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iNOS inhibitors: Benzimidazole-coumarin derivatives to combat inflammation

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RESEARCH ARTICLE

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ABSTRACT

Inducible nitric oxide synthase (iNOS) plays an important role in the inflammatory processes *via* accelerating the production of nitric oxide (NO). The efforts to develop small molecules as selective inhibitors of iNOS are being reported across the globe. The current study explores varied benzimidazole-coumarin derivatives as anti-iNOS agents. Literature survey suggests 2-aminobenzimidazole, coumarin nucleus, and 4-atom linker as important structural components for iNOS inhibition. Target compounds were designed and synthesized by coupling 2-aminobenzimidazole with (un)substituted coumarin through different linkers. These were docked in iNOS (1QW4) and nNOS (1QW6) targets to ascertain their iNOS selectivity, and evaluated for NO and iNOS inhibitory activities *in vitro*. The most active inhibitors were subsequently evaluated for acute toxicity and anti-inflammatory activity using carrageenan-induced rat paw edema model *in vivo*. All compounds possessed moderate to good NO and iNOS inhibitory activities. Compounds 14a, 14b, 14d, and 14e were the most potent inhibitors *in vitro*. These were found to significantly reduce the inflammation. Compounds 14d and 14e have been identified as the most potent iNOS inhibitors to combat inflammation. These derivatives may serve as potential compounds as such against iNOS, or as leads for the development of novel anti-iNOS agents.

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1. Introduction

Any physical injury, alteration in the normal immune response or in general any pathological condition triggers a series of biochemical events that involve the release of several pro-inflammatory mediators. These mediators induce inflamemation, which is actually a protective mechanism to get rid of the pathological condition(s). However, prolonged or untreated inflammation causes associated tissue damage and/or modulation of normal immune system, culminating in several complex and multifactorial diseases. Alzheimer's disease, Parkinson's disease, rheumatoid arthritis and osteoarthritis, ulcerative colitis, and all cancers are such diseases that are mainly characterized by inflammation. Although non-steroidal anti-inflammatory drugs (NSAIDs) are the first line of drugs for the treatment of inflammation in general, but these have proven inefficient in completely manipulating the inflammatory pathological indications in these multifactorial diseases. Moreover, their prolonged usage is associated with several undesirable effects, and the most common of these include hepatotoxicity and gastric ulcers [1,2]. Some drugs of biological origin such as etanercept and infliximab that act at different inflammatory targets have offered novel treatments for such diseases. However, their use is limited because of their high cost and poor bioavailability [3]. These limitations leave the door wide open for continued efforts toward the discovery of small molecules to counter inflammatory mechanisms. There are several molecular targets identified from the inflammatory cascades that could be antagonized to block the output of these cascades. These include cyclooxygenases (COXs), lipooxygenases (LOXs), tumor necrosis factor (TNF), and nitric oxide synthases (NOSs). NOS catalyze the formation of nitric oxide that plays a pivotal role in the inflammatory progression. It exists in three isoforms, *i.e.,* nNOS, iNOS and eNOS. Of these, iNOS were identified as an important inflammatory target in the past few years [4]. Several molecules have been developed and reported as iNOS inhibitors by different research groups across the globe. Some of these molecules entered the clinical trials but none reach the market because of their toxicity, lack of selectivity, and/or poor pharmacokinetics [5]. Thus, the discovery of selective and druggable compounds capable of inhibiting iNOS is still a research area needing more exploration. An extensive and critical analysis of the literature on iNOS inhibitors by our research group has revealed that a compound having two appropriately substituted aryl or heteroaryl moieties that are connected to each other linked through nitrogen, sulfur or oxygen containing 3-5 atoms long linker has a probability of being a potent and selective inhibitor of iNOS [6]. In the current study, 2-aminobenzimidazole is selected as one heteronucleus because it is a wonderful mimic of guanidine moiety of arginine (the natural substrate of NOS). Coumarin is selected as the other heteronucleus because it is a well-reported free radical scavenger [7,8] (NO is a free radical). Therefore, 4-substituted coumarin is linked to a 5-(un)substituted-2-aminobenzimi-

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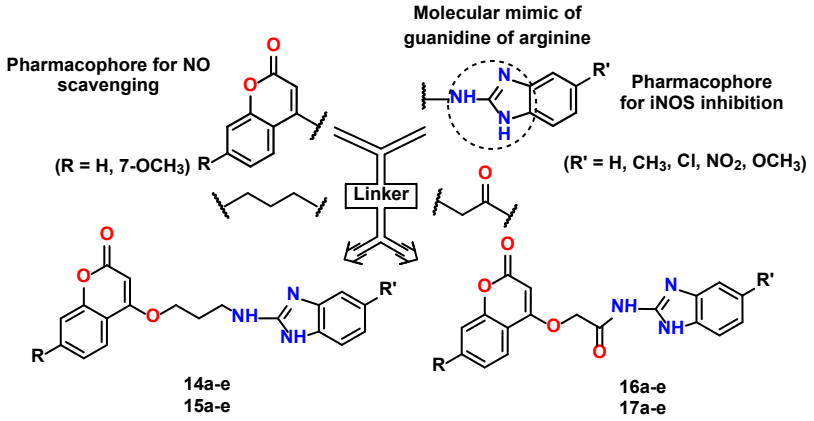

Figure 1. Designing of target compounds.

dazole through an alkoxy and amide linker to design target compounds **14a-e**, **15a-e**, **16a-e** and **17a-e** (Figure 1).

To assess the binding efficiency and selectivity, all synthesized compounds are docked into iNOS and nNOS taken as target proteins. The compounds are found to possess good docking scores towards iNOS. Results of docking analysis are supported by *in vitro* pharmacological evaluations (NO determination and iNOS enzyme assay). The compounds showing good potential *in vitro* are evaluated through an *in vivo* carrageenan-induced rat paw edema model using L-NAME and AG, respectively, as reference nonselective and selective iNOS inhibitors. Previously, our research group synthesized 3-substituted coumarin derivatives as anti-iNOS agents [9]. To check the effect of orientation of -OH group of coumarin on biological activity, the current study was designed.

2. Experimental

2.1. Instrumentation

Synthetic grade chemicals, reagents and solvents as procured from commercial sources including Loba Chem (India), SD-Fine Ltd. (India), Avra Synthesis Pvt. Ltd. (India), and Sigma Aldrich (India) were used for the synthesis of intermediates and target compounds. The solvents were dried, wherever required, by the standard methods. Pre-coated aluminum plates (Merck, Germany) were used for monitoring the progress of chemical reactions as well as to ascertain the purity of compounds. Melting points of all intermediates and target compounds were determined in open capillary tubes, and were uncorrected. IR spectrometer (Bruker Optik) was used for the analysis of IR spectra. ^{1}HNMR and ^{13}CNMR spectra were recorded in $CDCl_3$ and DMSO-d_6 solvents on a Bruker Avance II, 400 MHz spectrophotometer using tetramethylsilane (TMS) as an internal standard. Mass spectral analyses were performed using a Waters Q-TOF mass spectrometer. Docking study was performed with Schrodinger 2016-1 LLC (NY-USA) controlled with Maestro 10.5 software [10]. For the enzyme assay, an iNOS kit (ELISA) was procured from Krishgen Biosystems (Mumbai). Wistar rats of either sex weighing 190-200 g were used for *in vivo* anti-inflammatory activity evaluation of target compounds. The animals were taken care of out as per the guidelines issued by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, India). The animals were housed in cages at ambient temperature 22-27°C with 12 h light/dark cycle in the animal house of Punjabi University, Patiala. They had free access to a standard laboratory diet (Ashirwad Industries, Chandigarh, India) and water *ad libitum*. Both feed and water were withdrawn 12 h prior to the experiment. The compounds and reference drugs were administered through intraperitoneal route as suspension in 0.5% sodium carboxymethylcellulose solution (SCMC).

2.2. Chemistry

For the synthesis of the designed compounds, 5-(un)substituted-2-aminobenzimidazole intermediate (**13a-e**) was coupled with (un)substituted coumarin-based intermediates (**7**-**8** and **11**-**12**) by using the methods already reported in literature by our research group [9]. Intermediate **3** was procured commercially and used as such. Other intermediates were synthesized by the methods reported in literature with slight modifications [11] (Figure 2).

2.2.1. Synthesis of 1-(2,4-dihydroxyphenyl)ethan-1-one (2)

Compound **2** was synthesized by using the Nencki's reaction which involves acetylation with acetic acid in the presence of $ZnCl_2$ [10] with some modifications. Briefly, the fused and powdered $ZnCl_2$ (0.24 mol) was added in glacial acetic acid, and the mixture was heated up to boiling. Dried resorcinol (0.2 mol) was added to the mixture with stirring at 140 °C. The mixture was heated until it turns blood red in color. The temperature was maintained at 150 °C for 20 min. The solution was acidified with dilute HCl (1:1), and cooled. The separated product was filtered, washed with dil. HCl (1:3), and re-crystallized from hot water containing a little amount of hydrochloric acid as brownish small needles (Figure 2).

1-(2,4-Dihydroxyphenyl)ethan-1-one (**2**): Color: Orange. Yield: 78%. M.p.: 144-145 °C.

2.2.2 Synthesis of 2-hydroxy-4-methoxyacetophenone (4)

A solution of intermediate **2** (0.099 mol) in dry acetone was refluxed with dimethylsulphate (0.116 mol) and anhydrous potassium carbonate for 4 h under anhydrous conditions. The inorganic salts were filtered and washed with hot acetone. The filtrate and the washings were combined, and the solvent was recovered under reduced pressure. The left-over oily residue was macerated with ice-cold water, and extracted with solvent ether. The ethereal layer was washed with water, potassium carbonate solution (5%, w/v), and finally extracted with aq. sodium hydroxide (5%, w/v). The clear alkaline solution was acidified with dilute HCl and cooled at 8 °C. The separated product was filtered, washed with water, and re-crystallized from alcohol as colorless long needles (Figure 2).

2-Hydroxy-4-methoxyacetophenone (**4**): Color: Orange. Yield: 75%. M.p.: 53-54 °C.

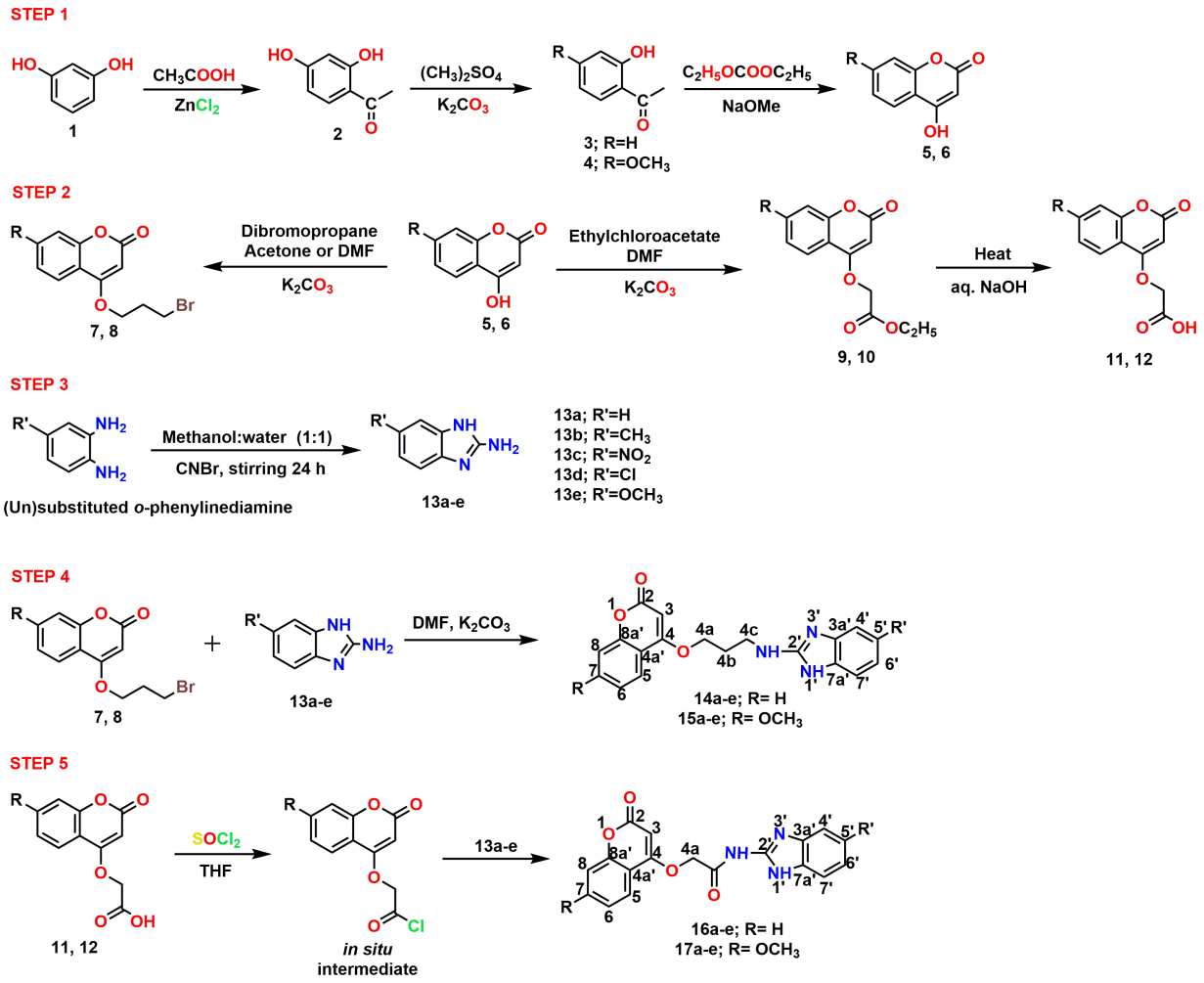

Figure 2. Synthetic scheme employed for the synthesis of target compounds.

2.2.3. Synthesis of 4-hydroxycoumarin (5) and 4-hydroxy-7-methoxycoumarin (6)

Intermediate **3** was procured commercially and used as such. A solution of compounds **3** (0.0147 mol) or **4** (0.006 mol) in dry diethyl carbonate (10-15 mL) was added to a suspension of sodium methoxide (0.0185 mol) in diethyl carbonate (10 mL). The reaction mixture was heated on a steam bath under anhydrous conditions for 5 h. It was cooled, diluted with water (25 mL) and extracted with solvent ether (2×40 mL) to remove the residual diethyl carbonate. The aqueous layer was acidified with hydrochloric acid. The separated product was filtered, washed with water, and re-crystallized from ethanol as white powder (Figure 2).

4-Hydroxycoumarin (**5**): Color: White powder. Yield: 80%. M.p.: 210-211 °C. ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 12.31 (s, 1H, OH), 7.82 (d, 1H, H-8, *J* = 1.54, Ar-H), 7.59 (t, 1H, H-6, *J* = 7.82 Hz, Ar-H), 7.30 (m, 2H, H-7, H-5, Ar-H), 5.61 (s, 1H, H-3, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 166.1 (C-4), 163.1 (C-2), 152.5 (C-8a'), 128.3 (C-7), 125.4 (C-6), 123.2 (C-5), 116.4 (C-4a', C-8), 91.1 (C-3).

4-Hydroxy-7-methoxycoumarin (**6**): Color: White powder. Yield: 37%. M.p.: 252-253 °C. ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 12.28 (s, 1H, OH), 7.70 (d, 1H, H-5, *J* = 6.1 Hz, Ar-H), 6.88 (m, 2H, H-6, H-8, Ar-H), 5.45 (s, 1H, H-3, Ar-H), 3.85 (s, 3H, OCH_3). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 165.8 (C-4), 162.4 (C-2), 152.3 (C-8a'), 160.3 (C-7), 124.3 (C-5), 111.0 (C-6), 108.5 (C-4a'), 100.5 (C-8), 88.7 (C-3), 55.8 (OCH_3).

2.2.4. Synthesis of 4-(3-bromopropoxy) coumarin (7) and 4-(3-bromopropoxy)-7methoxycoumarin (8)

Anhydrous potassium carbonate (1.2 mmol) was added to a solution of compounds **5** or **6** (0.99 mmol) in 40 mL of acetone or DMF, respectively. Following this, dibromopropane (1 mmol) was added, and the mixture was stirred under reflux until the reaction is complete (4-5 h). Solvent was partly recovered under vacuum, and the concentrated reaction mixture is transferred into water to get the white colored powder. This product was used for the next step of synthesis without any further purification (Figure 2).

4-(3-Bromopropoxy)coumarin (**7**): Color: White powder. Yield: 52%. M.p.: 90-92 °C. R_f: 0.64 in Petroleum ether:Ethyl acetate (5:5). ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): 7.79 (d, 1H, H-8, *J* = 7.92 Hz, Ar-H), 7.56 (t, 1H, H-6, *J* = 7.82 Hz, 1.58 Hz, Ar-H), 7.29 (m, 2H, H-7, H-5, Ar-H), 5.73 (s, 1H, H-3, Ar-H), 4.30 (t, 2H, H-4a, *J* = 5.84 Hz), 3.63 (t, 2H, H-4c, *J* = 6.32 Hz), 2.46 (q, 2H, H-4b, *J*= 6.08 Hz). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 168.56 (C-4), 162.31 (C-2), 152.50 (C-8a'), 128.2 (C-7), 123.2 (C-5), 125.2 (C-6), 116.17 (C-4a', C-8), 88.5 (C-3), 66.73 (C-4a), 34.17 (C-4c), 30.7 (C-4b). MS (*m/z*): 283.98 $[M+H]^+$.

4-(3-Bromopropoxy)-7-methoxycoumarin (**8**): Color: Off white powder. Yield: 35%. M.p.: 105-106 °C. R_f: 0.45 in Chloroform:Methanol (8:2). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 7.74 (d, 1H, H-5,*J* = 8.84), 6.92 (m, 2H, H-6, H-8), 5.79 (s, 1H, H-3), 4.42 (t, 2H, H-4a, *J* = 5.9 Hz), 3.85 (s, 3H, OCH_3), 2.38 (t, 2H, H-4c, *J* = 6 Hz), 1.94 (m, 2H, H-4b). ^{13}C NMR (100 MHz,

DMSO-d_6, δ, ppm): 168.54 (C-4), 162.25 (C-2), 150.12 (C-8a'), 159.5 (C-7), 111.0 (C-6), 123.6 (C-5), 108.5 (C-4a'), 100.3 (C-8), 88.9 (C-3), 65.9 (C-4a), 55.8 (OCH_3), 34.17 (C-4c), 30.5 (C-4b). MS (*m/z*): 313.97 $[M+H]^+$.

2.2.5. Synthesis of ethyl-2-((2-oxo-2H-chromen-4-yl)oxy) acetate (9) and ethyl 2-((7-methoxy-2-oxo-2H-chromen-4-yl)oxy)acetate (10)

A mixture of anhydrous potassium carbonate (5.5 mmol) and a solution of compounds **5** or **6** (5 mmol) in DMF was stirred at room temperature for 30-45 minutes. Thereafter, ethyl chloroacetate (5.2 mmol) was added drop-wise with stirring. After the addition was complete, temperature of the mixture was raised to 90 °C, and stirred for another 2-3 h. After the reaction was complete, the temperature of reaction mixture was brought to ambient conditions. Addition of water to the reaction mixture afforded the product as cream-colored precipitates, which are filtered, washed with water, and dried for further use (Figure 2).

Ethyl-2-((2-oxo-2H-chromen-4-yl)oxy)acetate (**9**): Color: Off white powder. Yield: 85%. M.p.: 110-111 °C. R_f: 0.55 in Petroleum ether:Ethyl acetate (5:5). ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): 7.91 (d, 1H, H-5, *J* = 7.9 Hz), 7.57 (t, 1H, H-7, *J* = 7.8 Hz), 7.29 (m, 2H, H-6, H-8), 5.58 (s, 1H, H-3), 4.77 (s, 2H, H-4a), 4.31 (q, 2H, CH_2), 1.33 (t, 3H, CH_3, *J* = 7.14 Hz). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 169.7 (C=O, C-4), 162.4 (C-2), 152.12 (C-8a'), 128.3 (C-7), 125.4 (C-6), 123.3 (C-5), 116.2 (C-4a', C-8), 88.5 (C-3), 62.5 (C-4a), 60.9 (CH_2), 15.2 (CH_3). MS (*m/z*): 249.07 $[M+H]^+$.

Ethyl 2-((7-methoxy-2-oxo-2H-chromen-4-yl)oxy)acetate (**10**): Color: White powder. Yield: 20%. M.p.: 129-130 °C. R_f: 0.68 in Petroleum ether:Ethyl acetate (5:5). ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): 7.79 (d, 1H, H-5, *J* = 6.8 Hz), 6.85 (d, 1H, H-6, *J* = 7.0 Hz), 6.80 (s, 1H, H-8), 5.44 (s, 1H, H-3), 4.74 (s, 2H, 4a), 4.31 (q, 2H, CH_2), 3.87 (s, 3H, OCH_3), 1.32 (t, 3H, CH_3, *J* = 5.8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 169.6 (C=O, C-4), 162.3 (C-2), 160.2 (C-7), 152.10 (C-8a'), 123.6 (C-5), 111.0 (C-6), 108.5 (C-4a'), 100.7 (C-8), 87.3 (C-3), 62.5 (C-4a), 60.0 (CH_2), 14.1 (CH_3). MS (*m/z*): 279.08 $[M+H]^+$.

2.2.6. 2-((2-Oxo-2H-chromen-4-yl)oxy)acetic acid (11) and 2-((7-methoxy-2-oxo-2H-chromen-4-yl)oxy)acetic acid (12)

Compounds **9** or **10** (0.5 mol) was refluxed with aqueous NaOH (40 g/160 mL) in a round bottom flask for 15-20 min to hydrolyze the ester reactant. An equal volume of water was added to the reaction mixture; the dilute mixture was cooled to room temperature, and poured with vigorous stirring into conc. HCl (125 mL). Cooling of the acidic solution to room temperature afforded the crude product that was filtered, washed with water, and re-crystallized from 1% HCl (Figure 2).

2-((2-Oxo-2H-chromen-4-yl)oxy)acetic acid (**11**): Color: White powder. Yield: 36%. M.p.:155-156 °C. R_f: 0.21 in Chloroform: Methanol (8:2). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 13.29 (brs, 1H, OH), 7.87 (m, 2H, H-5, H-8), 7.67 (t, 1H, H-7, *J* = 7.8 Hz), 7.52 (t, 1H, H-6, *J* = 7.75 Hz), 5.88 (s, 1H, H-3), 4.99 (s, 2H, H-4a). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 173.9 (C=O), 169.8 (C-4), 162.5 (C-2), 152.15 (C-8a'), 128.5 (C-7), 125.6 (C-6), 123.3 (C-5), 116.2 (C-4a', C-8), 87.5 (C-3), 64.9 (C-4a). MS (*m/z*): 221.04 $[M+H]^+$.

2-((7-Methoxy-2-oxo-2H-chromen-4-yl)oxy)acetic acid (**12**): Color: White powder. Yield: 10%. M.p.: 167-168 °C. R_f: 0.35 in Chloroform:Methanol (8:2). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 13.20 (brs, 1H, OH), 7.80 (d, 1H, H-5, *J* = 7.0 Hz), 6.84 (d, 1H, H-6, *J* = 6.8 Hz), 6.78 (s, 1H, H-8), 5.39 (s, 1H, H-3), 4.72 (s, 2H, CH_2), 3.87 (s, 3H, OCH_3). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 173.8 (C=O), 169.7 (C-4), 162.4 (C-2), 160.1 (C-7), 152.12 (C-8a'), 122.8 (C-5), 111.2 (C-6), 108.2 (C-4a'), 101.2 (C-8), 87.3 (C-3), 64.7 (C-4a), 55.8 (OCH_3). MS (*m/z*): 251.05 $[M+H]^+$.

2.2.7. Synthesis of 2-aminobenzimidazole intermediates (13a-e)

These were prepared by using a method already optimized in the laboratory. An aqueous solution of methanol (50%, v/v) was divided into two parts. To the first part CNBr (0.03 mol), and to the second part substituted diaminophenylenes (0.02 mol) were added. The solutions were poured into 100 mL RBF followed by stirring at 30-35 °C for 24-48 h. Thereafter methanol was distilled in vacuum; the solution was cooled to the room temperature and made alkaline by adding aq. ammonia solution. The precipitates were separated by filtration and re-crystallized from aq. ethanol (Figure 2).

2-Aminobenzimidazole (**13a**): Color: Brown crystal. Yield: 71%. M.p.: 229-230 °C. ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 7.11 (m, 2H, H-4 and H-7), 6.85 (m, 2H, H-6, H-5), 6.07 (s, 2H, NH_2), 5.57 (s, H, NH).

2-Amino-5-methylbenzimidazole (**13b**): Color: Light brown powder. Yield: 75%. M.p.: 198-199 °C. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): 7.01 (d, 1H, H-7, *J* = 7.8 Hz), 6.95 (s, 1H, H-4), 6.71 (d, 1H, H-6, *J* = 7.6 Hz), 5.90 (s, 2H, NH_2), 2.33 (s, 3H, CH_3).

2-Amino-5-nitrobenzimidazole (**13c**): Color: Yellow powder. Yield: 83%. M.p.: 133-134 °C. ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 7.96 (s, 1H, H-4), 7.85 (dd, 1H, H-6, *J* = 8.6 Hz, 1.4Hz), 7.18 (d, 1H, H-7, *J* = 8.2 Hz), 6.92 (s, 3H, 1-NH, NH_2).

2-Amino-5-chlorobenzimidazole (**13d**): Color: Yellow brown powder. Yield: 48%. M.p.: 169-171°C. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): 6.14 (d, 1H, H-4, *J* = 2 Hz), 6.10 (d, 1H, H-7, *J* = 8.12 Hz), 5.87 (dd, 1H, H-6, *J* = 8.24 Hz, 2.04 Hz), 5.31 (s, 2H, NH_2).

2-Amino-5-methoxybenzimidazole (**13e**): Color: Light brown powder. Yield: 42%. M.p.: 170-171 °C. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): 7.33 (d, 1H, H-7, *J* = 8 Hz), 6.93 (s, 1H, H-4), 6.79 (d, 1H, H-6, *J* = 7.6 Hz), 6.51 (s, 2H, NH_2), 3.83 (s, 3H, OCH_3).

2.2.8. Synthesis of target molecules (14a-e and 15a-e)

Several 3-substituted compounds were synthesized by our research group [9] to produce the target molecules. A suspension of dried potassium carbonate (2.5 mmol) and a solution of compounds **7** or **8** (0.5 mmol) in DMF (10 mL) was treated with 5-(un)substituted-2-aminobenzimidazole **13a-e** (1 mmol). During the 24 h reaction, the mixture was stirred at 60 °C. A vacuum was used to recover the solvent, and then cold water was poured over the concentrated mixture. With chloroform and methanol as mobile phases, the precipitates were further purified by column chromatography (silica gel (60-120 mesh) column) (Figure 2).

4-(3-(1H-Benzimidazol-2yl)aminopropoxy)coumarin (**14a**): Color: Brown. Yield: 28%. M.p.: 230-232 °C. R_f: 0.39 in Chloroform:Methanol (8:2). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 7.75 (d, 1H, H-8, *J* = 7.52 Hz), 7.59 (t, 1H, H-6, *J* = 7.2 Hz), 7.30 (m, 2H, H-7, H-5), 7.12 (m, 2H, H-4', H-7'), 6.90 (m, 2H, H-5', H-6'), 6.24 (s, 1H, NH), 5.72 (s, 1H, H-3), 4.43 (t, 2H, H-4a, *J* = 5.91 Hz), 4.24 (t, 2H, H-4c, J = 6.32 Hz), 2.32 (m, 2H, H-4b). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 164.64 (C-4), 161.29 (C-2), 154.30 (C-8a'), 152.45 (C-2'), 133.77 (C-3a'), 132.00 (C-7a'), 123.50 (C-5'), 122.80 (C-7), 122.50 (C-6), 120.07 (C-6'), 118.03 (C-5), 116.15 (C-7'), 116.02 (C-8), 115.00 (C-4a'), 114.57 (C-4'), 106.75 (C-3), 90.08 (C-4a), 66.56 (C-4c), 27.30 (C-4b). FT-IR (KBr, ν, cm^{-1}): 3478 (NH str), 3130 (Ar C-H str), 2815 (Al C-Hstr), 1710 (C=O lactone str), 1625 (C=N str), 1600 (C=C str), 1055 (C-O str). HRMS (ESI^+, *m/z*) calcd. for $C_{19}H_{17}N_3O_3$ $[M+H]^+$ 336.1303, found 336.1302 $[M+H]^+$.

4-(3-(5-Methyl-1H-benzimidazol-2yl)aminopropoxy) coumarin (**14b**): Color: Reddish brown. Yield: 25%. M.p.: 160-162 °C. R_f: 0.27 in Chloroform:Methanol (8:2). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 7.74 (d, 1H, H-8, *J* = 7.50 Hz), 7.56 (t, 1H, H-6, *J* = 7.21 Hz), 7.20 (m, 2H, H-7, H-5), 7.01 (d, 1H,H-7', *J* = 7.81),

6.85 (s, 1H, H-4'), 6.70 (m, 2H, H-6'), 6.19 (s, 1H, NH), 5.38 (s, 1H, H-3), 4.20 (t, 2H, H-4a, *J* = 5.90 Hz), 3.24 (t, 2H, H-4c, *J* = 6.23 Hz), 2.33 (s, 3H, CH_3), 2.16 (m, 2H, H-4b). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 164.32 (C-4), 161.00 (C-2), 154.15 (C-8a'), 152.17 (C-2'), 133.50 (C-3a'), 131.89 (C-7a'), 123.28 (C-5'), 122.53 (C-7), 122.29 (C-6), 119.99 (C-6'), 118.00 (C-5), 116.02 (C-7'), 115.92 (C-8), 114.97 (C-4a'), 114.50 (C-4'), 106.68 (C-3), 90.00 (C-4a), 66.37 (C-4c), 27.26 (C-4b),21.3 (CH_3). FT-IR (KBr, ν, cm^{-1}): 3320 (NH str), 3133 (Ar C-H str), 2815 (Al C-Hstr), 1715 (C=O lactone str), 1610 (C=N str), 1605 (C=C str), 1050 (C-O str). HRMS (ESI^+, *m/z*) calcd. for $C_{20}H_{19}N_3O_3$ $[M+H]^+$ 350.1460, found 350.4159 $[M+H]^+$.

4-(3-(5-Nitro-1H-benzimidazol-2yl)aminopropoxy)couma rin (**14c**): Color: Yellow brown. Yield: 22%. M.p.: 239-240 °C. R_f: 0.5 in Chloroform:Methanol (8:2). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 8.20 (s, 1H, H-4'), 8.02 (m, 1H,H-6'), 7.76 (m, 1H, H-6), 7.75 (m, 1H, H-8), 7.36 (m, 2H, H-7, H-5), 7.26 (m, 1H, H-7'), 6.69 (s, 1H, H-3), 5.98 (s, 1H, NH), 5.21 (m, 4H, H-4a, 4c), 2.19 (m, 2H, H-4b). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 164.77 (C-4), 161.58 (C-2), 152.71 (C-8a'), 152.00 (C-2'), 133.66 (C-3a'), 131.97 (C-7a'), 123.65 (C-5'), 122.78 (C-7), 122.55 (C-6), 120.14 (C-6'), 118.87 (C-5), 116.10 (C-7'), 115.98 (C-8), 115.52 (C-4a'), 114.96 (C-4'), 106.79 (C-3), 90.28 (C-4a), 65.84 (C-4c), 27.25 (C-4b). FT-IR (KBr, ν, cm^{-1}): 3485 (NH str), 3130 (Ar C-H str), 2818 (Al C-H str), 1710 (C=O lactone str), 1620 (C=N str), 1610 (C=C str), 1055 (C-O str). HRMS (ESI^+, *m/z*) calcd. for $C_{19}H_{16}N_4O_5$ $[M+H]^+$ 381.1154, found 381.1153 $[M+H]^+$.

4-(3-(5-Chloro-1H-benzimidazol-2yl)aminopropoxy) couma rin (**14d**): Color: Grey. Yield: 32%. M.p.: 220-222 °C. R_f: 0.4 in Chloroform:Methanol (8:2). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 7.78 (d, 1H, H-8, *J* = 7.9 Hz), 7.66 (m, 1H, H-6), 7.35 (m, 2H, H-7, H-5), 7.17 (m, 1H, H-4'), 7.07 (m, 1H, H-7'), 6.84 (m, 1H, H-6'), 6.66 (s, 1H, H-3), 6.61 (s, 1H, NH), 4.21 (m, 4H, H-4a, 4c), 2.21 (m, 2H, H-4b). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 164.84 (C-4), 161.57 (C-2), 154.62 (C-8a'), 152.72 (C-2'), 134.00 (C-3a'), 132.32 (C-7a'), 123.76 (C-5'), 123.06 (C-7), 122.84 (C-6), 120.27 (C-6'), 118.05 (C-5), 116.17 (C-7'), 116.07 (C-8), 115.12 (C-4a'), 114.79 (C-4'), 106.99 (C-3), 90.28 (C-4a), 66.73 (C-4c), 27.39 (C-4b). FT-IR (KBr, ν, cm^{-1}): 3487 (NH str), 3135 (Ar C-H str), 2820 (Al C-Hstr), 1715 (C=O lactone str), 1620 (C=N str) 1615 (C=C str), 1050 (C-O str). HRMS (ESI^+, *m/z*) calcd. for $C_{19}H_{16}ClN_3O_3$ $[M+H]^+$ 370.0958, found 370.0983 $[M+H]^+$ and 372.0941 $[(M+2)+H]^+$.

4-(3-(5-Methoxy-1H-benzimidazol-2-yl)amino propoxy) cou marin (**14e**): Color: Dark brown powder. Yield: 27%. M.p.: 253-254 °C. R_f: 0.32 in Chloroform:Methanol (8:2). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 7.75 (d, 1H, H-8, *J* = 7.50 Hz), 7.57 (t, 1H, H-6, *J* = 7.21 Hz), 7.20 (m, 2H, H-7, H-5), 7.01 (d, 1H, H-7', *J* = 7.81), 6.85 (s, 1H, H-4'), 6.69 (m, 2H, H-6'), 6.19 (s, 1H, NH), 5.38 (s, 1H, H-3), 4.20 (t, 2H, H-4a, *J* = 5.90 Hz), 3.83 (s, 3H, OCH_3), 2.24 (t, 2H, H-4c, *J* = 6.23 Hz), 2.16 (m, 2H, H-4b). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 164.03 (C-4), 160.89 (C-2), 154.08 (C-8a'), 152.07 (C-2'), 133.37 (C-3a'), 131.70 (C-7a'), 123.14 (C-5'), 122.37 (C-7), 122.12 (C-6), 119.87 (C-6'), 117.99 (C-5), 116.00 (C-7'), 115.87 (C-8), 114.92 (C-4a'), 114.42 (C-4'), 106.62 (C-3), 89.97 (C-4a), 66.35 (C-4c), 56.3 (OCH_3), 27.22 (C-4b). FT-IR (KBr, ν, cm^{-1}): 3325 (NH str), 3130 (Ar C-H str), 2810 (Al C-Hstr), 1705 (C=O lactone str), 1610 (C=N str), 1605 (C=C str), 1050 (C-O str). HRMS (ESI^+, *m/z*) calcd. for $C_{20}H_{19}N_3O_4$ $[M+H]^+$ 366.1376, found 366.1375 $[M+H]^+$.

4-(3-(1H-Benzimidazol-2-ylamino)propoxy)-7-methoxy cou marin (**15a**): Color: Light green. Yield: 35%. M.p.: 210-211 °C. R_f: 0.39 in Chloroform:Methanol (8:2). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 7.73 (d, 1H, H-5, *J* = 7.50 Hz), 7.10 (m, 2H, H-4', H-7'), 6.87 (m, 2H, H-6, H-8), 6.85 (m, 2H, H-6', H-5'), 6.24 (s, 1H, NH), 5.79 (s, 1H, H-3), 4.41 (t, 2H, H-4a, *J* = 5.9 Hz), 3.83 (s, 3H, OCH_3), 2.19 (t, 2H, H-4c, *J* = 6 Hz), 2.13 (m, 2H, H-4b). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 164.29 (C-4), 161.00 (C-2), 153.80 (C-8a'), 152.07 (C-2'), 133.57 (C-3a'), 131.98 (C-7a'), 123.42 (C-5'), 122.59 (C-7), 122.27 (C-6), 120.00 (C-6'), 117.95 (C-5), 116.02 (C-7'), 115.84 (C-8), 114.98 (C-4a'), 114.50 (C-4'), 106.59 (C-3), 90.00 (C-4a), 66.34 (C-4c), 55.4 (OCH_3), 27.22 (C-4b). FT-IR (KBr, ν, cm^{-1}): 3460 (NH str), 3125 (Ar C-H str), 2810 (Al C-Hstr), 1690 (C=O lactone str), 1615 (C=N str). 1605 (C=C str), 1045 (C-O str). HRMS (ESI^+, *m/z*) calcd. for $C_{20}H_{19}N_3O_4$ $[M+H]^+$ 366.1409, found 366.1408 $[M+H]^+$.

7-Methoxy-4-(3-((5-methyl-1H-benzimidazol-2-yl) amino) propoxy)coumarin (**15b**): Color: Brown powder. Yield: 40%. M.p.: 180-181 °C. R_f: 0.24 in Chloroform:Methanol (8:2). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 7.74 (d, 1H, H-5, *J* = 7.50 Hz), 7.01 (d, 1H, H-7', *J* = 7.8 Hz), 6.89 (m, 2H, H-6, H-8), 6.82 (s, 1H, H-4'), 6.71 (d, 1H, H-6', *J* = 7.6 Hz), 6.19 (s, 1H, NH), 5.73 (s, 1H, H-3), 4.40 (t, 2H, H-4a, *J* = 5.9 Hz), 3.83 (s, 3H, OCH_3), 2.30 (s, 3H, CH_3), 2.18 (t, 2H, H-4c, *J* = 6 Hz), 2.12 (m, 2H, H-4b). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 164.32 (C-4), 161.00 (C-2), 154.15 (C-8a'), 152.17 (C-2'), 133.50 (C-3a'), 131.89 (C-7a'), 123.28 (C-5'), 122.53 (C-7), 122.29 (C-6), 119.99 (C-6'), 118.00 (C-5), 116.02 (C-7'), 115.92 (C-8), 114.97 (C-4a'), 114.50 (C-4'), 106.68 (C-3), 90.00 (C-4a), 66.37 (C-4c), 55.7 (OCH_3), 27.26 (C-4b),21.3 (CH_3). FT-IR (KBr, ν, cm^{-1}): 3310 (NH str), 3125 (Ar C-H str), 2810 (Al C-Hstr), 1695 (C=O lactone str), 1615 (C=N str), 1610 (C=C str), 1050 (C-O str). HRMS (ESI^+, *m/z*) calcd. for $C_{21}H_{21}N_3O_4$ $[M+H]^+$ 380.1566, found 380.1565 $[M+H]^+$.

7-Methoxy-4-(3-((5-nitro-1H-benzimidazol-2-yl) amino) pro poxy)coumarin (**15c**): Color: Yellow brown. Yield: 38%. M.p.: 243-244 °C. R_f: 0.45 in Chloroform:Methanol (8:2).^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 8.20 (s, 1H, H-4'), 7.84 (dd, 1H, H-6', *J* = 8.6 Hz, 1.4 Hz), 7.71 (d, 1H, H-5, *J* = 7.50 Hz), 7.18 (d, 1H, H-7', *J* = 8.2 Hz), 6.89 (m, 2H, H-6, H-8), 6.70 (s, 1H, NH), 5.73 (s, 1H, H-3), 4.40 (t, 2H, H-4a, *J* = 5.9 Hz), 3.89 (s, 3H, OCH_3), 2.16 (t, 2H, H-4c, *J* = 6 Hz), 2.09 (m, 2H, H-4b). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 164.70 (C-4), 161.51 (C-2), 152.67 (C-8a'), 151.96 (C-2'), 133.59 (C-3a'), 131.90 (C-7a'), 123.58 (C-5'), 122.71 (C-7), 122.49 (C-6), 120.07 (C-6'), 118.80 (C-5), 116.02 (C-7'), 115.91 (C-8), 115.45 (C-4a'), 114.89 (C-4'), 106.72 (C-3), 90.21 (C-4a), 65.76 (C-4c), 55.7 (OCH_3), 27.22 (C-4b). FT-IR (KBr, ν, cm^{-1}): 3470 (NH str), 3125 (Ar C-H str), 2815 (Al C-Hstr), 1700 (C=O lactone str), 1620 (C=N str), 1605 (C=C str), 1050 (C-O str). HRMS (ESI^+, *m/z*) calcd. for $C_{20}H_{18}N_4O_6$ $[M+H]^+$ 411.1260, found 411.1259 $[M+H]^+$.

4-(3-((5-Chloro-1H-benzimidazol-2-yl)amino) propoxy)-7-methoxycoumarin (**15d**): Color: Light brown. Yield: 45%. M.p.: 272-273 °C. R_f: 0.4 in Chloroform:Methanol (8:2). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 8.34 (s, 1H, H-4'), 7.83 (dd, 1H, H-6', *J* = 8.6 Hz, 1.4Hz), 7.71 (d, 1H, H-5,*J* = 7.50 Hz), 7.18 (d, 1H, H-7', *J* = 8.2 Hz), 6.89 (m, 2H, H-6, H-8), 6.70 (s, 1H, NH), 5.70 (s, 1H, H-3), 4.40 (t, 2H, H-4a, *J* = 5.9 Hz), 3.89 (s, 3H, OCH_3), 2.17 (t, 2H, H-4c, *J* = 5.8 Hz), 2.10 (m, 2H, H-4b). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 164.80 (C-4), 161.50 (C-2), 154.60 (C-8a'), 152.66 (C-2'), 134.00 (C-3a'), 132.30 (C-7a'), 123.69 (C-5'), 123.00 (C-7), 122.78 (C-6), 120.19 (C-6'), 118.00 (C-5), 116.09 (C-7'), 116.01 (C-8), 115.06 (C-4a'), 114.70 (C-4'), 106.91 (C-3), 90.21 (C-4a), 66.67 (C-4c), 55.7 (OCH_3),27.32 (C-4b). FT-IR (KBr, ν, cm^{-1}): 3482 (NH str), 3130 (Ar C-H str), 2815 (Al C-Hstr), 1710 (C=O lactone str), 1620 (C=N str), 1610 (C=C str), 1050 (C-O str). HRMS (ESI^+, *m/z*) $C_{20}H_{18}ClN_3O_4$ $[M+H]^+$ 400.0956, found 400.980 $[M+H]^+$ and 402.0937 $[(M+2)+H]^+$.

7-Methoxy-4-(3-((5-methoxy-1H-benzimidazol-2-yl) amino) propoxy)coumarin (**15e**): Color: Light brown. Yield: 39%. M.p.: 225-226 °C. R_f: 0.35 in Chloroform:Methanol (8:2). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 7.69 (d, 1H, H-5, *J* = 7.6 Hz), 7.01 (d, 1H, H-7', *J* = 7.8 Hz), 6.90 (s, 1H, H-4'), 6.89 (m, 2H, H-6, H-8), 6.73 (d, 1H, H-6', *J* = 7.6 Hz), 6.19 (s, 1H, NH), 5.73 (s, 1H, H-3), 4.40 (t, 2H, H-4a, *J* = 5.9 Hz), 3.83 (s, 6H, OCH_3), 2.24 (t, 2H, H-4c, *J* = 6 Hz), 2.13 (m, 2H, H-4b). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 164.00 (C-4), 160.78 (C-2), 154.01 (C-8a'), 152.01 (C-2'), 133.29 (C-3a'), 131.61 (C-7a'), 123.10 (C-5'), 122.31 (C-7), 122.07 (C-6), 119.79 (C-6'), 117.92 (C-5), 116.01 (C-7'), 115.85 (C-8),

114.88 (C-4a'), 114.37 (C-4'), 106.56 (C-3), 89.91 (C-4a), 66.27 (C-4c), 56.27 (OCH_3), 27.23 (C-4b). FT-IR (KBr, ν, cm^{-1}): 3300 (NH str), 3125 (Ar C-H str), 2810 (Al C-H str), 1690 (C=O lactone str), 1610 (C=N str), 1605 (C=C str), 1047 (C-O str). HRMS (ESI^+, *m/z*) calcd. for $C_{21}H_{21}N_3O_5$[M+H]$^+$ 396.1515, found 396.1514 [M+H]$^+$.

2.2.9. Synthesis of target molecules (16a-e and 17a-e)

Previously, our research team reported a method for preparing these compounds [9]. A solution of compounds **11** or **12** (0.25 mol) in THF (10 mL) was added to an excess of $SOCl_2$ (2-5 mL) and allowed to heat at 40 °C for 30 min. In the reaction mixture, 5-substituted-2-aminobenzimidazole solutions (**13a-e**, 0.25 mol) were poured slowly over 1 hour. During the reaction, the mixture was stirred at 40-50 °C for 12-24 hours. By using a rotary vacuum evaporator, the solvent was recovered after the reaction. In order to get the target compounds in pure form, the crude solid was fractionated by column chromatography using a gradient solvent system (Petroleum ether:ethyl acetate) (Figure 2).

N-(1H-Benzimidazol-2-yl)-2-((2-oxo-2H-chromen-4-yl) oxy) acetamide (**16a**): Color: Yellow brown. Yield: 25%. M.p.: 205-206 °C. R_f: 0.55 in Petroleum ether:Ethyl acetate (5:5). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 12.60 (s, 1H, NH), 7.91 (d, 1H, H-5, *J* = 6.4 Hz), 7.57 (t, 1H, H-7, *J* = 6.2 Hz), 7.27 (m, 2H, H-6, H-8), 6.96 (m, 2H, H-5', H-6'), 6.89 (m, 2H, H-4', H-7'), 5.60 (s, 1H, H-3), 4.80 (s, 2H, H-4a). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 169.3 (C-4b, C-4), 162.3 (C-2), 152.5 (C-8a'), 146.7 (C-2'), 136.4 (C-3a', C-7a'), 128.3 (C-7), 125.4 (C-6), 123.3 (C-5, C-5', C-6'), 116.2 (C-4a', C-8), 115.2 (C-4', C-7'), 87.5 (C-3), 64.6 (C-4a). FT-IR (KBr, ν, cm^{-1}): 3360 (NH str), 3100 (Ar C-H str), 2900 (Al C-H str), 1720 (C=O, lactone str), 1705 (C=O), 1680 (NH bending), 1056 (C-O str). HRMS (ESI^+, *m/z*) calcd. for $C_{18}H_{13}N_3O_4$ [M+H]$^+$ 336.0940, found 336.0938 [M+H]$^+$.

N-(5-Methyl-1H-benzimidazol-2-yl)-2-((2-oxo-2H-chromen-4-yl)oxy)acetamide (**16b**): Color: Brown. Yield: 21%. M.p.: 165-166 °C. R_f: 0.60 inPetroleum ether: Ethyl acetate (5:5). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 12.59 (s, 1H, NH), 7.91 (d, 1H, H-5, *J* = 6.4 Hz), 7.57 (t, 1H, H-7, *J* = 6.2 Hz), 7.26 (m, 2H, H-6, H-8), 7.01 (d, 1H, H-7', *J* = 7.8 Hz), 6.98 (s, 1H, H-4'), 6.75 (d, 1H, H-6', *J* = 7.6 Hz), 5.60 (s, 1H, H-3), 4.82 (s, 2H, H-4a), 3.54 (s, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 168.2 (C-4b, C-4), 162.0 (C-2), 152.3 (C-8a'), 146.2 (C-2'), 136.4 (C-3a'), 133.5 (C-7a'), 132.7 (C-5'), 128.28 (C-7), 125.4 (C-6), 123.0 (C-5), 120.8 (C-6'), 116.19 (C-4a', C-8), 113.8 (C-4', C-7'), 87.3 (C-3), 64.3 (C-4a), 21.1 (CH_3). FT-IR (KBr, ν, cm^{-1}): 3355 (NH str), 3095 (Ar C-H str), 2890 (Al C-H str), 1715 (C=O, lactone str), 1705 (C=O), 1678 (NH bending), 1050 (C-O str). HRMS (ESI^+, *m/z*) calcd. for $C_{19}H_{15}N_3O_4$ [M+H]$^+$ 350.1096, found 350.1095 [M+H]$^+$.

N-(5-Nitro-1H-benzimidazol-2-yl)-2-((2-oxo-2H-chromen-4-yl)oxy)acetamide (**16c**): Color: Brown. Yield: 16%. M.p.: 245-246 °C. R_f: 0.47 in Petroleum ether:Ethyl acetate (5:5).^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 12.60 (s, 1H, NH), 7.95 (s, 1H, H-4'), 7.91 (d, 1H, H-5, *J* = 5.8 Hz), 7.85 (dd, 1H, H-6', *J* = 8.6 Hz, 1.4Hz), 7.58 (t, 1H, H-7, *J* = 6.0 Hz), 7.30 (m, 2H, H-6, H-8), 7.17 (d, 1H, H-7', *J* = 8.2 Hz), 5.60 (s, 1H, H-3), 4.79 (s, 2H, H-4a). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 169.6 (C-4b, C-4), 162.4 (C-2), 152.57 (C-8a'), 146.8 (C-2'), 145.5 (C-3a'), 144.2 (C-5'), 142.7 (C-7a'), 128.33 (C-7), 125.46 (C-6), 123.5 (C-5), 122.0 (C-6'), 116.23 (C-4a', C-8, C-7'), 114.4 (C-4'), 87.8 (C-3), 66.2 (C-4a). FT-IR (KBr, ν, cm^{-1}): 3355 (NH str), 3105 (Ar C-H str), 2905 (Al C-H str), 1720 (C=O, lactone str), 1680 (NH bending), 1050 (C-O str). HRMS (ESI^+, *m/z*) calcd. for $C_{18}H_{12}N_4O_6$ [M+H]$^+$ 381.0790, found 381.0788 [M+H]$^+$.

N-(5-Chloro-1H-benzimidazol-2-yl)-2-((2-oxo-2H-chromen-4-yl)oxy)acetamide (**16d**): Color: Light green. Yield: 27%. M.p.: 233-234 °C. R_f: 0.50 in Petroleum ether:Ethyl acetate (5:5). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 12.59 (s, 1H, NH), 7.97 (d, 1H, H-5, *J* = 7.8 Hz), 7.71 (t, 1H, H-7, *J* = 7.8 Hz), 7.58 (m, 2H, H-6', H-7'), 7.41 (m, 2H, H-6, H-8), 7.32 (d, 1H, H-4', *J* = 1.74 Hz), 6.02 (s, 1H, H-3), 5.30 (s, 2H, H-4a). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 169.0 (C-4b, C-4), 161.9 (C-2), 152.52 (C-8a'), 146.5 (C-2'), 139.7 (C-7a'), 135.3 (C-3a'), 133.8 (C-5'), 128.3 (C-7), 125.4 (C-6), 123.1 (C-5), 121.6 (C-6'), 116.2 (C-4a', C-8, C-7'), 114.7 (C-4'), 87.5 (C-3), 65.2 (C-4a). FT-IR (KBr, ν, cm^{-1}): 3360 (NH str), 3105 (Ar C-H str), 2900 (Al C-H str), 1715 (C=O, lactone str), 1675 (NH bending), 1052 (C-O str). HRMS (ESI^+, *m/z*) calcd. for $C_{18}H_{12}ClN_3O_4$ [M+H]$^+$370.0568, found 370.0598 [M+H]$^+$ and 372.0556 [(M+2)+H]$^+$.

N-(5-Methoxy-1H-benzimidazol-2-yl)-2-((2-oxo-2H-chromen-4-yl)oxy)acetamide (**16e**): Color: Light brown. Yield: 12%. M.p.: 178-179 °C. R_f: 0.58 in Petroleum ether:Ethyl acetate (5:5). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 11.69 (s, 1H, NH), 7.89 (d, 1H, H-5, *J* = 6.2 Hz), 7.54 (t, 1H, H-7, *J* = 6.0 Hz), 7.23 (m, 2H, H-6, H-8), 6.97 (d, 1H, H-7', *J* = 7.2 Hz), 6.90 (s, 1H, H-4'), 6.65 (d, 1H, H-6', *J* = 7.6 Hz), 5.58 (s, 1H, H-3), 4.76 (s, 2H, H-4a), 3.87 (s, 3H, OCH_3). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 168.7 (C-4b, C-4), 162.0 (C-2), 155.7 (C-5'), 152.49 (C-8a'), 146.0 (C-2'), 135.0 (C-3a'), 128.9 (C-7a', C-7), 125.40 (C-6), 123.3 (C-5), 116.19 (C-8, C-7'), 113.7 (C-6'), 108.0 (C-4a'), 105.8 (C-4'), 87.3 (C-3), 64.3 (C-4a), 55.3 (OCH_3). FT-IR (KBr, ν, cm^{-1}): 3357 (NH str), 3090 (Ar C-H str), 2892 (Al C-H str), 1718 (C=O, lactone str), 1678 (NH bending), 1050 (C-O str). HRMS (ESI^+, *m/z*) calcd. for $C_{19}H_{15}N_3O_5$ [M+H]$^+$ 366.1045, found 366.1044 [M+H]$^+$.

N-(1H-Benzimidazol-2-yl-)-2-((7-methoxy-2-oxo-2H chromen-4-yl)oxy)acetamide (**17a**): Color: Green. Yield: 22%. M.p.: 233-234 °C. R_f: 0.57 in Petroleum ether:Ethyl acetate (5:5). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 8.57 (s, 1H, NH), 7.78 (d, 1H, H-5, *J* = 6.8 Hz), 6.91 (m, 2H, H-5', H-6'), 6.82 (m, 2H, H-4', H-7'), 6.77 (d, 1H, H-6, *J* = 7.0 Hz), 6.74 (s, 1H, H-8), 5.35 (s, 1H, H-3), 4.71 (s, 2H, H-4b), 3.87 (s, 3H, OCH_3). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 169.3 (C-4b, C-4), 162.4 (C-2), 159.2 (C-7), 154.50 (C-8a'), 146.7 (C-2'), 136.6 (C-3a', C-7a'), 123.6 (C-5, C-5', C-6'), 115.8 (C-4', C-7'), 111.0 (C-6), 108.9 (C-4a'), 101 (C-8), 87.5 (C-3), 65.9 (C-4a), 55.8 (OCH_3). FT-IR (KBr, ν, cm^{-1}): 3353 (NH str), 2955 (Ar C-H str), 2895 (Al C-H str), 1715 (C=O lactone str), 1678 (NH bending), 1052 (C-O str). HRMS (ESI^+, *m/z*) calcd. for $C_{19}H_{15}N_3O_5$ [M+H]$^+$ 366.1045, found 366.1041 [M+H]$^+$.

2-((7-Methoxy-2-oxo-2H-chromen-4-yl)oxy)-N-(5-methylbenzimidazol-2-yl)acetamide (**17b**): Color: Red brown. Yield: 20%. M.p.: 203-204 °C.R_f: 0.62 in Petroleum ether:Ethyl acetate (5:5). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 8.56 (s, 1H, NH), 7.79 (d, 1H, H-5, *J* = 6.4 Hz), 7.01 (d, 1H, H-7', *J* = 7.6 Hz), 6.95 (s, 1H, H-4'), 6.79 (d, 2H, H-6, *J* = 6.8 Hz), 6.77 (s, 1H, H-8), 6.73 (d, 1H, H-6', *J* = 7.8 Hz), 5.40 (s, 1H, H-3), 4.70 (s, 2H, H-4b), 3.87 (s, 3H, OCH_3), 3.54 (s, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 168.0 (C-4b, C-4), 162.1 (C-2), 159.8 (C-7), 153.42 (C-8a'), 146.2 (C-2'), 138.4 (C-3a'), 133.4 (C-7a'), 131.4 (C-5'), 125.3 (C-6'), 123.2 (C-5), 114.8 (C-4', C-7'), 110.3 (C-6), 108.3 (C-4a'), 99.7 (C-8), 87.3 (C-3), 64.3 (C-4a), 55.7 (OCH_3), 21.3 (CH_3). FT-IR (KBr, ν, cm^{-1}): 3355 (NH str), 2952 (Ar C-H str), 2895 (Al C-H str), 1710 (C=O lactone str), 1700 (C=O), 1675 (NH bending), 1048 (C-O str). HRMS (ESI^+, *m/z*) calcd. for $C_{20}H_{17}N_3O_5$ [M+H]$^+$ 380.1202, found 380.1201 [M+H]$^+$.

2-((7-Methoxy-2-oxo-2H-chromen-4-yl)oxy)-N-(5-nitrobenzimidazol-2-yl)acetamide (**17c**): Color: Yellow brown. Yield: 25%. M.p.: 246-247 °C. R_f: 0.49 in Petroleum ether:Ethyl acetate (5:5). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 8.57 (s, 1H, NH), 7.97 (s, 1H, H-4'), 7.92 (d, 1H, H-5, *J* = 5.8 Hz), 7.85 (dd, 1H, H-6', *J* = 8.0 Hz, 1.2 Hz), 7.79 (d, 1H, H-7', *J* = 7.8 Hz), 7.52 (m, 2H, H-6, H-8), 5.40 (s, 1H, H-3), 4.78 (s, 2H, H-4a), 3.89 (s, 3H, OCH_3). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 169.3 (C-4b, C-4), 162.4 (C-2), 160.2 (C-7), 152.5 (C-8a'), 146.7 (C-2'), 145.7 (C-3a'), 144.1 (C-5'), 142.7 (C-7a'), 123.5 (C-5), 120.6 (C-6'), 113.9 (C-4'), 111.3 (C-6, C-7'), 108.3 (C-4a'), 100.5 (C-8), 87.5 (C-3), 65.9 (C-4a), 55.7 (OCH_3). FT-IR (KBr, ν, cm^{-1}): 3360 (NH str), 2950 (Ar C-H str), 2890 (Al C-H str), 1710 (C=O lactone str), 1680 (NH

bending), 1050 (C-O str). HRMS (ESI+, *m/z*) calcd. for $C_{19}H_{14}N_4O_7$ $[M+H]^+$ 411.0896, found 411.0895 $[M+H]^+$.

N-(5-Chlorobenzimidazol-2-yl-)-2-((7-methoxy-2-oxo-2H-chromen-4-yl)oxy)acetamide (**17d**): Color: Brown. Yield: 20%. M.p.: 267-268 °C. R_f: 0.51in Pet ether: Ethyl acetate (5:5). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 8.58 (s, 1H, NH), 7.95 (s, 1H, H-4'), 7.85 (d, 1H, H-7', *J* = 7.8 Hz), 7.75 (dd, 1H, H-6', *J* = 7.8 Hz, 1.2 Hz), 7.69 (d, 1H, H-5, *J* = 6.2 Hz), 7.52 (m, 2H, H-6, H-8), 5.39 (s, 1H, H-3), 4.75 (s, 2H, H-4a), 3.87 (s, 3H, OCH_3). ^{13}CNMR (100 MHz, DMSO-d_6, δ, ppm): 169.1 (C-4b, C-4), 161.4 (C-2), 160.1 (C-7), 152.51 (C-8a'), 145.7 (C-2'), 140.3 (C-3a'), 134.7 (C-7a', C-5'), 124.1 (C-6'), 123.6 (C-5), 116.4 (C-7'), 113.8 (C-4'), 111.2 (C-6), 108.3 (C-4a'), 100.5 (C-8), 87.5 (C-3), 65.0 (C-4a), 55.8 (OCH_3). FT-IR (KBr, ν, cm^{-1}): 3358 (NH str), 2955 (Ar C-H str), 2895 (Al C-H str), 1715 (C=O lactone str), 1678 (NH bending), 1048 (C-O str). HRMS (ESI+, *m/z*)calcd. for $C_{19}H_{14}ClN_3O_5$ $[M+H]^+$ 400.0562, found 400.0590 $[M+H]^+$ and 402.0939 $[(M+2)+H]^+$.

N-(5-Methoxybenzimidazol-2-yl-)-2-((7-methoxy-2-oxo-2H-chromen-4-yl)oxy)acetamide (**17e**): Color: Brown. Yield: 18%. M.p.: 289-290 °C. R_f: 0.60 in Petroleum ether:Ethyl acetate (5:5). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 8.55 (s, 1H, NH), 7.69 (d, 1H, H-5, *J* = 6.2 Hz), 7.52 (m, 2H, H-6, H-8), 7.29 (d, 1H, H-7', *J* = 8.0 Hz), 6.90 (s, 1H, H-4'), 6.75 (dd, 1H, H-6', *J* = 7.6 Hz, 1.2 Hz), 5.39 (s, 1H, H-3), 4.75 (s, 2H, H-4a), 3.82 (s, 6H, OCH_3). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 168.3 (C-4b, C-4), 162.0 (C-2), 159.1 (C-7, C-5'), 152.23 (C-8a'), 146.1 (C-2'), 135.3 (C-3a'), 128.9 (C-7a'), 123.6 (C-5), 116.19 (C-7'), 111.2 (C-6, C-6'), 100.8 (C-4', C-8, C-4a'), 87.0 (C-3), 64.2 (C-4a), 55.8 (OCH_3). FT-IR (KBr, ν, cm^{-1}): 3353 (NH str), 2945 (Ar C-H str), 2885 (Al C-H str), 1715 (C=O lactone str), 1675 (NH bending), 1050 (C-O str). HRMS (ESI+, *m/z*) calcd. for $C_{20}H_{17}N_3O_6$ $[M+H]^+$ 396.1151, found 396.1150 $[M+H]^+$.

2.3. Docking analysis

2.3.1. Computer and software

Specifications of the workstation used for molecular modelling studies are Intel® Core™i7-4558V@280GHz, 2801 MHz, 2 cores, SMBIOS Version 2.7 (Samsung-A740). Schrodinger 2016-1 LLC (NY-USA) controlled with Maestro 10.5 software [10] was employed for docking studies. Chemical structures of the designed molecules were drawn in ChemBioDraw Ultra-12.0 [12]. Windows 10 and Centos-6.6 operating systems were used for all computational analyses.

2.3.2. Molecular docking and ADME studies

All compounds were sketched and cleaned in Maestro using the molecular modeling workspace followed by energy minimization in *LIGPREP* program of Schrodinger software [13] using OPLS_2005 force field [14] at pH = 7.4. Structures of both the protein (1QW4 and 1QW6) with co-crystalline ligand 3AR (*N*-propyl-*L*-arginine) were downloaded from the protein data bank [15]. For the docking analysis, protein optimization was done for both proteins that include the addition of hydrogen atoms, deletion of water molecules, completion of bond orders, assignment of hydrogen bonds, and complex minimization using 3AR. Extra precision (XP) docking mode of '*glide*' was used for docking. The results were analysed on the basis of glide docking. *QIKPROP* program [16] of Maestro was used for calculating the theoretical absorption, distribution, metabolism, and excretion (ADME) properties of target compounds.

2.4. In vitro evaluations

2.4.1. Nitrite determination assay

Splenocytes suspension (100 µL), Roswell Park Memorial Institute (RPMI) media (700 µL) and lipopolysaccharide (LPS, 100 µL) were transferred into each of the test tubes marked as control, test, standard L-N^G-nitro arginine methyl ester (L-NAME), and standard aminoguanidine (AG). A 100 µL aliquot of RPMI media, test compound (10 µM concentration), L-NAME and AG was transferred into the respective test tubes. All tubes were incubated at 37 °C for 2 h. Thereafter, the contents in each tube were mixed well by gentle shaking, and a solution of arginine (20 µL; 0.034 g arginine in 10 mL normal saline) was added to each tube. The tubes were incubated in a CO_2 chamber for 24 h. Finally, Griess reagent was transferred in each test tube, the contents are mixed well, and the tubes are kept in the dark for 10 min. Each tube was shaken gently to mix the content, and the absorbance (OD) is read at 540 nm taking Griess reagent as blank [9,17,18]. NO inhibitory activity of the test and standard compounds was calculated using the Equation (1):

$$\% \text{ Inhibition of NO} = [(\text{Control OD} - \text{Test OD}) \div \text{Control OD}] \times 100 \quad (1)$$

2.4.2. iNOS assay

Inducible nitric oxide synthase (iNOS) inhibitory activity of standard as well as test compounds was evaluated through a rat iNOS enzyme-linked immunosorbent assay (ELISA) kit using a 96-well microtiter plate by the method as provided by the kit manufacturer.

2.5. In vivo evaluations

2.5.1. Acute toxicity study

Acute toxicity studies were performed for test compounds **14a**, **14b**, **14d**, and **14e** by on rats using acute toxic class limit test dose in accordance with guidelines 425 of Organization for Economic Co-operation and Development (OECD 2001) [19]. The control group animals were administered 0.5% sodium carboxymethyl cellulose (SCMC) *i.p.,* whereas the test group animals received the test compounds (300 mg/kg body weight, *i.p.*). The toxicological effects were assessed in terms of mortality and behavioral changes that occur during 48 h after the administration of compounds.

2.5.2. Anti-inflammatory activity

Wistar rats of either sex weighing 200-250 g were used for the evaluation of anti-inflammatory activity of the test and standard compounds using carrageenan-induced rat paw edema model as reported by Salvemini *et al*. [20]. The rats were randomly divided into control, test-treated, AG-treated, and L-NAME-treated groups, with five rats in each group. SCMC (0.5%, used as vehicle for test and standard compounds) was administered to control group. The four test-treated groups were administered compounds **14a**, **b**, **d** and **e**. The AG- and L-NAME-treated groups were administered AG and L-NAME, respectively. Dose of each test and standard compound was 30 mg/kg *i.p.* After 1h of administration of test or standard compound, carrageenan 1.0% w/v suspension in normal saline was injected in right paw of each rat in each group. The paw volume was measured just before the injection of carrageenan as well as at hourly intervals for 10 h in each animal in all groups with a plethysmometer. Anti-inflammatory activity of each compound was determined from a plot obtained by using reduction in the paw volume as ordinate and time as abscissa with the help of Graphpad prism software [21].

3. Results and discussion

3.1. Chemistry

Target compounds were synthesized by using the methods optimized earlier in our laboratory as well as those reported in the literature [9,11,22]. The structural characterization of all

Table 1. Docking scores of the target compounds and the amino acids in iNOS (1QW4), and nNOS (1QW6) involved in interactions with compounds.

Compound	Docking scores 1QW4/1QW6	Amino acid residues interacting with 1QW4	Amino acid residues interacting with 1QW6
14a	-6.37/-3.30	Heme900, Glu371, Tyr367, Ash376, Arg382	Heme900, Arg481, Glu592
14b	-6.38/-2.66	Heme900, Glu371, Tyr367, Ash376, Arg382, Arg260	Heme900, Arg603, Glu592
14c	-4.41/-3.04	Heme900, Ash376, Tyr367	Asn569, Tyr706, Gln478, Tyr588
14d	-6.46/-2.53	Heme900, Glu371, Tyr367, Ash376, Arg382, Arg260	Heme900, Asn569, Gln478, Tyr588
14e	-6.15/-1.39	Heme900, Glu371, Arg260, Ash376, Arg382	Heme900, Glu592
15a	-4.82/-3.31	Heme900, Glu371, Arg260, Asn348	Heme900, Arg603, Ash597
15b	-4.94/-4.00	Heme900, Glu371, Asn115	Heme900, Glu592, Arg603
15c	-4.49/-4.14	Heme900, Gln486, Ash376, Tyr367	Tyr588, Ash597, Tyr706
15d	-4.76/-4.51	Heme900, Tyr485, Ash376, Tyr367, Glu486	Heme900, Tyr706, Tyr588, Ash597
15e	-5.35/-4.32	Heme900, Tyr485, Ash376, Tyr367	Heme900, Glu592, Arg481
16a	-5.00/-4.32	Heme900, Glu371, Tyr367, Ash376, Arg382, Arg260	Tyr588, Ash597
16b	-4.64/-3.38	Tyr367, Ash376, Arg382	Gln478, Tyr588, Ash597
16c	-4.70/-4.63	Heme900, Glu371, Tyr367, Ash376	Heme900, Arg596, Gln478, Ash597
16d	-4.85/-4.11	Heme900, Glu371, Tyr367, Ash376, Arg260	Tyr588, Ash597
16e	-5.22/-0.92	Heme900, Glu371, Tyr367, Ash376, Arg260	Heme900, Glu592, Arg481, Arg603, Gln478
17a	-4.97/-4.09	Heme900, Glu371, Tyr367	Tyr588, Ash597
17b	-4.25/-4.28	Heme900, Glu371, Asn348	Tyr588, Ash597, Gln478
17c	-4.10/-2.07	Heme900, Tyr485, Glu488, Ash376, Tyr367	Tyr706, Tyr588, Ash597
17d	-3.94/-2.66	Tyr367, Ash376, Arg260, Asn348	Heme900, Arg596, Ash597, Tyr588
17e	-0.71/-4.42	Tyr367, Tyr485	Tyr588, Ash597, Arg596
AG	-4.80/-5.01	Heme900, Glu371, Trp366	Heme900, Glu592
NAME	-5.59/-4.65	Heme900, Glu371, Trp366, Ash376, Tyr367	Heme900, Glu592, Trp587, Tyr588, Ash597
3AR	-6.86/-5.65	Heme900, Glu371, Ash376, Arg260	Heme900, Glu592, Gln478, Tyr588, Ash597

compounds was carried out using IR, NMR, and mass spectrometry. In ^{1}H NMR spectra of intermediates **5** and **6**, all protons of the coumarin ring were detected in the range of δ 5-8 ppm, the OH protons appeared as singlets at δ 12.31 (**5**) and 12.28 ppm (**6**), and the OCH_3 protons were detected as a 3-proton singlet at δ 3.85 ppm (**6**). Intermediates **7** and **8** were synthesized by o-alkylation of compounds **5** and **6**, respectively in the presence of a base. Initial experiments used cesium carbonate as a basic catalyst [22], but the yields were poor. A second base, K_2CO_3, and solvents (Acetonitrile, acetone, and DMF) were used to optimize the reaction [23]. At the end, DMF as a solvent and potassium carbonate as a catalyst produced good yields. In ^{1}H NMR analyses, aromatic protons were observed in a range of δ 7.79-5.73 ppm, while aliphatic protons were observed in a range of δ 4.30-2.46 ppm. Protons in compound **8** were slightly upfield than those in compound **7**. This is the result of the presence of an electron-releasing group (-OCH_3) on the 7th position of the coumarin nucleus. Both intermediates were also analysed by mass spectrometry. Through proton NMR spectroscopy, aromatic protons were observed in the range of δ 7.91-5.58 and 7.79-5.44 ppm, respectively, in intermediates **9** and **10**. The aliphatic protons of compounds **9** and **10** appear in the range of δ 4.31-1.32 ppm. A peak of the OH group was observed in intermediates **11** and **12** (δ 13.29 and 13.20 ppm, respectively). Benzimidazole intermediates (**13a-e**) were synthesized by reacting different 4-(un)substituted phenylenediamines with CNBr in the presence of aq. methanol. The aromatic protons of all these intermediates were noted at the δ values as reported in literature [24]. The chemical shifts were observed upfield or downfield according to the electronic nature of the functional group present at 5th position of benzimidazole nucleus in compounds **13a-e**. To synthesize the target compounds **14a-e** and **15a-e** intermediates **7** or **8**, and **13a-e** were coupled in the presence of K_2CO_3. IR, NMR (^{1}H NMR and ^{13}C NMR), and HRMS spectral analyses were performed. IR spectra showed a signal of NH in the range of 3300-3478 cm^{-1} in all the target compounds. Appearance of a single NH signal confirmed the coupling of compounds **13a-e** intermediates with coumarin intermediates (**7** and **8**). ^{1}H NMR spectra of the target compounds showed a singlet of one proton of NH in the range of δ 5.98-7.00 ppm for the target compounds **14a-e** and **15a-e**. Observed ^{13}C NMR signals of these target compounds were in the predicted ranges. HRMS analysis showed that the mass of all target compounds did not deviate by more than 0.005 Da. In order to synthesize compounds **16a-e** and **17a-e** (series with amide linkage), intermediates **11**/**12** were used. In situ conversion of these intermediates into the corresponding acid, chloride was performed by dissolving them in THF and adding an excess of $SOCl_2$. Thereafter, a solution of compounds **13a-e** in THF was added to the reaction mixture containing the acid chloride to form the corresponding amidic compound. In IR spectra of these compounds, amide stretch is observed above 3350 cm^{-1} for all the target compounds. Protons and carbons of the compounds were detected at δ values almost similar to the δ values of corresponding compounds **14a-e** and **15a-e**. Masses of these compounds were also found to deviate by not more than 0.005 Da during HRMS analysis. These spectral data confirmed that all target compounds are synthesized as designed.

3.2. Docking analysis

The docking scores of all the target compounds and the amino acid residues with which they are interacting in the active sites of both iNOS (1QW4) and neuronal nitric oxide synthase (nNOS) (1QW6) are summarized in Table 1.

In these studies, it was discovered that all designed compounds might act as selective anti-iNOS agents. According to literature reports on selective iNOS inhibitors, ligand H-bonding with Glu371 or Glu592 determines the ligand's selectivity toward iNOS or nNOS [25]. Interactions with synthesized compounds (docking score > -6 kcal/mol, Figure 3) indicate that their 2-aminobenzimidazole moiety occupies a pocket lined by Phe363, Asn364, Gly365, Trp366, Tyr367, and Met368, and interacts with Glu371 *via* a hydrogen bond and Heme900 *via* a co-ordinate bond.

3AR exhibited a docking score of -6.868 and -5.652 kcal/mol for 1QW4 and 1QW6, respectively, with RMSD (Root mean square deviation) < 1 Å. This observation suggested that both the target proteins are validated and suitable for predicting the binding affinity of test compounds. All compounds, except compounds **17d** and **17e**, exhibited docking scores equivalent to or greater than that of AG and L-NAME for 1QW4. Conversely, docking scores with 1QW6 were significantly lower than that of AG and L-NAME.

Coumarin nucleus in all test compounds occupies the other pocket(s) in binding site and affects the binding pattern, which may be responsible for the lower docking scores of all test compounds vis-a-vis 3AR. Nevertheless, the coumarin nucleus incurs hydrophobicity to the compounds, which is conducive to better oral absorption. This statement is in agreement with ADME properties of the compounds predicted by the *QIKPROP* module (Table 2). In addition to this, free-radical scavenging activity of coumarin nucleus is predicted to enhance therapeutic potential of the compounds [7,26].

Table 2. ADME properties * of 3AR, AG, NAME, and target compounds **14a-e**, **15a-e**,**16a-e** and **17a-e**.

Compound	Mol wt	Donor, HB	Acceptor, HB	QP, Po/w	QP, Logs	QPP, Caco	QPlog, BB	QPPMDCK	% Oral Abs.
14a	335.36	2	5.25	3.26	-4.88	550.49	-1.164	259.49	95.12
14b	349.38	2	5.25	3.60	-5.55	549.22	-1.225	258.85	100.00
14c	380.35	2	6.25	2.60	-5.14	68.78	-2.311	27.40	75.04
14d	369.80	2	5.25	3.76	-5.65	573.69	-1.010	667.01	100.00
14e	365.38	2	6.00	3.41	-5.28	549.60	-1.287	259.04	96.34
15a	365.38	2	6.00	3.36	-5.11	550.05	-1.258	259.27	96.91
15b	379.41	2	6.00	3.70	-5.79	548.85	-1.318	258.66	100.00
15c	410.38	2	7.00	2.73	-5.45	68.72	-2.448	27.38	75.78
15d	399.83	2	6.00	3.85	-5.72	641.06	-1.021	752.09	100.00
15e	395.41	2	6.75	3.50	-5.42	584.68	-1.336	276.96	96.81
16a	335.31	0	4.25	2.78	-3.67	183.76	-1.366	79.26	84.63
16b	349.34	0	4.25	3.45	-4.89	184.64	-1.502	79.68	86.07
16c	380.31	1	7.25	1.70	-4.45	31.49	-2.450	11.77	63.72
16d	369.76	0	4.25	3.64	-5.08	184.42	-1.323	196.22	87.06
16e	365.34	0	5.00	2.83	-3.80	183.69	-1.476	79.23	84.43
17a	365.34	0	5.00	2.78	-3.56	185.17	-1.414	79.92	84.80
17b	379.37	0	5.00	3.13	-4.33	183.35	-1.518	79.07	86.58
17c	410.34	1	8.00	1.77	-4.63	31.46	-2.571	11.76	51.34
17d	399.79	0	5.00	3.64	-5.04	183.76	-1.405	187.05	87.63
17e	395.37	0	5.75	2.88	-3.89	183.71	-1.573	79.24	85.40
3AR	201.26	4	4.00	1.29	-2.41	37.37	-1.80	18.02	62.67
AG	74.08	6	3.00	-2.64	1.92	16.56	-0.87	6.50	20.30
NAME	233.22	5	7.50	-1.92	-0.35	2.53	-2.40	0.85	22.90

* MW (Molecular weight in Da); Donor HB (hydrogen bond that can be donated, 0.0 to 6.0); Acceptor HB (hydrogen bond that can be accepted, 2.0 to 20.0); $QPLogP_{o/w}$ (Predicted octanol/water partition coefficient, –2.0 to 6.5); QPLogS (Predicted aqueous solubility, log S, S in mol/dm^3, -6.5 to -0.5); QPPCaco (Predicted apparent Caco-2 cell permeability, nm/sec, <25 poor and >500 good); QPLogBB (Predicted brain/blood partition coefficient,–3.0 to –1.2); QPPMDCK (Predicted apparent Madin-Darby canine kidney (MDCK) cell permeability, nm/sec, <25 poor and >500 great); %HOA (Predicted human oral absorption on 0 to 100% scale).

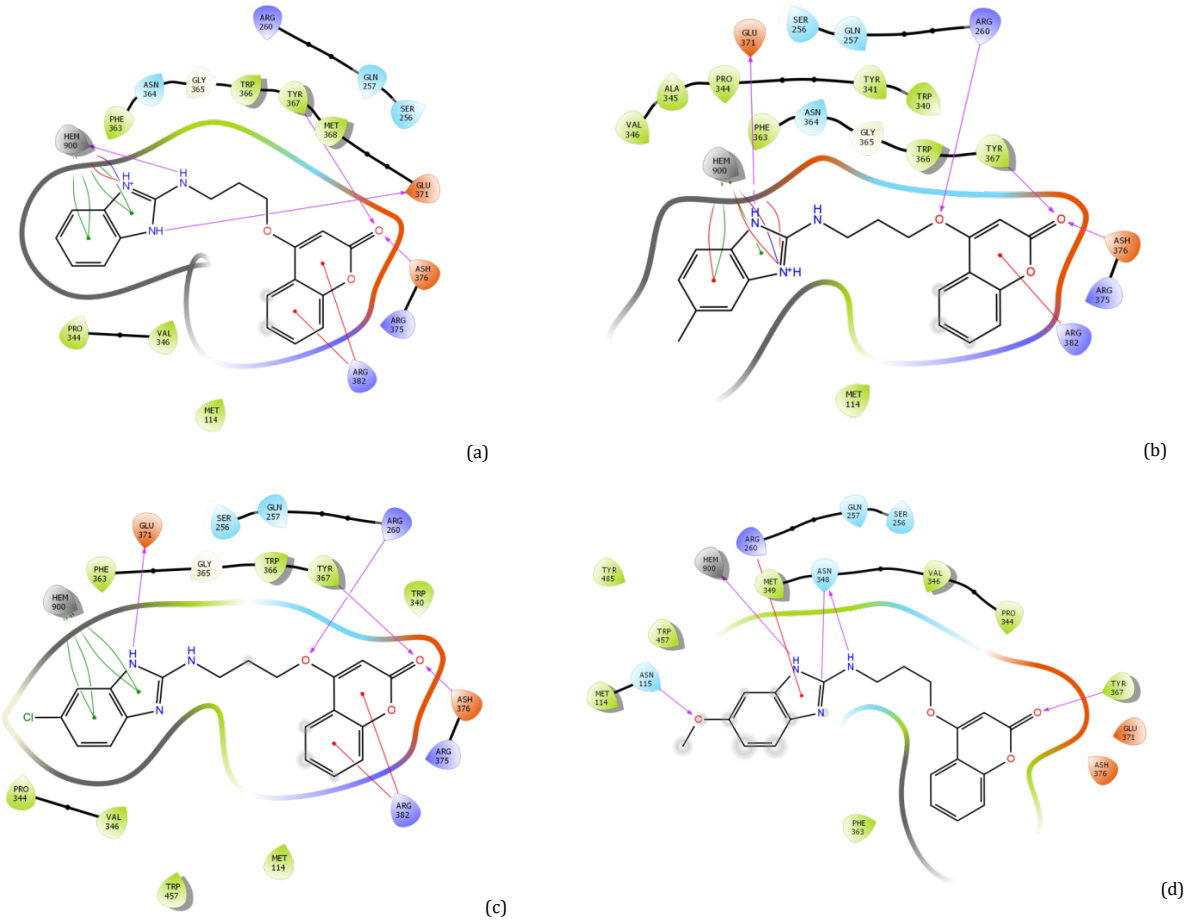

Figure 3. Interactions of compounds **14a** (a), **14b** (b), **14d** (c) and **14e** (d) with iNOS.

3.3. In vitro evaluations

3.3.1. Nitrite assay

All test compounds significantly inhibited the LPS-induced NO production. Compounds **14a**, **b**, **d**, and **e** possessed docking score almost equivalent to 3AR (Table 3), and also showed NO inhibition in the range of 70.33-78.62%. Among all the test compounds, compounds**14d** (78.62% inhibition) and **14e** (73.74% inhibition) are maximally active. It is even more active than L-NAME (68.27%), and almost equipotent to AG (81.41%).

Table 3. NO and iNOS inhibition of target compounds *.

Compound	% Inhibition of NO radical (10 µM conc.)	% Inhibition of iNOS (10 µM conc.)
14a	70.33±0.63[a]	67.23±0.34[a,b]
14b	71.17±0.96[a,b]	63.10±3.32[a,b]
14c	52.30±0.86[a,b]	40.42±0.40[a,b]
14d	78.62±0.91[a,b]	77.19±1.99[a,b]
14e	73.74±0.74[a,b]	73.97±0.24[a,b]
15a	62.58±0.69[a,b]	44.10±1.16[a,b]
15b	63.65±0.97[a,b]	44.06±0.19[a,b]
15c	55.25±0.86[a,b]	38.94±0.41[a,b]
15d	61.41±0.73[a,b]	43.97±0.24[a,b]
15e	67.26±0.81[a]	59.97±1.09[a,b]
16a	67.48±0.72[a]	54.11±2.15[a,b]
16b	57.61±0.89[a,b]	48.69±1.18[a,b]
16c	60.28±0.92[a]	41.52±0.31[a,b]
16d	61.49±0.90[a,b]	47.28±0.43[a,b]
16e	65.75±0.68[a]	61.47±0.43[a,b]
17a	63.62±0.99[a,b]	58.93±0.37[a,b]
17b	55.20±0.99[a,b]	50.56±0.37[a,b]
17c	50.79±0.97[a,b]	42.04±0.45[a,b]
17d	50.98±0.95[a,b]	41.21±0.37[a,b]
17e	31.83±0.96[a,b]	28.49±0.47[a,b]
AG	81.41±0.93[b]	91.81±0.28[b]
NAME	40.17±0.84[a]	83.26±0.62[a]

* One-way ANOVA (Analysis of variance) followed by Tukey test were used for statistical analysis of the data. [a,b] Values are statistically different from AG and NAME, respectively, at $p<0.05$.

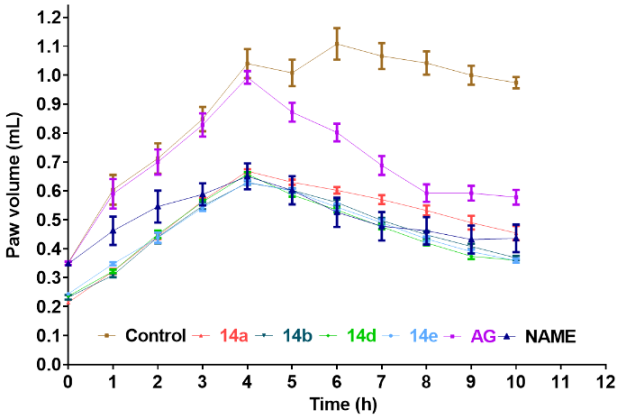

Figure 4. Paw volume is expressed as mean±S.E.M. from five rats and analysed by two-way ANOVA followed by Bonferroni's test.

3.3.2. iNOS assay

All test compounds were found to inhibit iNOS by 28.49 to 77.19%. However, the compounds are less active than both AG and NAME (91.82 and 87.58%, respectively) (Table 3).

Compounds **14a**, **b**, **d**, and **e** possessed the maximum docking score, and inhibited the NO production maximally, and also exhibited good iNOS inhibition. The maximum iNOS inhibitory activity was exhibited by compound **14d** (77.19% inhibition). These findings of biological activity evaluation are in concordance with the docking analysis of the compounds, indicating that 1QW4 can be used as a dependable target for the prediction of both NO production and iNOS inhibitory activities of new compounds.

3.4. In vivo evaluation

Based on the research studies reported in literature, *in vivo* activities of AG, NAME as well as test compounds **14a**, **b**, **d** and **e** were evaluated at a dose of 30 mg/kg.

3.4.1. Acute toxicity evaluation

The maximum inhibitory activity against NO production as well as iNOS was exhibited by compounds **14a**, **14b**, **14d** and **14e**. Hence, these were selected for evaluating *in vivo* activity. None of the selected test compounds showed any toxic effect in any animal at a dose level of 300 mg/kg. No mortality, no weight loss, and no abnormal behaviour were seen in any treated animal during 48 h of the study.

3.4.2. Anti-inflammatory activity

Inflammation response is usually divided into two phases, *i.e.,* early and delayed phases. The early phase inflammation can be attenuated by a non-selective iNOS inhibitor, and the delayed phase inflammation is characterized by an increased expression of iNOS. Therefore, a compound capable of inhibiting iNOS selectively would intervene in the delayed phase of inflammation [20]. All tested compounds were found to reduce the paw volume to significant levels and proved themselves as good anti-inflammatory agents. AG being a selective iNOS inhibitor starts inhibiting the inflammation in the late stage of inflammation whereas L-NAME which is a non-selective iNOS inhibitor starts suppressing the inflammation from an early-stage (up to 12.16% inhibition in initial 4 h). All tested compounds showed marked reduction in paw volume after 4 h of carrageenan administration along with some reduction in the early phase also (Figure 4).

Results of *in vivo* activity indicated that although these compounds are more potent inhibitors of iNOS than the reference drugs, but these may act as anti-inflammatory agents *via* acting on some other mechanisms also since they are showing inhibition in the early phase. Amongst all the test

compounds, compounds **14d** and **14e** were found to be the most potent rat paw edema inhibitors, and act as potential anti-inflammatory agents.

4. Conclusion

Compounds **14a-e**, **15a-e**, **16a-e**, and **17a-e** were synthesized by coupling of 5-(un)substituted-2-aminobenzimidazole with 7-(un)substituted coumarin through an alkoxy and amide linker (at 4th position of the coumarin nucleus). Docking analysis indicated that majority of the synthesized compounds are selective inhibitors of iNOS. The test compounds exhibited *in vitro* NO production and iNOS inhibitory activities complying with the findings of docking analysis. All compounds are found to be non-toxic. Compounds **14d** and **14e** were identified as the most potent inhibitors of NO production and iNOS enzyme. The reason for this may be their affinity for the iNOS enzyme, which explains their maximum docking score as well. The *in vivo* anti-inflammatory activity of the tested compounds indicated that all compounds possessed marked reduction in the late phase of inflammation along with some reduction in the initial phase also. In carrageenan-induced rat paw edema model compounds **14d** and **e** were found to be the potent anti-inflammatory agents. These findings support the hypothesis that the benzimidazole-coumarin hybrids can be potential drug candidates for multifactorial inflammatory disorders. Using structural activity relationships of these compounds, we have observed that the most potent iNOS inhibitors are found in the series in which the coumarin nucleus is unsubstituted. Additionally, five atoms separate two heteronuclear moieties. There is no relationship between the biological activity of the synthesized compounds and the electronic nature of the descriptor on the 5th position of the benzimidazole nucleus.

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Disclosure statement

Conflict of interest: The authors declare that they have no conflict of interest.
Ethical approval: All ethical guidelines have been adhered. This study was approved (Reference No. 107/GO/ReBi/S/99/CPCSEA/2019-7) by the institutional animal ethics committee of Punjabi University, Patiala (India).
Human and animal rights: No human subjects were used in this study. All the animal procedures were in accordance with the CPCSEA guidelines.
Sample availability: Samples of the compounds are available from the authors.

CRediT authorship contribution statement

Conceptualization: Richa Minhas, Yogita Bansal; Methodology: Richa Minhas, Yogita Bansal; Software: Raj Kumar; Validation: Richa Minhas; Formal Analysis: Richa Minhas; Investigation: Yogita Bansal; Resources: Punjabi University, Patiala, Department of Science and Technology (DST); Data Curation: Richa Minhas, Yogita Bansal; Writing - Original Draft: Richa Minhas; Writing - Review and Editing: Richa Minhas, Dr. Yogita Bansal; Visualization: Richa Minhas, Yogita Bansal; Funding acquisition: Richa Minhas; Supervision: Yogita Bansal; Project Administration: Yogita Bansal.

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