Research Article

Synthesis, docking, characterization and anti-inflammatory activity of novel phosphodiesterase-4 inhibitors

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Abstract

Objective: Phosphodiesterase-4 (PDE4) is the key enzyme involved in the hydrolysis of cyclic adenosine monophosphate to adenosine monophospahte in inflammatory and immunomodulatory cells, is the important target for treatment of various inflammatory conditions such as asthma, chronic obstructive pulmonary disease, rhinitis, dermatitis, multiple sclerosis, and rheumatoid arthritis. The objective of present study was to synthesize and evaluate various PDE4 inhibitors as potential anti-inflammatory agents. **Materials and Methods**: Coumarin belongs to a group of benzopyrones, and consists of a benzene ring joined to a pyrone nucleus. In the present study, the substituted coumarins were synthesized by reaction of substituted phenols and ethyl acetoacetate in the presence of concentrated sulphuric acid using substitution with aromatic & aliphatic groups. 7-hydroxy-4-methyl coumarin was reacted in the presence of sodium hydroxide with commercially available aliphatic & aromatic acids and halides to obtain the proposed esters & ketones. The compounds were evaluated by docking study in the binding site of PDE4 protein using AutoDock Vina and their dock scores were calculated. The newly synthesized compounds were further screened for the anti-inflammatory activity using carrageenan induced rat paw edema method. **Results**: Based on the docking studies, the compounds with good binding interactions with PDE4B enzyme were selected for evaluation of anti-inflammatory activity. The anti-inflammatory activity was measured by calculating the volume displaced and percent inhibition of edema and was further analyzed by two-way ANOVA**.** The value of anti-inflammatory activity indicated that some compounds exhibited good anti-inflammatory activities. Aromatic compound displayed appreciable anti-inflammatory activity compared to the standard drug (Rolipram). It was found that substitution with electronegative or aromatic groups enhanced the anti-inflammatory activity while substitution with aliphatic groups showed poor binding affinity for the enzyme as well as poor anti-inflammatory activity. **Conclusion:** Aromatic substituent's (Benzene, Phenol, Chlorobenzene & Benzoyl chloride) showed significant binding affinity in docking studies and also displayed good anti-inflammatory activities when compared to aliphatic groups (Propionyl chloride, Acetyl chloride, n-propyl, Carboxamide).

Keywords: PDE4B, inflammation, coumarin, phosphodiesterase, docking

Introduction

Phosphodiestrases (PDEs) have great clinical significance as they regulate the signal transduction mediated by second messengers like cAMP and cGMP, which further regulate many biological processes such as metabolism, growth, differentiation, smooth muscle relaxation and secretion (Kodimuthali at al., 2008; DeNinno, 2012). Based on substrate specificities, amino acid sequences, tissue distribution and regulatory properties, PDE family is subdivided into 11 different groups from PDE1 to PDE11. Out of these subtypes, Phosphodiesterase-4 (PDE4) is the important target for many drugs owing to its unique structure and tissue distribution. It is widely distributed in many cells and tissues including leukocytes, vascular

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endothelium, bronchial and vascular smooth muscle and brain (Keravis et al., 2012). In the past few decades, great attention has been focused on PDE4 due to their role in pathological process associated with inflammation, angiogenesis and various vascular and neurological disorders (Houslay et al., 2005). Selective inhibition of PDE4 prevents the release of inflammatory mediators from different cells and is a great therapeutic approach for various inflammation related disorders. Various novel PDE4 inhibitors have proved to be beneficial for a variety of disorders including asthma, chronic obstructive pulmonary disease, rheumatoid arthritis, Alzheimer's disease, encephalomyelitis and depression (Jeffery, 2005; O'Donnell et al., 2004; Spina D, 2004; O'Byrne et al., 2009; Sharma et al., 2011). Roflumilast is selective PDE4 inhibitor approved by FDA in 2011. After roflumilast, apremilast (FDA, 2014) was approved by FDA for psoriatic arthritis in 2014. Crisaborole (FDA, 2016), a selective PDE4B inhibitor has been recently approved by FDA for eczema. Several PDE4 inhibitors have shown promising results in clinical trials and still many have been discontinued from development due to their undesired side effects specifically nausea, emesis, abdominal pain and dyspepsia. It has been suggested that the side effects of PDE4 inhibitors are a result of their non-selectivity to all four PDE4 subtypes, and thus synthesis of new PDE4 inhibitors with subtype selectivity may provide clinical benefits by maintaining therapeutic efficacy and decreasing the side effects (Jin et al., 2012).

The study of coumarin began more than 200 years ago. The name of this chemical family is derived from Coumarouna Odourata Aube (Dipteryx Odourata). Coumarin is a widely occurring secondary metabolite that occurs naturally in several plant families & essential oils and has been used as a fragrance in food & cosmetic products. The coumarin ring corresponds to benzo-α-pyrone (2*H*-1-benzopiron-2-one) and is an effective inhibitor of a number of protein & enzyme functions. Therapeutic activity of coumarin is based upon the substitution of the ring with different moieties. Coumarins are catogerized into: simple coumarins, pyranocoumarins, furanocoumarins, biscoumarins, triscocoumarins and coumarinoligans (Borges et al., 2005). Coumarins have been used as anti-tumor, anti-coagulants, anti-microbial, anti-inflammatory, anti-oxidant, anti-viral and anti-alzheimer's agents (Manidhar et al., 2013; Vilar et al., 2006; Ronad et al., 2010; Ghate et al., 2005; Melagraki et al., 2009; Trivedi et al.,

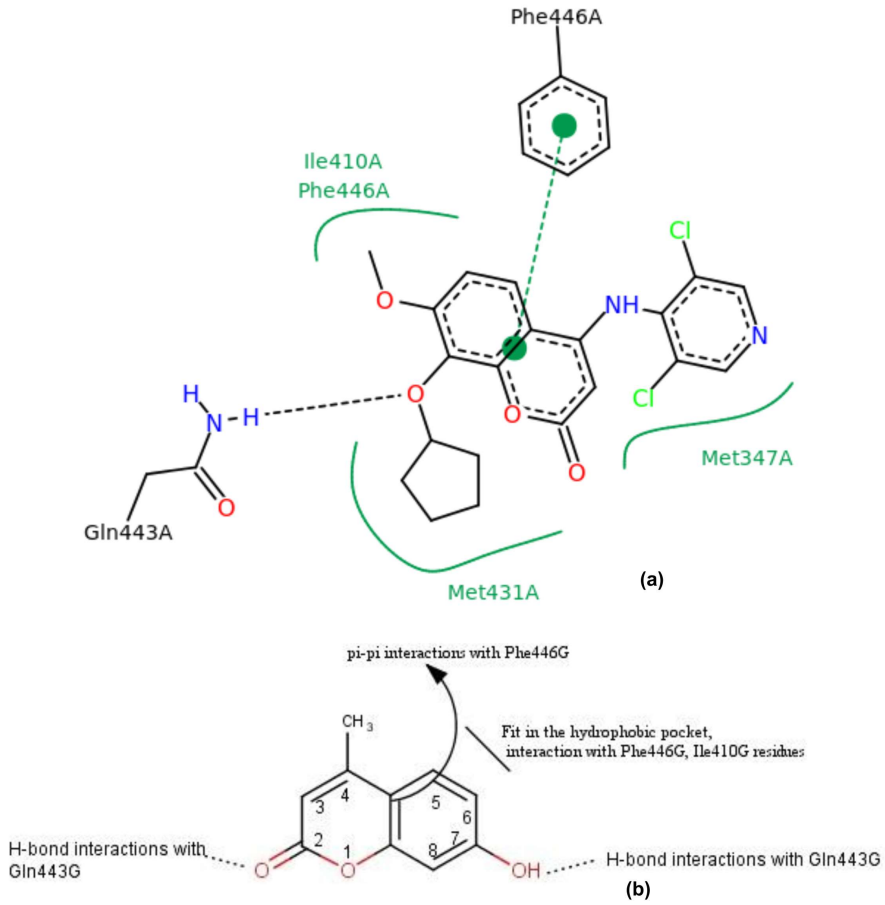

Figure 1(a): Binding interactions of co-crystallized PDE4B inhibitor showing H-bonding with the Gln443G residue; hydrophobic and π-π bonding with the Phe446G **1(b):** Structure of coumarin derivative designed as potential PDE4 inhibitors

2007; Heckman et al., 2015). Coumarins as PDE4 inhibitors were found to have potential to reduce the symptoms and masked the inflammation associated with dry eye (Govek et al., 2010). Coumarins have been intensively investigated for their anti-inflammatory activity to attenuate inflammatory response in multiple cell types (Spina D, 2008). Recently, benzoxazole and coumarin nuclei were coupled to synthesize novel benzoxazole-coumarin derivatives as safe and potent anti-inflammatory agents (Minhas et al., 2017).

Based on the available X-ray structures of PDE4 inhibitors in the PDB database, their interactions with PDE enzyme were seen and the pharmacophoric features required for PDE4 inhibitory activity were identified as shown in figure **1**. Attributed to the pharmacophoric features required for PDE4 inhibition and consideration of coumarins as selective PDE4 inhibitors, the novel coumarins were designed and synthesized as potential PDE4B inhibitors and evaluated as anti-inflammatory agents.

Material and methods

Chemistry

The chemicals were purchased from Loba chemie Pvt. Ltd. and Spectrochem Pvt. Ltd. and were utilized for the experimental work without further purification. The physical properties of the newly synthesized derivatives like molecular formula, molecular weight, melting point and retention factor were calculated (Table **1**). Melting point was determined in an open capillary tube on a melting point apparatus. IR spectra were recorded on FTIR spectrophotometer (Shimadzu) by KBr pellet method. ^{1}H-NMR spectra was recorded on Bruker Avance II at 400 MHz NMR spectrophotometer using DMSO-d6 & expressed in parts per million downfield from tetramethylsilane as an internal standard. ^{13}C NMR spectra were recorded on the same spectrophotometer at 100 MHz using same solvents.

Synthesis of coumarin derivatives

Briefly, 1.0 molar solution of substituted phenol in 1.0 molar of ethyl acetoacetate was contained in a 150 ml conical flask & an excess quantity of sulphuric acid was added. The mixture was heated on a water bath at 75-80º for 20 minutes. It was poured into excess ice water. The pale yellow solid was collected by suction filtration. The solid was washed with cold water & dried at 60ºC followed by recrystallization from ethanol or methanol. 7-hydroxy-4-methyl coumarin was heated in the presence of sodium hydroxide with commercially available aliphatic & aromatic acids, halides & aldehydes to obtain the novel esters, ethers & ketones respectively (Scheme **1**) (Vogel, 2004).

Molecular Docking

The catalytic domain of human Phosphodiesterase 4B in complex with a coumarin-based inhibitor was obtained from the protein data bank (PDB Code: 3LY2). The chemical structures were drawn using Marvin sketch and molecular docking studies were performed using Autodock vina (Trott et al., 2010). The protein 3D structure was cleaned by deleting the water molecules, cofactors & other ligands. This was followed by adding hydrogen atoms in their standard geometry, adjusting the bond orders & formal charges. Mole2 files were created for ligands followed by creating PDBQT files for both protein & ligands respectively. The compounds were finally analyzed in PyMOL to obtain the overlay & nature of bonding between the ligands.

http://www.rcsb.org/pdb/explore/explore.do?structureId=3LY2.

Characterization

1. 4-methyl-7-propoxy-2*H*-chromen-2-one: IR (KBr) νmax (cm^{-1}): 3437.15 (O-H str.), 1122.57(C-O str.), 1396.46 (C-H bending), 1639.49 (Ar C=C str.), 1674.21 (C=O str.) **^{1}H-NMR** ($CDCl_3$, δ, ppm): 2.286 (t, 3H, CH_3 of Propyl), 2.507 (m, 2H, CH_2 of Propyl), 3.562 (s, CH, C_4 of Ar-CH_3), 5.783 (s, 1H, CH C_8 of coumarin ring), 6.450 (d, 1H, CH C_5 of coumarin ring), 6.620 (d, 1H, CH C_6 of coumarin ring), 7.328 (s, 1H, CH C_3 of coumarin ring) **^{13}C-NMR** (400MHz, $CDCl_3$): 161.79 (C=O), 154.31 (Ar-CH), 125.76 (C=C), 39.94 (RCH_2R), 18.56 (R-CH_3).

2. 4-methyl-2-oxo-2H-chromen-7-yl acetate: IR

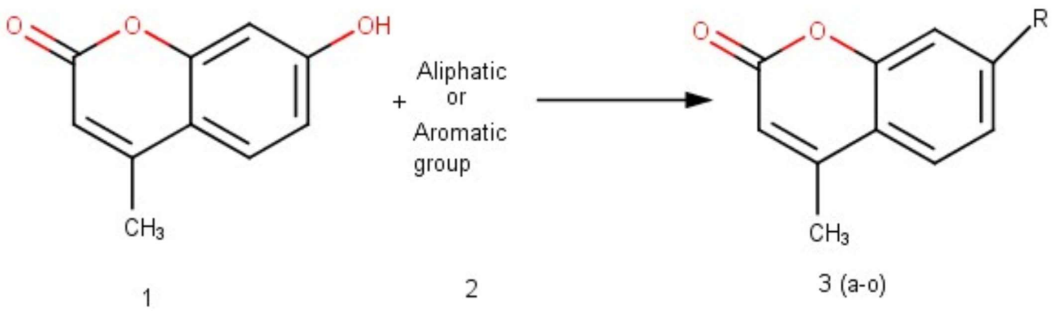

Figure 2. Synthesis of coumarin derivatives (Scheme-1)

(KBr) vmax (cm^{-1}): 3263.56 (O-H str.), 1141.86 (C-O str.), 1448.54 (C-H bending), 1683.86 (C=O str), 1612.49 (Ar C=C str.) **^{1}H-NMR** ($CDCl_3$, δ, ppm): 2.363 (m, 3H, CH, CH_3CO), 3.353 (s, 3H, CH, C_4 of Ar-CH_3), 6.122 (m, 1H, CH C_8 of coumarin ring), 7.526 (m, 1H, CH C_5 of coumarin ring), 9.348 (s, 1H, CH C_6 of coumarin ring), 10.503 (s, 1H, CH C_3 of coumarin ring) **^{13}C-NMR** (400MHz, $CDCl_3$): 161.62 (C=O), 155.31 (Ar-CH), 133.62(Ar –CH), 129.13 (Ar-CH), 113.32 (Ar-CH), 102.65 (C=C), 40.64 (C-O), 18.55 (R-CH_3).

3. 4-methyl-2-oxo-2*H*-chromen-7-yl propionate: IR (KBr) vmax (cm^{-1}): 3500.8 (O-H str.), 1670.35 (C=O str.), 1573.91, (C=C str.), 1390.68 (C-O str.) **^{1}H-NMR** ($CDCl_3$, δ, ppm): 2.024 (q, 3H, CH_3 of Propoxyl), 2.336 (d, 2H, CH_2 of propoxyl), 2.498 (s, 1H, CH C_8 of coumarin ring), 3.172 (q, 3H, CH C_4 of Ar-CH_3), 4.209 (s, 1H, CH C_5 of coumarin ring), 6.003 (d, 1H, CH C_6 of coumarin ring), 7.491 (m, 1H, CH C_3 of coumarin ring) **^{13}C-NMR** (400MHz, $CDCl_3$): 177.96 (C=O), 164.79 (Ar-CH), 154.16 (Ar –CH), 126.60 (Ar-CH), 109.21 (Ar-CH), 102.81 (C=C), 40.59 (C-O), 18.56 (R-CH_3).

4. 4-methyl-2-oxo-2H-chromen-7-yl benzoate: IR (KBr) vmax (cm^{-1}): 3215.34 (O-H str.), 1213.23 (C-O str.), 1419 (C-H bending), 1730 (C=O str.) **^{1}H-NMR** ($CDCl_3$, δ, ppm): 2.368 (s, 3H, CH, C_4 of Ar-CH_3), 2.507 (m, 5H, CH of C_6H_5), 3.334 (s, CH, Ar-CH_3), 6.128 (s, 1H, CH C_8 of coumarin ring), 6.424 (s, 1H, CH C_5 of coumarin ring), 7.359 (m, 1H, CH C_6 of coumarin ring), 8.156 (t, 1H, CH C_3 of coumarin ring) **^{13}C-NMR** (400MHz, $CDCl_3$): 167.77 (C=O), 155.32 (Ar-CH), 134.77(Ar –CH), 129.72 (Ar-CH), 119.00 (Ar-CH), 102.65 (C=C), 40.64 (C-O), 18.66 (R-CH_3).

Evaluation of anti-Inflammatory activity

Animals

Wistar rats (150-300 g) were obtained from the disease-free Animal House, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar (India). The rats were housed in standard polypropylene cages (two rats/cage) and maintained under controlled room temperature (22±2°C) and humidity (55±5%) with 12:12 h light and dark cycle. All the rats were provided with commercially available rat normal pellet diet (Ashirwad Feeds) and water ad libitum. The experimental protocol was approved by Institutional Animals Ethics Committee (Approval No. JCDMCOP/IAEC/06/16/35) and animal care was taken as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Govt. of India.

Acute toxicity studies

Acute toxicity studies were carried out to find out the safe dose of synthesized derivatives. The nine animals were divided into three groups of three animals each. Each group of animals were administered different doses (10, 100, 1000 and 2000 mg/kg) of test compound. The animals were placed under observation for 24 hours to monitor their behavior as well as mortality (Zinovieva et al., 2017).

Carrageenan induced paw edema in rats

The anti-inflammatory activity of the synthesized compounds was evaluated by carrageenan induced paw edema in rats. Rolipram was used as standard anti-inflammatory drug. The initial paw volume of animal was measured by digital plethysmometer. The animals were divided into different groups (control, standard and test groups), each consisting of six rats. The animals were starved over night and only water was given ad-libitium. The control group was treated with tween 80 (1% w/v) suspension. The standard group was treated with standard drug Rolipram (25 mg/kg) and the test group were treated with the suspension of test compounds (100 mg/kg, orally). After 30 minutes, the animals were injected with 0.1 ml of carrageenan (1% w/v) in the sub planter region of left hind paw of rats. The final paw volume was measured immediately after injection and at two-hour intervals till 04 hours.

Statistical Analysis

The data of the anti-inflammatory activity was statistically analyzed by two-way ANOVA and the value of $P<0.05$ was considered significant.

Results and discussion

The novel coumarin derivatives were synthesized as shown in Scheme **1**. Coumarin derivatives were obtained by reaction of substituted phenols with ethyl acetoacetate in the presence of concentrated sulphuric acid. Substituted coumarins were prepared by heating in the presence of sodium hydroxide with commercially available aliphatic & aromatic acids, halides & aldehydes to obtain the novel esters, ethers & ketones respectively. The physicochemical properties of the synthesized compounds are shown in table **1**.The purity of novel compounds was confirmed by their IR and NMR spectra.

The binding interactions of novel compounds in the active site of PDE4B protein were evaluated by docking studies. Initially, the co-crystallized PDB ligand was docked in the active site of PDE4B followed by docking of all the designed compounds. The compounds **3(g, h)** showed good binding interaction as determined by their H-bonding, binding affinity and docking score of best docked poses (Figure **4**). These compounds showed similar binding pattern in the active site of enzyme as that of the co-crystallized ligand. An interesting observation was made that aromatic group at different positions of the coumarin ring enhanced the binding affinity much more effectively.

Table 1. Physicochemical properties & docking score of the synthesized coumarin compounds

Sr. No.	Compound Name	MF	MW	*R_f	Dock Value
Co-crystallized PDB Ligand	8-(cyclopentyloxy)-4-[(3,5-dichloropyridin-4-yl)amino]-7-methoxy-2H-chromen-2-one	$C_{20}H_{18}Cl_2N_2O_4$	421	-	-9.3
3a	4-methyl-7-methoxy-2H-chromen-2-one	$C_{11}H_{10}O_3$	190	0.61	-5.9
3b	7-ethoxy-4-methyl-2H-chromen-2-one	$C_{13}H_{12}O_3$	216	0.64	-5.3
3c	4-methyl-7-propoxy-2H-chromen-2-one	$C_{14}H_{14}O_3$	230	0.63	-5.4
3d	4-methyl-7-butoxy-2H-chromen-2-one	$C_{15}H_{16}O_3$	244	0.65	-8.0
3e	4-methyl-2-oxo-2*H*-chromen-7-acetate	$C_{12}H_{10}O_4$	194	0.66	-8.0
3f	4-methyl-2-oxo-2*H*-chromen-7-propionate	$C_{13}H_{12}O_4$	308	0.70	-8.1
3g	4-methyl-2-oxo-2*H*-chromen-7-benzoate	$C_{17}H_{12}O_4$	264	0.72	-9.6
3h	4-methyl-2-oxo-2*H*-chromen-7-phenolate	$C_{16}H_{11}O_3$	251	0.73	-9.1
3i	4-methyl-7-phenyl-2*H*-chromen-2-one	$C_{16}H_{12}O_2$	236	0.74	-8.0
3j	7-(4-chlorophenyl)-4-methyl-2H-chromen-2-one	$C_{16}H_{12}O_2Cl$	271	0.69	-8.2
3k	Acetic acid-4-methyl-2-oxo-2*H*-chromen-7-yl-ester	$C_{12}H_{10}O_4$	194	0.71	-7.3
3l	Propionic acid acid-4-methyl-2-oxo-2*H*-chromen-7-yl-ester	$C_{13}H_{12}O_4$	308	0.75	-7.5
3m	Benzoic acid acid-4-methyl-2-oxo-2*H*-chromen-7-yl-ester	$C_{17}H_{12}O_4$	264	0.77	-8.2
3n	4-methyl-2-oxo-2*H*-chromene-3-carboxamide	$C_{11}H_9O_3N$	203	0.80	-8.5
3o	7-hydroxy-4-methyl-2-oxo-2*H*-chromene-3-carboxamide	$C_{11}H_{10}O_4N$	220	0.82	-8.9

*Toluene: Ether; 1:1

In the study, it was found that the substitution with aromatic group (e.g. Benzene, Phenol, Chlorobenzene & Benzoyl chloride) showed good binding affinity for the enzyme.

Based on the docking studies, the compounds with good binding interactions with PDE4B enzyme were selected for evaluation of anti-inflammatory activity. The acute toxicity study showed that dose given orally at 2,000 mg/kg caused no mortality after 24 hours The anti-inflammatory activity was measured by calculating the volume displaced and percent inhibition of edema was further analyzed by two-way ANOVA**.** The value of anti-inflammatory activity indicated that compounds **3 (g, h)** exhibited good anti-inflammatory activities. Compound 3g displayed appreciable anti-inflammatory activity compared to the standard drug (Rolipram) as shown in figure **3**. It was found that aromatic substitution (e.g. Benzene, Phenol, Chlorobenzene & Benzoyl chloride) enhanced the anti-inflammatory activity while aliphatic substitution (e.g. Propionyl chloride, Acetyl chloride, n-propyl) showed poor binding affinity for the enzyme as well as poor anti-inflammatory activity. In contrast, aliphatic substitution with carboxamide group leads to better binding & anti-inflammatory activities as shown in figure **4a**.

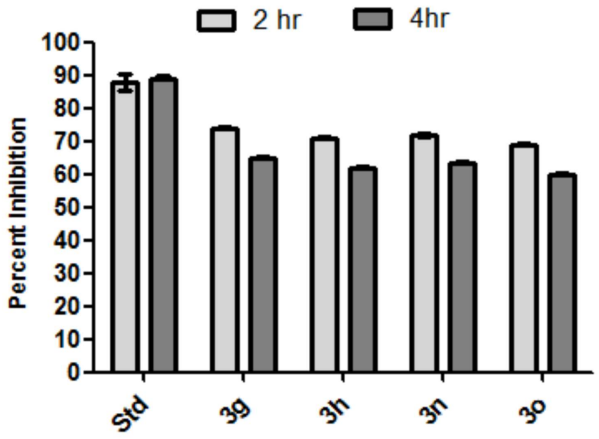

Figure 3. Anti-inflammatory activity (% inhibition of paw edema) of selected molecules at different time intervals. The values are mean of the six measurements (n=6). Data were statistically analyzed by two-way ANOVA ($P<0.05$).

Conclusion

Novel coumarin derivatives were synthesized and evaluated as potent inflammatory agents through the inhibition of PDE4 enzyme. Molecular docking was performed to find out the binding affinity and binding interactions of novel compounds with the enzyme. Based on good binding affinity in docking studies compounds were selected for further anti-inflammatory studies. Out of these derivatives, compounds with aromatic group showed good binding affinity for the PDE4B enzyme as well as anti-inflammatory activity and compounds with aliphatic group

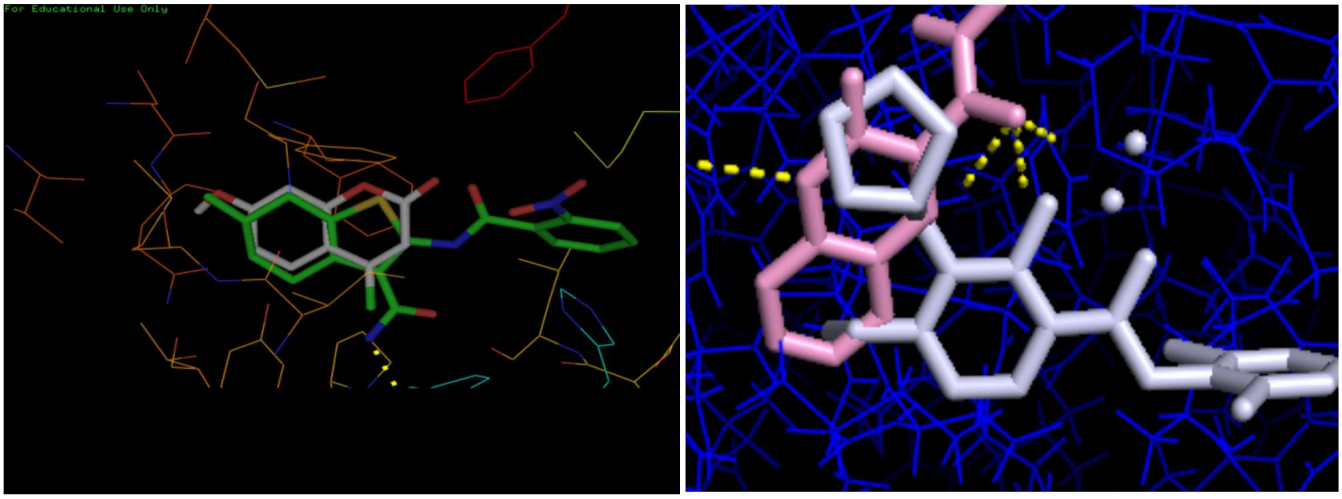

Figure 4(a): Overlay of coumarin compound (3a) docked in the binding site of PDE4B in complex (3HMV) with a coumarin-based inhibitor showing H-bond interactions. **4(b):** Overlay of coumarin compound (3n) docked in the binding site of PDE4B in complex (3LY2) with a coumarin-based inhibitor showing H-bond interactions

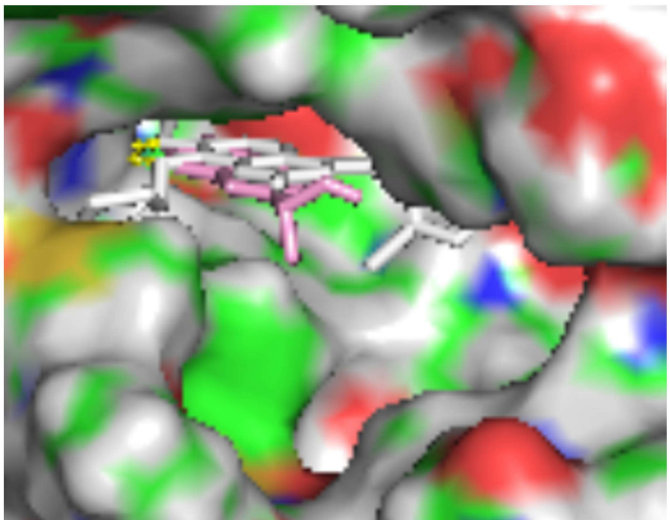

Figure 5. Compound 3o (in pocket) docked with catalytic domain of human PDE4B in complex (3LY2) with a coumarin-based inhibitor

showed poor binding affinity for the PDE4B enzyme as well as anti-inflammatory activity. Compounds **3 (g, h)** showed significant binding affinity in docking studies and also displayed good anti-inflammatory activities. Based on docking studies, it has also been observed that compounds **3 (n, o)** having substitution at 3 positions on coumarin ring leads to better binding affinity (Figure **5**).

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Conflict of interest

The authors declare that they have no conflict of interest.

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