anti-inflammatory activity of synthesized compounds **PH (1-6)** was carried out on Wister rats of either sex (150-200 g). These animals were reared with robust health by providing pellet diet and water *ad libitum* in the animal house under standard environmental conditions of temperature, relative humidity and dark/light cycle. After randomization into various groups and before initiation of experiment, the animals were acclimatized for one week. The animal experiments were previously approved by Institutional Animal Ethical Committee (IAEC) and followed CPCSEA guidelines.

Experimental

1. In silico Screening or Molecular Docking

A.* Protein file selection*: The criteria for the selection of protein file (pdb) were that it should be from the human source and should have a resolution between 0.5-2.00 A. Thus PDB file of COX -2 (1CX2) enzyme was selected. To explore the probable mechanism and interaction of synthesized compounds with COX 2, molecular docking studies were performed on X-ray crystal structure of COX-2 enzyme (obtained from organism *Mus musculus*) (PDB code: 1CX2; resolution 3.00 A^0) using V-life MDS 3.5 software. The PDB was downloaded from RCSB protein data bank. The allowable and disallowable regions were checked from Ramchandran plot and errat report of PDB. The water molecules were removed from the monomer and the reference ligand was extracted. The hydrogens were added in the protein molecule and energy was minimized using Merck Molecular Force Field (MMFF). The batch grid docking was performed at cavity 1 after generating conformers of the target compounds by using Biopredicta module. The ligand enzyme complex was again minimized and the interactions of the compounds with the amino acid residues present in the cavity were noted. The results are presented in Table 4

B. Validation of Protein PDB

The protein PDB was validated by Ramchandran plot and errat report obtained from NIH MBI server online.

C. Ligand preparation

Ligands from the series of 2-(6-substituted benzo[*d*]thiazol-2-yl) isoindoline-1, 3-diones PH(1-6) were drawn and converted from the 2-D structures to 3-D mol files. 2D structure of ligand were prepared and converted to 3D by Chem Draw Ultra 8.0 software. The 3D structure was energetically minimized using molecular mechanics followed by Merck Molecular Force Field (MMFF). Conformers of the compound were generated by systemic search method. All the conformers were then energetically minimized up to the rms gradient of 0.001 and saved in separate folder. MMFF was used for optimizing molecule and optimize geometry of molecule from by minimizing energy. Parameters used here are MMFF, FF, Gasteiger Marsili charge and dielectric properties were kept constant. Gradient type used was analytical with maximum number of cycles were 1000. The results of the docking are given in Table 4.

2. Synthesis

The title compounds were synthesized as outlined in scheme-1.

Synthesis of 2-amino benzothiazoles B(1-6)

The 2-amino benzothiazoles B(1-6) were prepared as per reported method.[8]

Procedure for synthesis of 2-(6-substituted benzo[*d*]thiazol-2-yl) isoindoline-1, 3-dione PH (1-6)

Equimolar quantities of phthalic anhydride and 6- substituted 2-amino benzothiazoles **B (1-6)** were taken in a beaker and heated. The mixture was stirred occasionally and phthalic anhydride which sublimed was pushed down into the reaction mixture, till the fusion complete. The mixture was kept undisturbed for 5 minutes, when the liquid mass solidified. The solid mass was suspended in water to remove unreacted anhydride. The solid obtained was filtered, dried and recrystallized from 50% ethanol. The physical and spectral data are presented in Table 1 and Table 2 respectively.

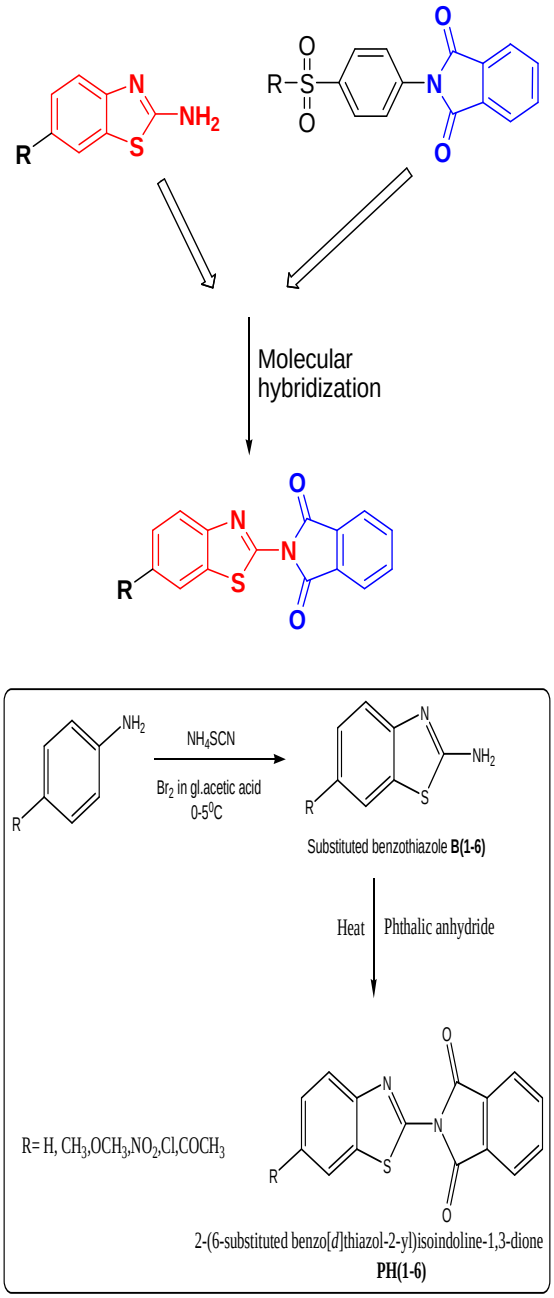
3. Pharmacological Screening

A. Acute oral toxicity studies:

Oral acute toxicity studies were carried out on Swiss albino mice of either sex as per OECD 425 guidelines. None of the synthesized compounds showed any mortality up to 3500 mg/kg body weight dose level.

B. *In vivo* anti-inflammatory COX-2 inhibition assay

The synthesized compounds were evaluated for their anti-inflammatory potential. The anti-inflammatory activity was carried out using carrageen induced rat paw edema with diclofenac as the reference drug. The model employed is the standard battery test for *in vivo* estimation of COX-2 inhibition or it can be said that this model is mechanistic *in vivo* model for COX-2 inhibition. The animals were divided into three groups containing 6 animals in each group. One group served as control, another served as a standard (diclofenac) and the rest of the group was used for the test drugs. The animals were fasted for 18 hours prior to the experiment. Test compounds and the standard were suspended in 0.5% of sodium carboxy methyl cellulose mucilage, which was used as vehicle. The rats were dosed orally at 100 mg/kg body weight for the test and 30 mg/kg body weight standard groups. Control group only received vehicle. After 30 minutes of dosing, 0.1ml of 1% carrageenan in normal saline was injected into the sub planter region of left hind paw of all groups. Volume of the injected paw was measured with a plethysmometer at zero hour immediately after injecting carrageenan. The same procedure was repeated at 1Hr, 2Hr and 3Hr. The difference between zero hour and subsequent readings was taken as actual paw edema volume. The results are presented in Table 3



Scheme1: Synthetic route for target compounds

RESULT AND DISCUSSION

1. In silico screening or molecular docking

The suitability of PDB of COX-2 (1CX2) collected from RCSB protein data bank was checked from Ramchandran plot which indicated that 82.1% of residues give favorable region, 14.2% of residues were found in allowed region and 3.7% residues were in outlier region. The overall quality factor was found to be 83.980 from errat report. The docking study was then carried out with the synthesized compounds, PH (1-6) as well as reference ligand, SC558. The results (Table 4) of the docking study indicate that SC558 has a dock score of -5.916 kcal/ mol and forms hydrogen bond with Tyr-115 (2.06A^0) and Lys-83 (2.50 A^0, 1.68 A^0, 1.629A^0). Compound, **PH-5** showed a dock score of -5.25 kcal/mol and was held at the active site through non bonded vdW forces. **PH-5** was surrounded by Lys-83, Val-89, Pro-84, Leu-93, Ile-112, Tyr-115, Val-116, Ser-119, Arg120, and Ser-471 amino acid residues. The compound, **PH-6** showed -5.120 kcal/mol dock score and one hydrogen bond with Lys-83 (2.41A^0). Compounds, **PH-6** and **PH-4** have shown hydrophobic interactions with the amino acid residues, such as Ser-471, and Lys-83; however compound, **PH-3** showed poor dock score. The same compound has also displayed a weak *in-vivo* anti-inflammatory activity. (**Figure 1)** All the compounds showed comparable inhibition scores as compared with reference ligand (SC558) employed in the docking trials and hence to evaluate the hypothesis all the molecules were taken up for synthesis and evaluated *in vivo* for anti-inflammatory activity by COX-2 inhibition *in vivo* mechanistic model.

2. Synthesis and Characterization

The target compounds PH(1-6) were prepared as per scheme 1 procedure. The intermediates 2-aminobenzothiazoles were fused with phthalic anhydride. The structures of target compounds were assigned by ^{1}H NMR, IR and mass analysis. The solid state spectra (KBr cm^{-1}) of PH **(1-6)** revealed characteristic aromatic C-H stretch between 3027-3134cm^{-1}.

The C=N group present in the benzothiazole ring reveals peak between 1647-1600 cm^{-1} while the sharp carbonyl (C=O) peak of isoindoline-1, 3-dione was seen at 1770-1720 cm^{-1}. The IR spectra of target compounds showed the absence of primary amino group at 3200–3300 cm^{-1} seen with the substituted 2-amino benzothiazoles. The absence of broad band of phthalic anhydride at 3050-2900 cm^{-1} also confirmed the assigned structure. Absence of any peak of NH_2 proton and appearance of multiplets for aromatic protons of isoindoline-1, 3-diones between 7.100 – 8.04 ppm confirms the formation of target isoindoline-1, 3-diones PH (1-6). The aromatic protons of benzothiazole ring display peaks between 7.00 to 8.65 ppm. The substituent at 6th position of benzothiazole ring have shown singlet for CH_3, OCH_3, $COCH_3$ at 2.51, 3.895 and 2.711 ppm respectively. Mass spectra were found in agreement with the assigned chemical structures of synthesized compounds. The physical and spectral data of the compounds is given in **Table 1 and 2**, respectively.

3. Pharmacological Screening

A. Acute Oral toxicity studies (AOT)

No mortality was found in mice upto 3500gm/kg body weight dose. 100mg/kg was selected as anti inflammatory dose.

B. Anti-inflammatory activity

The compounds **PH (1-6)** were screened for anti-inflammatory activity using carrageenan induced rat paw edema model. All the results are expressed as mean ± SEM. Statistical evaluation was performed using analysis of variance followed by Dunnett's test for subgroup comparison. The inhibition of swelling in carrageenan induced edema in rat paw brought about by oral administration of the synthesized compounds is shown in **Table 3**. The percentage of swelling by the compound was calculated using following equation.

Inhibition % = {[(V_t- V_o) control-(V_t-V_o) treated]/ (V_t- V_o) control} x 100

From statistical data it was observed that diclofenac treated group showed statistically significant (P<0.001) reduction in paw volume compared to control. Compound **PH-5** and **PH-6** showed statistically significant (P<0.001) reduction in rat paw volume as compared to control while compounds **PH-1**, **PH-2** and **PH-4** showed less significant (P<0.01) reduction in paw volume. However, no significant difference was observed for **PH-3.** Thus, compound **PH-5** and **PH-6** can be said to have optimum structural features amongst the series to show promising *in- vivo* anti-inflammatory activity.

CONCLUSIONS

A series of 2-(6-substituted benzo[*d*]thiazol-2-yl) isoindoline-1, 3-diones were synthesized and screened for their anti-inflammatory activity. Compounds, **PH-5** and **PH-6** were found to possess comparable anti-inflammatory activity to that of diclofenac, the reference drug used in the study. Significant correlation between the dock score and the *in-vivo* anti-inflammatory activity was observed in this study. In conclusion, compounds, PH-5 and PH-6 hold promise as lead compounds for further development as anti-inflammatory agents and that docking procedure can be useful in predicting *in-vivo* anti-inflammatory activity of any designed molecules.

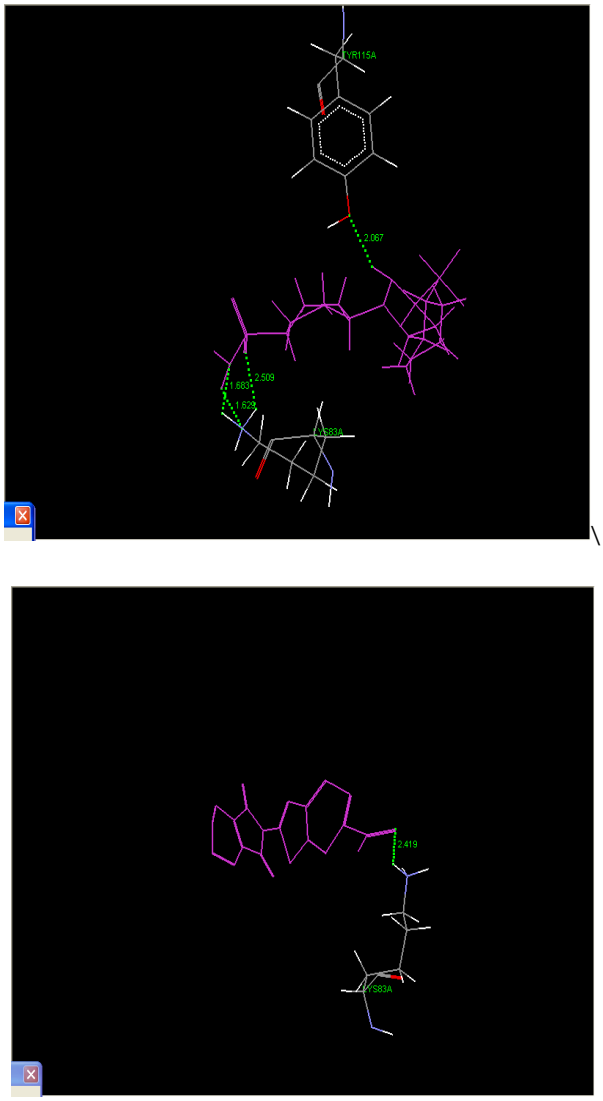


Fig. 1: Hydrogen bonding of SC558 and PH-6 within active site of COX-2 enzyme

Table 1: Physical Data of Synthesized Compounds (PH 1-6)

Compound	R	Yield %	M.P. (^{0} C)	R_f
PH-1	H	71	159-163	0.69
PH-2	-CH3	67	162-165	0.68
PH-3	-OCH3	70	201-204	0.56
PH-4	$-NO_2$	65	196-199	0.70
PH-5	-Cl	67	212-214	0.66
PH-6	$-COCH_3$	60	111-114	0.78

*All melting points are uncorrected, Mobile phase: Toluene: methanol = 8:2, Recrystallisation solvent: 50% Ethanol

Table 2: Spectral Data of Target Compounds (PH 1-6)

Compound	R	IR (KBr) cm-1	^{1}H NMR($CDCl_3$) δ ppm	MS(m/z)
PH-1	H	3020(C-H), 1748(C=O), C=N(1642)	7.402(t, 2H,Ar), 7.406(t, 2H,Ar), 7.862-8.08 (m,3H, Ar), 8.13(d, 1H, Ar)	282(M+2)
PH-2	-CH3	1720(C=O), 3130(C-H), C=N (1631)	2.51(s,3H, CH_3), 7.699(d,1H, Ar), 7.318, (d, 1H, Ar), 8.004(s, 1H, Ar), 7.88(m, 2H, Ar), 8.031(m, 2H,Ar)	294.71(M+), 104
PH-3	-OCH3	1722(C=O), 3034(C-H), C=N(1639	3.895(s, 3H, OCH_3), 7.100(d, 1H, Ar), 7.342(s, 1H, Ar), 7.84-7.99(m, 2H, Ar), 8.02-8.04(m, 3H, Ar)	
PH-4	$-NO_2$	1765(C=O), 3063(C-H), C=N (1647)	8.65(s, 1H, Ar), 8.41(d, 1H, Ar), 7.93-8.07(m, 5H, Ar, 1H of benzothiazole)	
PH-5	-Cl	1748(C=O), 3134(C-H), C=N(1633), C-Cl (701)	7.463 (d, 1H, Ar, J=10.5), 7.88-7.90(m, 3H, Ar), 8.03-8.08(m, 3H, Ar)	
PH-6	$-COCH_3$	1728(C=O), 3032(C-H), C=N(1631)	2.711(d, 3H, CH_3), 7.61(d, 1H, Ar), 7.80-8.19(m, 5H, Ar), 8.570 (s, 1H, Ar)	

Table 3: Anti-inflammatory activity of target compounds (PH 1-6)

Experimental Group	% Inhibition of paw edema			
	30 min	**60 min**	**120 min**	**180 min**
Control	0%	0%	0%	0%
Diclofenac (30mg/kg)	44.37 ± 4.96***	44.53 ±2.19***	58.97 ± 1.04***	59.73± 0.15***
PH-1 (100mg/kg)	17.45 ± 0.90***	19.25 ±0.91***	23.48±0.78***	26.12±0.73***
PH-2 (100mg/kg)	10.89 ± 1.38**	16.90 ±0.44**	21.46 ±1.59**	27.87±1.31**
PH-3 (100mg/kg)	7.04 ± 0.99 ns	12.32 ± 0.84 ns	15.01 ± 0.93 ns	18.58 ± 0.21 ns
PH-4 (100mg/kg)	14.00 ±0.36**	16.88 ± 1.32**	19.46 ± 1.48**	22.55 ± 2.25**
PH-5 (100mg/kg)	31.52± 3.69***	39.95 ± 1.21***	51.45 ± 1.89***	53.51 ± 1.80***
PH-6 (100mg/kg)	29.15 ± 0.06**	38.39 ± 0.31**	50.95 ± 0.10**	51.31 ±0.07**

All values are expressed as mean ± SEM. n = 6. All data are subjected to One Way ANOVA followed by Dunnett's tests. *** Values are significant at $p < 0.001$

Table 4: Dock scores of target compounds PH (1-6)

Compounds	Dock score for COX-2 docking study (kcal/mol)	Binding energy	Interactions observed
Reference ligand SC558	-5.916	-2.723	H-bond- Tyr-115, Lys-83A
PH-1	5.09	-7.27	vdW- Lys-83, Pro-84, Val-89, Leu-9315, Ser-119, Arg120, Ser-471
PH-2	-5.08	-20.13	Hydrophobic- Ile-92, Leu-93 Charge interaction- Lys-83
PH-3	-4.863	-11.36	Hydrophobic- Lys-83, Ser-471
PH-4	-5.102	-19.15	H- bond with Tyr-115A
PH-5	-5.25	-12.45	vdW- Lys-83, Pro-84, Val-89, Leu-93, Ile-112, Tyr-115, Ser-119, Arg120, Ser-471.
PH-6	-5.120	-6.20	H- bond with Lys-83A Hydrophobic- Ser-471, Lys-83A

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