Analgesic, Anti-inflammatory and Antidiarrheal Properties of Synthesized Benzimidazole Derivatives via *In vivo* and *In silico* Methods

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ABSTRACT: The present communication reports the pharmacological investigation of a few synthesized benzimidazole derivatives by *in vivo* and *in silico* approaches. The *in vivo* peripheral analgesic, central analgesic, anti-inflammatory and antidiarrheal potentialities of the synthesized analogs were examined in animal model. The peripheral analgesic experiment revealed noticeable effect for compounds **1** and **2** with writhing inhibition values of 79.66 and 83.05 %, respectively at a dose of 50 mg/kg in comparison with standard aceclofenac (85.59 % writhing inhibition at 25 mg/kg dose). After 60 minutes of oral administration, compounds **1** and **2** demonstrated higher tail-flicking time of 2.96 ± 0.018 min and 2.83 ± 0.011 min at 50 mg/kg body weight dose compared to that of morphine (2.95 ± 0.13 min). Promising anti-inflammatory effects were observed for compounds **1** and **2** with 87.72 and 85.96 % paw edema inhibition in contrast to the standard aceclofenac showing 92.98 % reduction of rat paw edema. Concerning antidiarrheal proficiency, compounds **1** and **2** inhibited the frequency of defecation by 84.35 and 88.69 %, respectively at 50 mg/kg dose as compared to standard loperamide (85.22 % diarrheal inhibition at 25 mg/kg dose). Additionally, the synthesized analogs were subjected to molecular docking analysis against a variety of analgesic, anti-inflammatory and antidiarrheal target proteins namely cyclooxygenase-2, phospholipase A2, interleukin-1 receptor associated kinase-4 and μ-opioid receptor. Among the test compounds, **1** and **2** displayed outstanding binding affinities to all molecular targets which reconfirm their superior analgesic, anti-inflammatory and antidiarrheal attributes shown during *in vivo* analysis.

Key words: Benzimidazole, peripheral analgesic, central analgesic, antidiarrheal, anti-inflammatory, molecular docking.

INTRODUCTION

Pain sensation is a series of complex interplay between the central nervous system and the peripheral nervous system which is regulated by various excitatory and inhibitory neurotransmitters secreted in response to physical or physiological stimuli.[1] Although pain is a body defense mechanism essential for survival, persistent pain may lead to anxiety and depression, greatly reducing the quality of life. Again, a significant percentage of patients undergoing cancer chemotherapy report incompetent management of pain. NSAIDs and opioids, the backbone of current pain management are reported to possess minimal safety and efficiency and are overloaded with various side effects.[1,2] Inflammation can be described as a protective response of the body's immune system which is mounted against noxious stimuli including pathogen, toxin, stress, injury or infection. This well-orchestrated phenomenon involves a chain of molecular and cellular events and interactions acting in concert to eliminate the damaging pathogens or toxins. Unresolved inflammation may promote the progression of potentially threatening inflammatory disorders such as autoimmune disease, chronic inflammation, asthma, glomerulonephritis, reperfusion injury, inflammatory bowel disease,

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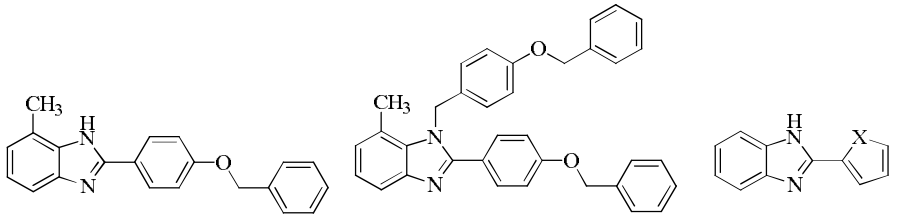
pelvic inflammatory disease, atherosclerosis, hypersensitivities, rheumatoid arthritis, hay fever etc. Although the past decades have witnessed the emergence of several analgesic and anti-inflammatory molecules to mitigate disease severity, many of them have demonstrated either hazardous effects or drug resistance.[1,2] Hence, the discovery of new analgesic and anti-inflammatory entities has generated huge appeal among organic and medicinal chemists. Diarrhea is a gastrointestinal disorder characterized by raised gastrointestinal motility and secretion and a reduction in the absorption of fluids and electrolytes. According to WHO reports updated in May 2017, childhood diarrheal disease has become a social burden with nearly 1.7 billion cases per year.[3] Again, diarrhea has raised enormous concern in developing countries like ours as it claims the lives of approximately 525 children below the age of 5 each year.[4] Conventional antidiarrheal drugs such as loperamide, opiates, diphenoxylate, astringents, adrenergic agonists etc. don't provide satisfactory therapeutic benefit.[3] Therefore, massive efforts need to be directed towards the design and development of novel analgesic, anti-inflammatory and antidiarrheal agents with enhanced clinical outcome and minimal detrimental effects.

Benzimidazole, one of the privileged heterocyclic motifs, has immensely appealed the medicinal chemists due to its ability to interact with diverse proteins and enzymes.[5] This promising scaffold has been reported to have myriads of therapeutic attributes including anti-cancer, antiviral, antimicrobial, anti-inflammatory, antidiarrheal, analgesic, anthelmintic, antihypertensive, anti-emetic and antiulcer.[6] Years of relentless efforts of researchers to design and optimize the core benzimidazole structure by introducing various substituents have yielded a large number of medicinal agents such as omeprazole, esomeprazole, lansoprazole, rabeprazole, pantoprazole as proton pump inhibitors; thiabendazole, albendazole, mebendazole, nocodazole as anthelmintic agents; enviradine as antiviral; bendamustine as anticancer; etonitazene, clonitazene as analgesics; pimobendam as antidiabetics; chloromidazole, carbendazim as antifungal agents; candesartan cilexitil, telmisartan as antihypertensives and a lot of lead compounds in different therapeutic areas.[7,8] The medicinal versatility of benzimidazole skeleton has prompted us to synthesize some benzimidazole derivatives and evaluate their pharmacological potentials.[9-13] In our earlier report, we have portrayed the synthetic route and investigated the ADMET properties and SARS-CoV-2 inhibitory activities of four synthesized benzimidazoles through molecular docking and molecular dynamic simulation study.[14] A great deal of articles over the past few years revealed how benzimidazole nucleus emerged as the "pharmacophore of choice" for designing and developing analgesic, anti-inflammatory and antidiarrheal drugs.[11-13,15,16] Hence, the present communication will pursue the evaluation of analgesic, anti-inflammatory and antidiarrheal properties of some recently synthesized benzimidazole derivatives[14] (Figure 1) by *in vivo* and *in silico* methods.



Compound **1** Compound **2** Compound **3**; X= O
Compound **4**; X= S

Figure 1. Synthesized mono- and di-substituted benzimidazoles. Compound **1**: 2–(4–(benzyloxy) phenyl)–4–methyl–1*H*–benzimidazole; Compound **2**: 1-(4-(benzyloxy) benzyl)-2-(4-(benzyloxy) phenyl)-4-methyl-1H-benzimidazole; Compound **3**: 2-(Furan-2-yl)-1H-benzimidazole; Compound **4**: 2-(Thiophen-2-yl)-1H-benzimidazole.

MATERIALS AND METHODS

Pharmacological investigations. All the synthesized benzimidazoles were screened *in vivo* for several pharmacological activities including peripheral, analgesic, anti-inflammatory and antidiarrheal activities in animal model. The study protocol was ethically approved by the Ethical Review Committee, Faculty of Biological Sciences (Ref. number. 208/Biol. Sci. December 20, 2022), University of Dhaka.

Experimental animals. After being raised for four to five weeks, Swiss-albino mice (*Mus musculus*) and Wistar rats (Rattus norvegicus) of both sexes, weighing roughly 25 to 30 grams and 100 to 150 grams, respectively, were taken from the Jahangirnagar University's animal house in Bangladesh. Prior to the experiment in the animal house of the Institute of Food and Nutrition, University of Dhaka, the animals were kept in conventional polypropylene cages for a period of seven days, with proper maintenance of a controlled environment (24 ± 2°C temperature; 60–70% relative humidity). The mice were given mouse food and water that had been prepared by the International Centre for Diarrheal Diseases and Research, Bangladesh (ICDDR,B). Because the animals are sensitive to changes in their surroundings, they were housed in the experiment site for a minimum of three to four days prior to the experiment's start. Twelve hours prior to the start of the trial, the animals were fed nothing but water. The minimal number of animals were used in the research, and all ethical guidelines and standards advised by the Swiss Academy of Medical Sciences were strictly adhered to.[17]

Assessment of peripheral analgesic activity. The synthesized products were screened for their peripheral anti-nociceptive property by acetic acid induced writhing method as reported by Koster *et al* (1959).[18] The experiment began with the random selection of fifty experimental animals which were subsequently divided into ten separate groups. 0.9% NaCl was administered to the group of negative control. Aceclofenac (25 mg/kg body weight) was used as the standard which was administered to the group of positive control.[19] The synthesized compounds **1**, **2**, **3** and **4** (25 mg/kg and 50 mg/kg doses) were administered to the remaining eight groups.[11-13,19,20] The rodents were handled with utmost care during the analgesic property screening. They didn't excrete wet feces out of fear during the administration of standard drugs and test samples. The mean writhing counts of the groups receiving standard drugs and the test compounds were compared against that shown by the negative control group to determine the percent inhibition of writhing responses.

Assessment of central analgesic activity. The tail-immersion method was utilized to evaluate the central analgesic efficiency of the synthesized products as described by Ben Bassat *et al* (1959)[21] and later the method went through modification by Grotto and Sulman (1967).[22] The experiment was carried out with ten groups of mice formed randomly. As the animals were exposed to heat stress, they tried to flee by flicking their tail aside in pain. The mice's tail-withdrawal time was noted down as a measure of central analgesia. The percentage of mice in each group who had their tails flicked longer than the negative control was determined. There will be more central analgesic activity in the group whose tail flicking time elongation percentage is higher.

Assessment of anti-inflammatory property. To test the synthetic derivatives' anti-inflammatory properties, Winter *et al* (1962)[23] employed the carrageenan-induced hind paw edema method in rats. Six groups of thirty Wister rats each, consisting of five animals each, were randomly assigned. Aceclofenac (100 mg/kg body weight) and 0.9% NaCl were given to the positive and negative control groups, respectively. Test chemicals were administered to the test groups at a dose of 100 mg/kg body weight.[11, 12, 20, 24-25] Following an oral administration of one hour, each rat in each group received an injection of 0.1 ml of 1% carrageenan solution into the subplantar region of its right hind paw, causing edema due to localized inflammation.

No defecation of liquid feces by the animals was observed during the experimental process. An estimate of the mean increase in paw volume was made and compared with the control group to assess the anti-inflammatory activity of the studied compounds.

Assessment of antidiarrheal property. The synthesized derivatives' antidiarrheal effect was evaluated employing a slightly altered method stated by Awouters *et al* (2011).[26] This study aimed at investigating the antidiarrheal potential of the test compounds on castor oil induced diarrhea. For this experiment, the animals were divided into a total of ten groups consisting of five mice each. The positive control animal group received loperamide, but those in the control group were only given normal saline (1% tween 80 in normal saline). To guarantee adequate absorption, a 30-minute break was allowed after oral sample administration. The mice in each group were then given 0.5 mL of castor oil. For four hours, the mice were under observation, and during that time, the frequency and consistency of their feces were noted. The observations of the experimental groups were compared with those of the control and standard groups in order to examine the antidiarrheal proficiency of the test samples.

Statistical analysis. Each experimental value was expressed as the mean ± standard error of the mean (SEM), and the data analysis was carried out using GraphPad software. A one-way analysis of Variance (ANOVA) and a subsequent Dunnett's test were performed. Statistical measurements were considered significant at $p< 0.05$.

Molecular docking analysis. The structure of target proteins cyclooxygenase-2 (COX-2) (PDB ID: 3LN1), phospholipase A2 (PLA2) (PDB ID: 4UY1), interleukin-1 receptor associated kinase-4 (IRAK-4) (PDB ID: 5KX7), μ-opioid receptor (PDB ID: 5C1M) was obtained from Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB).[27] The target macromolecules were prepared and energy minimized using pyMOL[28] and Swiss-PDB viewer.[29] Optimization of ligands (synthesized products and reference compounds) was performed in Avogadro[30] using MMFF94 force field. The docking process was validated by re-docking of the native ligands in the binding pockets of respective macromolecular targets. AutoDock Vina (version 1.1.2)[31] was used to execute docking of the synthesized drugs and the reference standards against some crucial target proteins. A grid box having the dimensions of 25.00, 25.00, 25.00 Å was generated surrounding the active site residues of the molecular targets during the analysis. Discovery Studio Client 2024 was used to analyze the interaction patterns of ligands with target macromolecules.

RESULT AND DISCUSSION

Assessment of peripheral analgesic activity. Peripheral anti-nociception activity of the synthesized compounds was evaluated by acetic acid induced writhing method. As depicted in table 1, compounds **1** and **2** manifested promising peripheral analgesic properties with writhing inhibition values of 72.03 and 72.88 %, respectively at a dose of 25 mg/kg which were comparable to that shown by standard aceclofenac (25 mg/kg; 85.59 %). At 50 mg/kg dose, compounds **1** and **2** demonstrated a writhing inhibition of 79.66 and 83.05 %, respectively. The remaining two compounds (**3** and **4**) displayed moderate peripheral analgesia at 50 mg/kg dose (% inhibition values 52.54 and 55.08 %, respectively) and mild analgesic activity at 25 mg/kg dose (% inhibition values 29.66 and 31.36 %, respectively). Statistical evaluation of the data guaranteed the significance of the analgesic potentiality of the test samples.

Assessment of central analgesic activity. The test compounds prolonged the reaction time in a dose dependent manner when they were subjected to screening of central analgesic property by tail immersion method. The data were summarized in table 2. Following 30 min, 60 min and 90 min of oral administration, superior central analgesic activity was manifested by compounds **1** (% elongation values 256.00, 289.47 and 338.46 %, respectively) and **2** (% elongation values 228.00, 272.37 and 311.54 %, respectively) at a dose of 50 mg/kg as compared to

the standard. Moderate central analgesia was observed for these two compounds at a lower dose of 25 mg/kg. The other synthesized analogs (compounds **3** and **4**) displayed mild to moderate central analgesic effect at both 25 mg/kg and 50 mg/kg body weight doses.

Table 1. Investigation of peripheral analgesic property of synthesized benzimidazole derivatives by acetic acid-induced writhing reaction in mice.

Sample ID	No. of writhing (Mean ± SEM)[a]	Writhing (%)	Inhibition (%)
CS	23.6 ± 0.75	100	-
SS_A	3.4 ± 0.51***	14.40	85.59
1 (d_1)	6.6 ± 0.75***	27.97	72.03
1 (d_2)	4.8 ± 0.80***	20.34	79.66
2 (d_1)	6.4 ± 0.81***	27.12	72.88
2 (d_2)	4.0 ± 0.55***	16.95	83.05
3 (d_1)	16.6 ± 0.93***	70.34	29.66
3 (d_2)	11.2 ± 1.28***	47.46	52.54
4 (d_1)	16.2 ± 0.86***	68.64	31.36
4 (d_2)	10.6 ± 1.03***	44.92	55.08

[a] The values indicate the Mean ± SEM for n = 5, with ***p < 0.001, **p < 0.01, and *p < 0.05 when compared to the control group using One-way ANOVA and Dunnett's test. 0.9% NaCl is control sample (CS), aceclofenac, 25 mg/kg b.w., is the standard sample (SS_A); (d_1) is the lower dose (25 mg/kg b.w.); (d_2) is the higher dose (50 mg/kg b.w.).

Table 2. Assessment of central analgesic property of synthesized benzimidazole derivatives by tail-immersion method.

Sample ID	Tail flicking time (Mean ± SEM)[a]				% Elongation time			
	0 min.	30 min.	60 min.	90 min.	0 min.	30 min.	60 min.	90 min.
CS	0.72 ± 0.059	0.75 ± 0.089	0.76 ± 0.071	0.78 ± 0.087	-	-	-	-
SS_M	1.54 ± 0.13***	3.25 ± 0.16***	2.95 ± 0.13***	2.13 ± 0.12***	113.89	333.33	288.16	173.08
1 (d_1)	1.52 ± 0.018***	1.98 ± 0.019***	2.11 ± 0.015***	2.35 ± 0.022***	111.11	164.00	177.63	201.28
1 (d_2)	1.62 ± 0.014***	2.67 ± 0.033***	2.96 ± 0.018***	3.42 ± 0.023***	125.00	256.00	289.47	338.46
2 (d_1)	1.43 ± 0.013***	1.79 ± 0.038***	2.41 ± 0.011***	1.96 ± 0.017***	98.61	138.67	217.11	151.28
2 (d_2)	1.55 ± 0.026***	2.46 ± 0.027***	2.83 ± 0.025***	3.21 ± 0.014***	115.28	228.00	272.37	311.54
3 (d_1)	1.02 ± 0.035**	1.47 ± 0.04***	1.46 ± 0.04***	1.28 ± 0.02***	41.67	96.00	92.11	64.10
3 (d_2)	1.48 ± 0.01***	2.33 ± 0.02***	2.20 ± 0.02***	1.64 ± 0.04***	105.56	210.67	189.47	110.26
4 (d_1)	0.83 ± 0.034	1.77 ± 0.02***	1.70 ± 0.02***	1.32 ± 0.014***	15.28	136.00	123.68	69.23
4 (d_2)	0.84 ± 0.051	2.08 ± 0.02***	1.88 ± 0.02***	1.39 ± 0.036***	16.67	177.33	147.37	78.21

[a] The values indicate the Mean ± SEM for n = 5, with ***p < 0.001, **p < 0.01, and *p < 0.05 when compared to the control group using One-way ANOVA and Dunnett's test. 0.9% NaCl is control sample (CS), morphine, 2 mg/kg b.w., is the standard sample (SS_M); (d_1) is the lower dose (25 mg/kg b.w.); (d_2) is the higher dose (50 mg/kg b.w.)

Assessment of anti-inflammatory activity. In the evaluation of anti-inflammatory activity by the carrageenan-induced hind paw edema method, compounds **1** and **2** were found to exhibit considerable anti-inflammatory activity with significant reduction in paw edema at 100 mg/kg dose compared to the control group. At the 1st, 2nd, 3rd and 4th hour, the % paw edema inhibition values of compounds **1** (55.32, 76.92, 83.33 and 87.72 %, respectively) and **2** (51.06, 67.31, 80.70 and 85.96 %, respectively) were highly comparable to those manifested by the standard drug (61.70, 80.77, 90.74 and 92.98 %, respectively). Mild to moderate anti-inflammatory effects were observed for the remaining two benzimidazoles (compounds **3** and **4**) in contrast to the standard. The mean paw volume (ml) and percentage of paw edema inhibition by the test compounds are portrayed in table 3.

Assessment of anti-diarrheal activity. For evaluating antidiarrheal property of the synthesized products, castor oil-induced diarrhea method was employed. Among the test samples, compounds **1** and **2** demonstrated encouraging antidiarrheal potentiality with 68.69 and 71.30 % inhibition of wet feces at a dose of 25 mg/kg when compared against standard loperamide with 85.22 % diarrheal inhibition. At 50 mg/kg dose, significantly enhanced inhibition of defecation was observed for compounds **1** and **2** with 84.35 and 88.69 % inhibitions, respectively. The other two derivatives (**3** and **4**) showed moderate inhibition of wet feces at both 25 mg/kg and 50 mg/kg body weight dose as epitomized in table 4. Among the synthesized compounds, disubstituted benzimidazole derivative (compound **2**) produced the strongest antidiarrheal effect which is consistent with our previous findings using different disubstituted analogs.[13]

Table 3. Screening of anti-inflammatory property of benzimidazole derivatives in rat model.

Sample ID	Paw volume (ml) (Mean ± SEM)[a]				% Paw edema inhibition			
	1st hr	2nd hr	3rd hr	4th hr	1st hr	2nd hr	3rd hr	4th hr
CS	0.76 ± 0.027	0.81 ± 0.025	0.83 ± 0.019	0.86 ± 0.023	-	-	-	-
SS_A	0.48 ± 0.020***	0.40 ± 0.014***	0.35 ± 0.013***	0.34 ± 0.014***	61.70	80.77	90.74	92.98
1	0.48 ± 0.020***	0.39 ± 0.009***	0.36 ± 0.011***	0.034 ± 0.016***	55.32	76.92	83.33	87.72
2	0.52 ± 0.017***	0.46 ± 0.015***	0.40 ± 0.029***	0.37 ± 0.018***	51.06	67.31	80.70	85.96
3	0.56 ± 0.024***	0.46 ± 0.024***	0.42 ± 0.023***	0.44 ± 0.012***	27.66	53.85	62.96	61.40
4	0.65 ± 0.017**	0.51 ± 0.019***	0.47 ± 0.018***	0.49 ± 0.024***	21.28	55.77	64.81	63.16

[a] The values indicate the Mean ± SEM for n = 5, with ***p < 0.001, **p < 0.01, and *p < 0.05 when compared to the control group using One-way ANOVA and Dunnett's test. 0.9% NaCl is control sample (CS), aceclofenac, 100 mg/kg b.w., is the standard sample (SS_A); (d_1) is the lower dose (25 mg/kg b.w.); (d_2) is the higher dose (50 mg/kg b.w.).

Table 4. Evaluation of anti-diarrheal activity of the synthesized benzimidazoles on castor oil induced diarrhea.

Sample ID	Number of defecated pellets of 5 mice in 4 h (Mean ± SEM)[a]	% Inhibition of defecation
CS	23.00 ± 0.71	-
SS	3.40 ± 0.24***	85.22
1 (d_1)	7.20 ± 0.58***	68.69
1 (d_2)	3.60 ± 0.51***	84.35
2 (d_1)	6.60 ± 0.51***	71.30
2 (d_2)	2.60 ± 0.24***	88.69
3 (d_1)	7.60 ± 0.51***	66.96
3 (d_2)	5.80 ± 0.58***	74.78
4 (d_1)	11.40 ± 0.51***	50.43
4 (d_2)	7.40 ± 0.40***	67.83

[a] The values indicate the Mean ± SEM for n = 5, with ***p < 0.001, **p < 0.01 and *p < 0.05 when compared to the control group using One-way ANOVA and Dunnett's test. 0.9% NaCl is control sample (CS), loperamide, 25 mg/kg b.w., is the standard sample (SS); (d_1) is the lower dose (25 mg/kg b.w.); (d_2) is the higher dose (50 mg/kg b.w.).

Molecular docking analysis. During the pharmacological investigation, the synthesized compounds demonstrated a varying degree of analgesic, anti-inflammatory and antidiarrheal activities. A molecular docking study was conducted to test hypotheses regarding the biological targets of the synthesized products and to corroborate the findings from biological research involving several proteins implicated in pain, inflammation and diarrhea. To confirm the *in vivo* analgesic and anti-inflammatory efficacy displayed by the test compounds, we have chosen and docked our

compounds against interleukin-1 receptor associated kinase-4 (IRAK-4)[20], cyclooxygenase-2 (COX-2)[32] and phospholipase A2 (PLA-2)[33]. Additionally, μ-opioid receptor was chosen as a target macromolecule for the confirmation of antidiarrheal activity shown by the compounds during *in vivo* analysis.[34,35] The binding energy values of the compounds against all macromolecular target proteins are summarized in table 5. Among the synthesized ligands, compounds **1** and **2** displayed higher binding affinities toward all the molecular targets. Compound **1** demonstrated stronger binding against COX-2, PLA-2, IRAK-4 and μ-opioid receptor with binding affinity values of -8.6, -9.2, -9.4 and -9.4 kcal/mol respectively in contrast to that shown by the reference ligand (-9.2, -7.6, -7.8 and -9.1 kcal/mol respectively). Compound **2** manifested excellent binding towards COX-2, PLA-2, IRAK-4 and μ-opioid receptor with the binding energy scores of -8.9, -9.8, -9.3 and -10.5 kcal/mol respectively which were quite lower than those displayed by the reference compound.

Table 5. Binding affinities of standard drugs and the synthesized compounds against some analgesic, anti-inflammatory and antidiarrheal target proteins.

Target proteins	Binding affinity (kcal/mol)				
	Compound **1**	Compound **2**	Compound **3**	Compound **4**	Reference compound[a]
COX-2 (PDB ID: 3LN1)	-8.6	-8.9	-7.8	-7.9	-9.2
PLA2 (PDB ID: 4UY1)	-9.2	-9.8	-7.0	-7.0	-7.6
IRAK-4 (PDB ID: 5KX7)	-9.4	-9.3	-6.7	-6.8	-7.8
μ-opioid receptor (PDB ID-5C1M)	-9.4	-10.5	-7.4	-7.2	-9.1

[a]Aceclofenac was used as the reference compound against COX-2, PLA2 and IRAK-4 while loperamide was considered as the reference ligand against μ-opioid receptor.

Consequently, interactions between compounds **1** and **2** and the afore-mentioned targets may account for the compounds' notable analgesic, anti-inflammatory, and antidiarrheal properties as observed in the *in vivo* tests. Compounds **1** and **2** appeared to have robust interactions with the target proteins' active site residues in figures 2-5, exhibiting a pattern resembling that of the reference ligand. The two benzimidazole derivatives (compounds **1** and **2**)'s docking scores and pattern of contacts support the findings of the *in vivo* screening, which validated the compounds' analgesic, anti-inflammatory and antidiarrheal properties. Again, the moderate binding affinities displayed by the other two benzimidazole analogs (compounds **3** and **4**) towards the molecular targets account for their mild to moderate analgesic, anti-inflammatory and antidiarrheal attributes manifested during *in vivo* analysis. The higher binding affinity of compounds **1** and **2** might be attributed to the presence of benzyl moiety which interacted with the respective targets via pi-pi stacking and hydrophobic forces (Figures 2-5).

Compounds **1** and **2** demonstrated stronger binding interactions towards cyclooxygenase-2 (COX-2)[32] and phospholipase A2 (PLA-2)[33] (two crucial target proteins in cyclooxygenase pathway) in the molecular docking study. Therefore, the analgesic and anti-inflammatory actions of these benzimidazole analogs might have evolved from their interference in cyclooxygenase pathway leading to the blockade of pain sensation and inflammatory response.

Again, μ-opioid receptors located in the brain and spinal cord have been highly implicated in the generation of analgesia.[36] In addition, large number of μ-opioid receptors expressed on the neurons of the enteric nervous system are responsible for governing majority of gastrointestinal effects.[37] The remarkable binding affinities of compounds **1** and **2** to μ-opioid receptor indicate that these ligands may possess μ-opioid receptors agonistic action resulting in antidiarrheal response. However, opioid agonist drugs may generate constipation as a side effect by hindering the propulsive motility patterns in the gastrointestinal tract. To perceive whether the

antidiarrheal action exhibited by our synthesized benzimidazoles **1** and **2** might be a side effect caused by their μ-opioid receptor agonistic activity, further extensive investigations need to be carried out.

Our future work will also include molecular dynamic simulation of the protein-ligand complexes with the best docking scores to gather information on the conformational stability and conformational flexibility of the complexes.

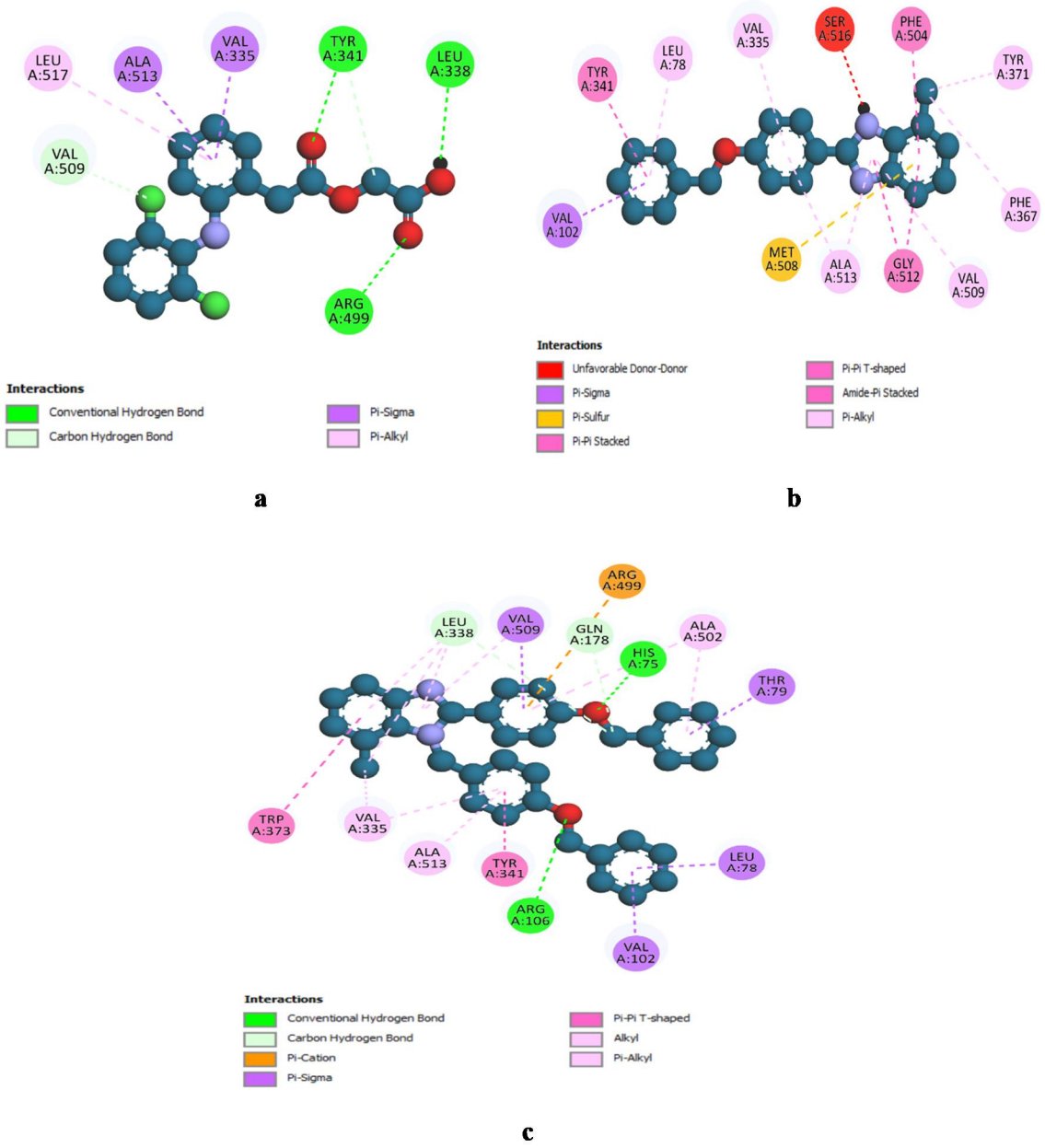


Figure 2. 2D interaction of the reference compound aceclofenac (**a**), compound **1** (**b**) and compound **2** (**c**) with COX-2 (PDB ID- 3LN1).

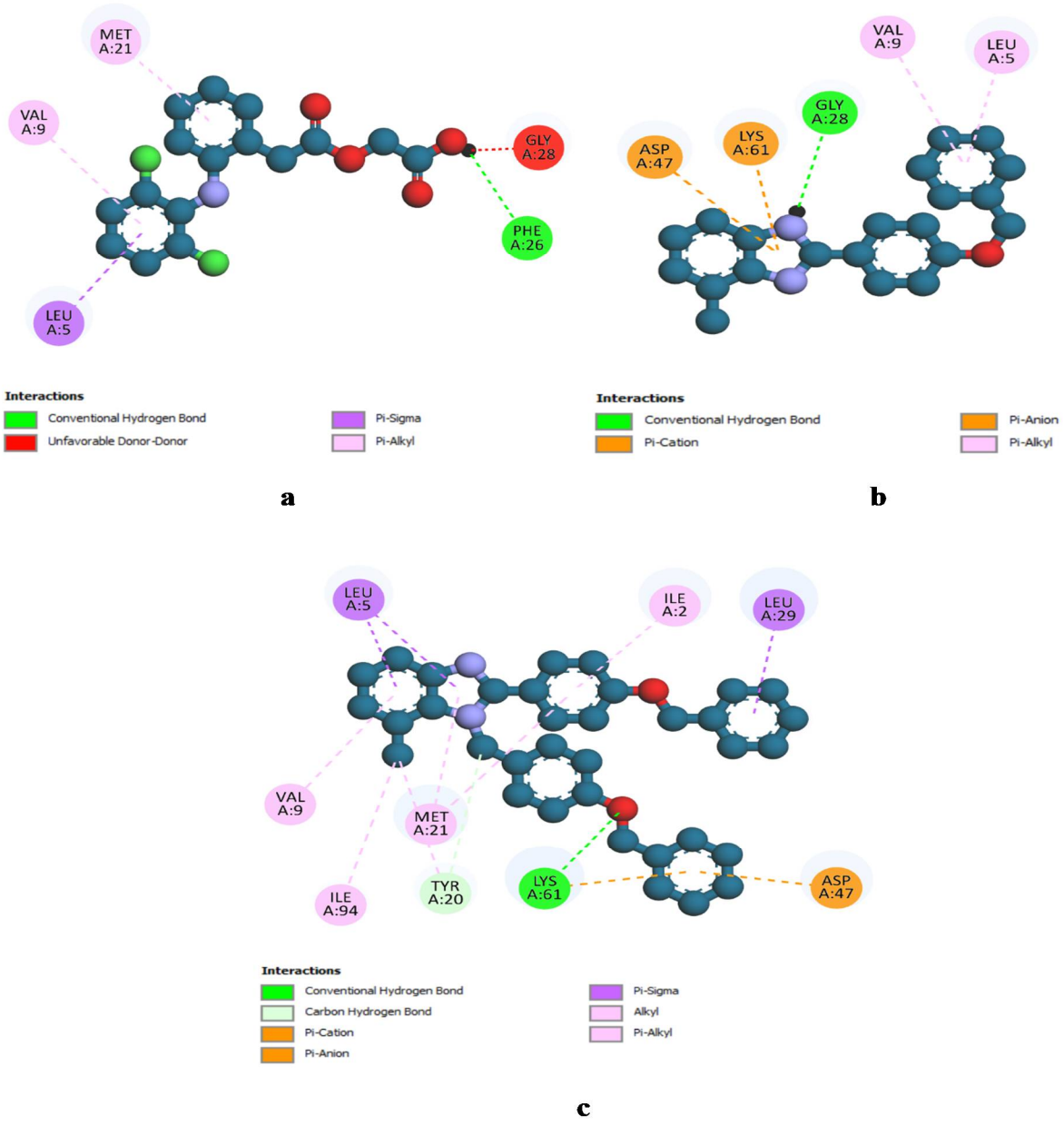


Figure 3. 2D interaction of the reference compound aceclofenac (**a**), compound **1** (**b**) and compound **2** (**c**) with PLA-2 (PDB ID- 4UY1)

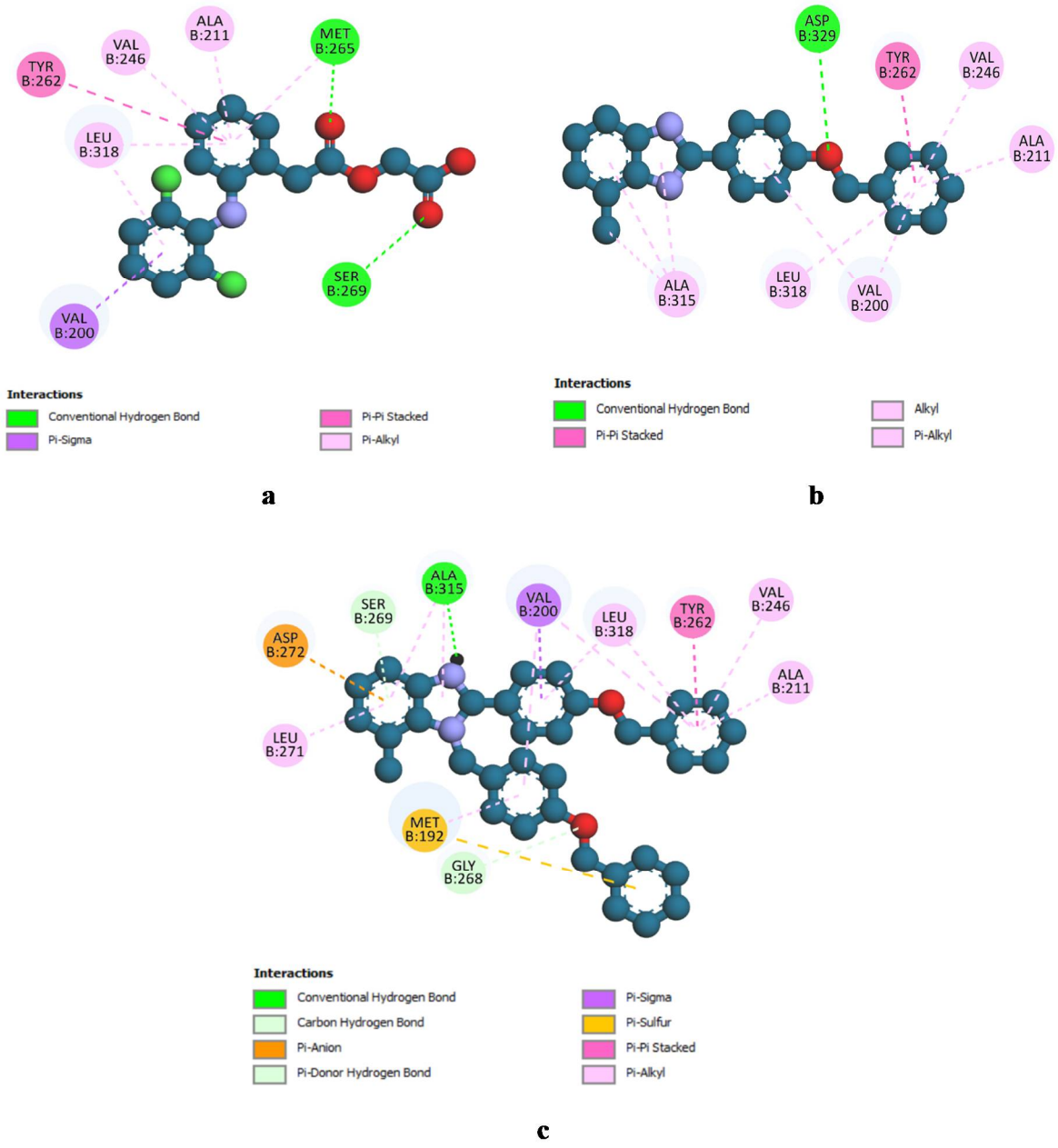


Figure 4. 2D interaction of the reference compound aceclofenac (**a**), compound **1** (**b**) and compound **2** (**c**) with IRAK-4 (PDB ID- 5KX7)

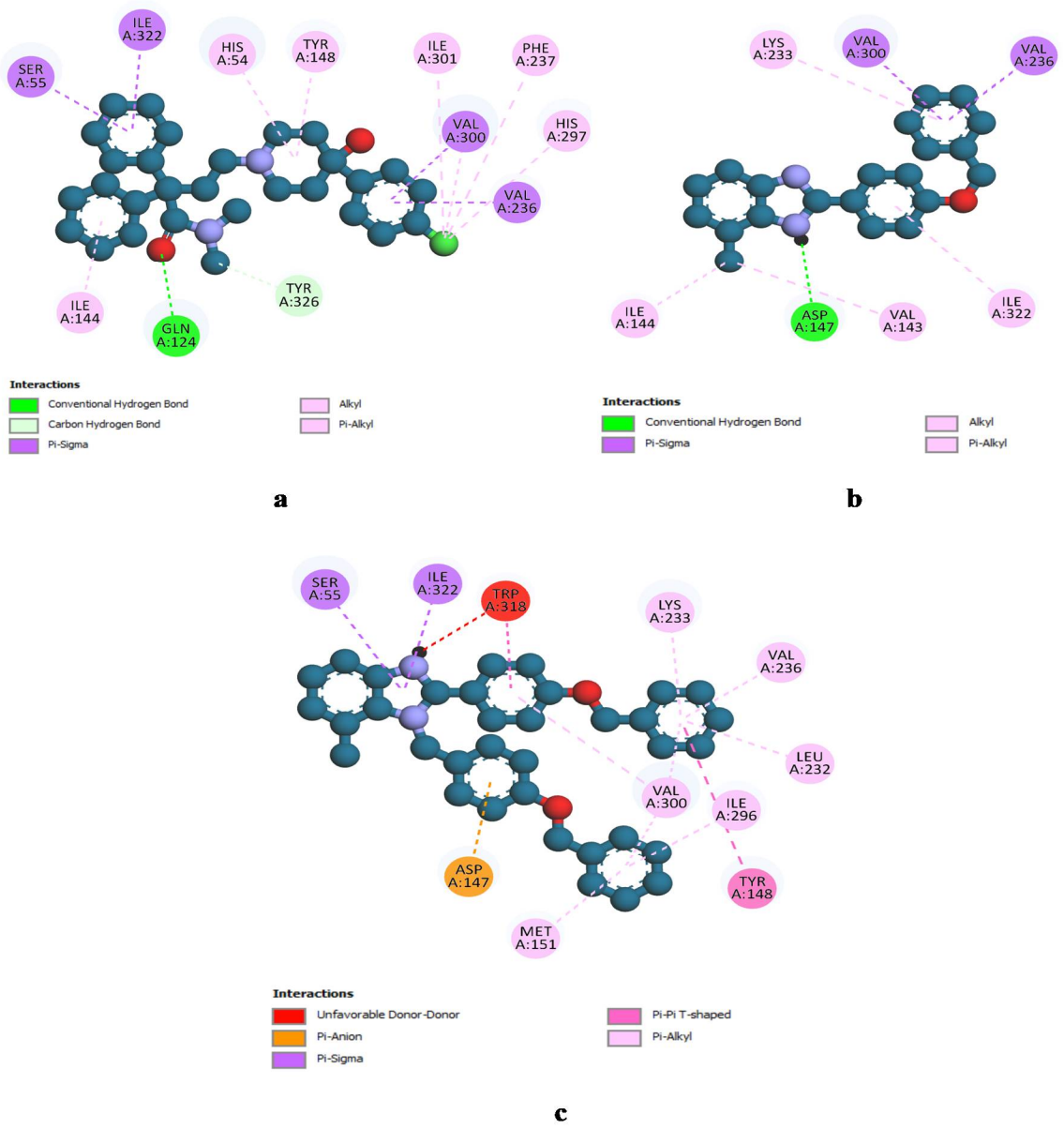


Figure 5. 2D interaction of the reference compound loperamide (a), compound **1** (b) and compound **2** (c) with μ-Opioid Receptor (PDB ID- 5C1M)

CONCLUSION

Four benzimidazole derivatives recently synthesized in our laboratory have been evaluated through some screening procedures for specific bio-properties such as peripheral analgesia, central analgesia, anti-inflammatory and antidiarrheal properties. Among the synthesized analogs, compounds **1** and **2** displayed strong analgesic, anti-inflammatory and antidiarrheal actions in animal model. The results from the molecular docking study also showed conformity to the *in vivo* data. However, these preliminary findings necessitate extensive and more vigorous investigations which can pave the way for newer and better analgesic, anti-inflammatory and antidiarrheal agents in the days to come.

AUTHOR CONTRIBUTIONS

Mokaddas Flora Ananta: Writing – original draft, methodology, investigation, formal analysis, data curation. Poushali Saha: Writing – review & editing, resources, formal analysis. Sabiha Enam Spriha: Writing – review & editing, methodology, formal analysis. S.M. Abdur Rahman: Conceptualization, writing – review & editing, supervision, project administration. A.K. Azad Chowdhury: Supervision, conceptualization, writing – review & editing. All authors have read cautiously and agreed to the published version of the manuscript.

REFERENCES

1. Azim, T., Wasim, M., Akhtar, M. S. and Akram, I. 2021. An *in vivo* evaluation of anti-inflammatory, analgesic and anti-pyretic activities of newly synthesized 1, 2, 4 triazole derivatives. *BMC Complement. Med. Ther.* **21**, 304. DOI:10.1186/s12906-021-03485-x.
2. Jin, Q., Zhao, Y. L., Liu, Y. P., Zhang, R. S., Zhu, P. F. and Zhao, L. Q. 2022. Anti-inflammatory and analgesic monoterpenoid indole alkaloids of *Kopsia officinalis*. *J. Ethnopharmacol.* **285**, 114848. DOI:10.1016/j.jep.2021.114848.
3. Li, Z., Li, J., Zhang, F., Zhu, N., Sha, Z. and Li, D. 2020. Antidiarrheal effect of *Sechang-Zhixie-San* on acute diarrhea mice and network pharmacology deciphering its characteristics and potential mechanisms. *Evid. Based Complement. Alternat. Med.* **2020**, 1-16.
4. Chen, M.R., Zhao, J., Fu, S.F., Yu, J.Q., Zhang, X., Zhang, Q.Y. and Zhou, Z.H. 2019. Clinical practice of Chinese medicine navel therapy for chronic diarrhea: a literature review. *J. Gastroenterol. Hepatol.* **34**, 643-649.
5. Taha, M., Ismail, N. H., Imran, S., Mohamad, M. H., Wadood, A. and Rahim, F. 2016. Synthesis, α-glucosidase inhibitory, cytotoxicity and docking studies of 2-aryl-7-methylbenzimidazoles. *Bioorg. Chem.* **65**, 100-109.
6. Bristy, S. R., Hossain, M. J., Khandakar. M. U., Faruque, M. R. I., Osman, H. and Rahman, S. M. A. 2021. A comprehensive account on recent progress in pharmacological activities of benzimidazole derivatives. *Front. Pharmacol.* **12**, 765807. DOI:10.3389/fphar.2021.762807.
7. Vasava, M. S., Bhoi, M. N., Rathwa, S. K., Jethava, D. J., Acharya, P.T. and Patel, D.B. 2020. Benzimidazole: a milestone in the field of medicinal chemistry. *Mini-Rev. Med. Chem.* **20**, 532-565.
8. Bansal, Y. and Silakari, O. 2012. The therapeutic journey of benzimidazoles: a review. *Bioorg. Med. Chem.* **20**, 6208-6236.
9. Podder, S. K., Saqueeb, N. and Rahman, S. M. A. 2016. Synthesis and biological evaluation of 2-methyl-1H benzimidazole and 1-H benzimidazole-2-yl-methanol. *Dhaka Univ. J. Pharm. Sci.* **15**, 83-87.
10. Bristy, S. R., Saha, P., Mahmud, Z. A. and Rahman, S. M. A. 2020. Synthesis and evaluation of analgesic and antioxidant activities of substituted benzimidazole derivatives. *Dhaka Univ. J. Pharm. Sci.* **19**, 37-46.
11. Raka, S. C., Rahman, A., Hussain, F. and Rahman, S. M. A. 2022. Synthesis, characterization and *in vitro*, *in vivo*, *in silico* biological evaluations of substituted benzimidazole derivatives. *Saudi J. Biol. Sci.* **29**, 239-250.
12. Saha, P., Brishty, S. R. and Rahman, S. A. 2021. Pharmacological screening of substituted benzimidazole derivatives. *Dhaka Univ. J. Pharm. Sci.* **20**, 95-102.
13. Saha, P., Brishti, S. R. and Rahman, S. M. 2020. Synthesis and evaluation of disubstituted benzimidazole derivatives as potential analgesic and antidiarrheal agents. *Indian J. Pharm. Sci.* **82**, 39-46.
14. Ananta, M. F., Saha, P., Rahman, F. I., Spriha, S. E., Chowdhury, A. K. A. and Rahman, S. M. A. 2024. Design, synthesis and computational study of benzimidazole derivatives as potential anti-SARS-CoV-2 agents. *J. Mol. Struct.* **1306**. DOI:10.1016/j.molstruc.2024.137940.
15. Gaba, M., Singh, S. and Mohan, C. 2014. Benzimidazole: an emerging scaffold for analgesic and anti-inflammatory agents. *Eur. J. Med. Chem.* **76**, 494-505.
16. Achar, K. C. S., Hosamani, K. M. and Seetharamareddy, H. R. 2010. *In-vivo* analgesic and anti-inflammatory activities of newly synthesized benzimidazole derivatives. *Eur. J. Med. Chem.* **45**, 2048-2054.
17. Charan, J. and Kantharia, N. D. 2013. How to calculate sample size in animal studies? *J. Pharmacol. Pharmacother.* **4**, 303-306.
18. Koster, R., Anderson, M. and De Beer, E. J. 1959. Acetic acid for analgesic screening. *Fed. Proc.* **18**, 412-417.
19. Hussain, F., Rahman, F. I., Saha, P., Mikami, A., Osawa, T., Obika, S. and Rahman, S. M. A. 2022. Synthesis of sugar and nucleoside analogs and evaluation of their anticancer and analgesic potentials. *Molecules* **27**, 3499. DOI:10.3390/molecules27113499.
20. Spriha, S. E., Rahman, F. I. and Rahman, S. M. 2021. Synthesis, *in vivo* and *in silico* analgesic and anti-inflammatory studies of α-D-ribofuranose derivatives. *Saudi Pharm. J.* **29**, 981-991.
21. Ben-Bassat, J., Peretz, E. and Sulman, F.G. 1959. Analgesimetry and ranking of analgesic drugs by the receptacle method. *Arch. Int. Pharmacodyn. Ther.* **122**, 434-447.
22. Grotto, M. and Sulman, F. G. 1967. Modified receptacle method for animal analgesimetry. *Arch. Int. Pharmacodyn. Ther.* **165**, 152-159.
23. Winter, C., Risley, E. and Nuss, G. 1962. Carrageenin-induced edema in hind paw of the rat as an assay for antiinflammatory drugs. *Exp. Biol. Med.* **111**, 544-547.
24. Ramesh, B. and Sumana, T. 2010. Synthesis and anti-inflammatory activity of pyrazolines. *J. Chem.* **7**, 514-516.

25. Rahman, S. M. A., Ahmed, T., Asaduzzaman, M., Rahman, F. I. and Chowdhury, A. K. A. 2024. Broad spectrum antimicrobial, anti-inflammatory and pheripheral analgesic activities of heteroaryl nitazoxanide analogs. *Egypt. Pharm. J.* **23**, 348-358.
26. Awouters, F., Niemegeers, C. J. E., Lenaerts, F. M. and Janssen, P. A. J. 2011. Delay of castor oil diarrhoea in rats: a new way to evaluate inhibitors of prostaglandin biosynthesis. *J. Pharm. Pharmacol.* **30**, 41-45.
27. Berman, H. M., Battistuz, T., Bhat, T. N., Bluhm, W. F., Bourne, P. E. and Burkhardt, K. 2002. The Protein Data Bank. *Acta Crystallogr. D Biol. Crystallogr.* **58**, 899-907.
28. Seeliger, D. and de Groot, B. L. 2002. Ligand docking and binding site analysis with PyMOL and Autodock/Vina. *J. Comput. Aided Mol. Des.* **24**, 417-422.
29. Guex, N. and Peitsch, M. C. 1997. SWISS-model and the Swiss-Pdb Viewer: An environment for comparative protein modeling. *Electrophoresis.* **18**, 2714-2723.
30. Hanwell, M. D., Curtis, D. E., Lonie, D. C., Vandermeersch, T., Zurek, E. and Hutchison, G. R. 2012. Avogadro: an advanced semantic chemical editor, visualization, and analysis platform. *J. Cheminform.* **4**, 17.
31. Trott, O. and Olson, A. J. 2010. AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J. Comput. Chem.* **31**, 455-461.
32. Orlando, B. J. and Malkowski, M. G. 2016. Substrate selective inhibition of cyclooxygenase-2 by fenamic acid derivatives is dependent on peroxide tone. *J. Biol. Chem.* **291**, 15069-15081.
33. Chen, H., Knerr, L., Akerud, T., Hallberg, K., Öster, L., Rohman, M., Österlund, K., Beisel, H. G., Olsson, T., Brengdhal, J., Sandmark, J. and Bodin, C. 2014. Discovery of a novel pyrazole series of group X secreted phospholipase A2 inhibitor (sPLA2X) via fragment based virtual screening. *Biorg. Med. Chem. Lett.* **24**, 5251-5255.
34. Koehl, A., Hu, H., Maeda, S., Zhang, Y., Qu, Q., Paggi, J. M., Latorraca, N. R., Hilger, D., Dawson, R., Matile, H., Schertler, G. F. X., Granier, S., Weis, W. I., Dror, R. O., Manglik, A., Skiniotis, G. and Kobilka, B. K. 2018. Structure of the μ-opioid receptor- Gi protein complex. *Nature* **558**, 547-552.
35. Paul, S. R., Saha, P., Rahman, F. I., Dhar, S. and Rahman, S. M. A. 2022. Preferential synthesis and pharmacological evaluation of mono and di-substituted benzimidazole derivatives. *ChemistrySelect* **7**, e202201710. DOI:10.1002/slct.202201710.
36. Fürst, S., Zádori, Z.S., Zádor, F., Király, K., Balogh, M., László, S.B., Hutka, B., Mohammadzadeh, A., Calabrese, C., Galambos, A.R., Riba, P., Romualdi, P., Benyhe, S., Timár, J., Schmidhammer, H., Spetea, M. and Al-Khrasani, M. 2020. On the role of peripheral sensory and gut Mu opioid receptors: peripheral analgesia and tolerance. *Molecules*. **25**, 2473. DOI:10.3390/molecules25112473.
37. Holzer, P. 2009. Opioid receptors in the gastrointestinal tract. *Regul. Pept.* **155**, 11–17.