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Revealing analgesic and anxiolytic potentials of synthetic benzimidazole analogues: An *in-vivo* and *in-silico* study

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Abstract: Certain drugs have potential to affect and alter individual's behavior. On the other hand, pain is a complex phenomenon with various treatment options; analgesic medicines are the primary source. Therefore, this study was based on examining some of the benzimidazole analogues for their analgesic as well as behavioral potential following Tail immersion test and Open field test respectively. In addition, molecular docking was performed to find the interaction of these compounds with the active site using AutoDock Vina which was further visualized through Discovery Studio Visualizer. It was seen that the cyano-methyl benzimidazole derivatives (CMB1-CMB3) showed relief in pain as compared to benzimidazole derivatives (BI1-BI3), CMB2 demonstrated highly potent analgesic effect. Likewise, all structures except BI1 displayed increase locomotion during open field test and can be offered as anxiolytic compounds. Almost all derivatives showed improve binding energies for the tested proteins where the high analgesic action of CMB2 might be correlated to its high binding affinity and interaction at μOR. It was also noticed that all structures except BI showed possible binding interaction with $GABA_A$ receptor and hence possessed anxiolytic like potential. Thus, this study offered benzimidazole analogues for further drug development of analgesic and anxiolytic like compounds.

Keywords: Benzimidazole, tail flick method, open field test, molecular docking.

INTRODUCTION

Anxiety reflects the thought about the future threat and could lead to physical, behavioral, emotional, cognitive and psychological symptoms (disorders) (Craske *et al.*, 2011) where in particular, the behavioral symptoms of anxiety reflect what individual do (or don't do) while being anxious. Anxiolytic compounds such as benzodiazepine etc. are the drugs used to treat anxiety, however all displayed undesirable side effects. So there is a growing need to discover new anxiolytic compounds with less side effects and improve efficacy than the currently existing drugs (Sirakanyan *et al.*, 2021).

Pain is a discomfort linked to real or potential tissue injury with sensory, emotive, cognitive and social elements and could be acute or chronic in nature (Williams and Craig, 2016). Both the non-steroidal anti-inflammatory drugs (NSAIDs) and synthetic non-opioid analgesics have been used to relief mild to moderate pain but the associated side effects warned their use in clinical practice (Zobdeh *et al.*, 2022). This also demands new better, safer and effective analgesic compounds.

Benzimidazole is one of most common heterocyclic, aromatic compound that constitutes a central skeleton of six-membered benzene ring joined to a five-membered Nitrogen based imidazole ring. The molecule gained wide attention in medicinal chemistry when this core structure was thought to possess an analogy with purine-like structures and also identified as a degradation product of vitamin B_{12} (Kamanna, 2019). Afterwards, benzimidazole remained a molecule of interest in research, where Mebendazole and Omeprazole emerged out as a result of this elbow grease and have been successfully used in clinical practice.

It has been observed from the literature that the synthetic derivatives of benzimidazole have analgesic (Cheretaev *et al.*, 2018; Brishty *et al.*, 2020; Nagesh *et al.*, 2022; Nardi *et al.*, 2023) and anxiolytic potentials (Dokuparthi *et al.*, 2018; Maltsev *et al.*, 2020; Spasov *et al.*, 2020). Keeping this in view, herein we tested our previously synthesized six benzimidazole (BI) and Cyanomethyl benzimdazole (CMB) derivatives (Asghar *et al.*, 2018) (table 1) for their potential in reducing pain and anxiety. In addition, molecular docking of these compounds was also carried out against target macromolecules such as Cyclooxygenase-1 (COX-1), Cyclooxygenase-2 (COX-2), μ-type opioid receptor (μOR) and Gamma-aminobutyric acid type A ($GABA_A$) receptor to explore the binding patterns for analgesic and anxiolytic like activities respectively.

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MATERIALS AND METHODS

Preparation of test solution

Eight mice about 6-7 weeks old, weighing 20-25g were taken for test and control group each to perform both the analgesic and anxiolytic activities. Test solutions of BI, BI1- BI3 and CMB, CMB1-CMB3 were prepared in 10% DMSO and water for injection (WFI) respectively. Activities were performed at the test dose of 50mg/kg body weight, where the test solutions were administered intraperitoneally. Control groups' mice were given only solvents. The study was approved by Board of Advanced Studies & Research, University of Karachi (BASR/01183/Pharm.).

In-vivo Analgesic activity

Tail immersion technique was used for screening of analgesic activity (Aziz *et al.,* 2019) . During this test, 1/3 of the mice tail was dipped in warm water (51˚C) and immersion time was monitored till mice withdrew its tail. Analgesic effects of the test compounds were noticed as of Tail Flick Latency Difference (TFLD) and Possible maximal analgesia percentages (PMAP) at 30, 60, 90, 120, 150 and 180 minutes of the study (Naseem *et al.*, 2023).

In-vivo anxiolytic activity

Open field test (OFT) (Asghar *et al.*, 2022) was performed to determine the anxiolytic behavior of the synthesized analogues. In this test, animal was allowed to move freely in a test apparatus (base of 25 equal squares surrounded by four sided walls as 76 x 76 cm^2 with walls 42 cm high). The response was examined by counting the number of squares travelled by mice in a specified time (5minutes); increase number indicated anxiolytic-like effects (Gadotti and Zamponi, 2019).

In-silico study (Molecular docking)

Software and programs

Python 3.9 language was obtained from www.python.com. Molecular graphics laboratory (MGL) tools (1.5.7) and AutoDock 4.2.6 and AutoDock Vina was obtained from The Scripps Research Institute. Biovia Discovery Studio Visualizer 2021 was downloaded from Dassault Systèmes.

Preparation of ligands

Chemdraw Ultra 8.0.3 was used for drawing 2D structures of the synthetic analogues (ligands). Chem3D Ultra version 8.0.3 was used for energy minimization and preparation of Ligand PDBs. AutoDock tool 1.5.7 (ADT) was first used to prepare ligand for combinations with non-polar hydrogens, additions of Gasteiger changes and rotatable bond. Later ADT was used for the conversion of respective PDB files to PDBQT formats (Trott and Olson, 2010; Afriza *et al.*, 2018).

Preparation of proteins

The three dimensional structures of the selected enzymes COX-1 (PDB ID: 1EQG) (Spriha *et al.*, 2021), COX-2 (PDB ID: 4COX) (James *et al.*, 2020), μOR (PDB ID: 5C1M) (Aljohani *et al.*, 2022) $GABA_A$ (PDB ID: 6X3X) (Kim *et al.*, 2020) were downloaded from the RCSB protein data bank (http://www.rcsb.org). Biovia Discovery Studio Visualizer 2021 was used to determine the binding pocket (SBD-site-sphere) around the native ligand and hence the configuration file. Later the ligands, water molecules (if present) and additional chains were removed, polar hydrogen were also added to save file as PDB. After that, ADT was used to assign Kollman and Gasteiger charges to protein structures to be prepared in formats required for AutoDock Vina and then saved as PDBQT format (Jaghoori *et al.*, 2016).

Molecular docking

The molecular interactions between ligands and selected enzymes were studied using AutoDock Vina program. During the *in-silico* study, each ligand was docked individually, where ligand was in a pliable state when interacting with macromolecules under fixed conditions. The size for SBD-site spheres of three proteins 1EQG, 4COX and 6X3X was set at 15x×15y×15z respectively. In case of 6X3X, x, y and z centers were adjusted at 89.473050, 125.991700 and 105.598150 dimensions respectively. The x, y and z centers in 4COX were set at 24.848720, 22.294640 and 18.546780 respectively. Similarly for SBD-site sphere of 1EQG, the x, y and z centers were set at 26.828200, 33.484333 and 200.848000 respectively. On the other hand, a size of 25x, 25y and 25z and center dimensions of 1.285656x, 16.447875y and -59.114250z were set for SBD-site sphere of enzyme 5C1M. The docking parameters were validated by re-docking the native ligand at the binding site of the respective proteins. The configuration files were operated by incorporating notepad to run AutoDock Vina. Binding affinity (ΔG) was used to report the ligand-protein interaction in kcal/mol as computed by AutoDock Vina scoring function (Trott and Olson, 2010; Afriza *et al.*, 2018). The investigation of the docking results was carried out using Discovery Studio on the basis of most energetically favorable conformation of each ligand (analogue) having RMSD value <3).

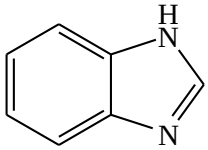
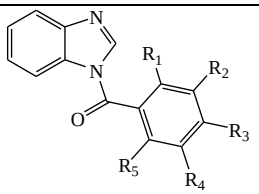
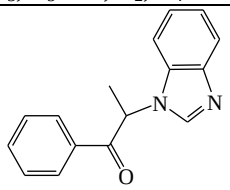
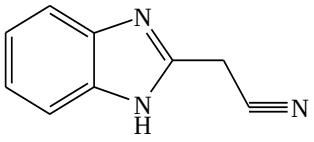
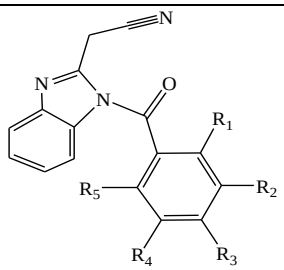
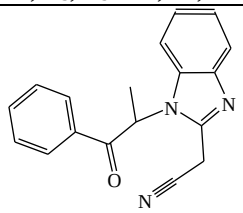
STATISTICAL ANALYSIS

Student's t-test was used to analyze the data statistically using SPSS software (version 20.0) and the values were presented as Mean ± SEM (standard error mean). Data was considered significant or highly significant on the basis of t-test values at $P<0.05$ and $P<0.01$ respectively

RESULTS

Analgesic drugs are extensively used to treat moderate to severe acute and chronic pain. Nonetheless, these medications have several potential disadvantages since they produce analgesic tolerance, which frequently leads

Table 1: Benzimidazole and its synthetic derivatives

Compound	Derivatives	IUPAC NAME
Benzimidazole (BI) 	 BI1: R_1, R_2, R_4, R_5 = H, R_3 = CH_3 BI2: R_1, R_3, R_5 = H, R_2, R_4= NO_2	BI1: (1H-benzo[d]imidazol-1-yl)(p-tolyl)methanone BI2: (1H-benzo[d]imidazol-1-yl)(3,5-dinitrophenyl)methanone
	 BI3	BI3:2-(1H-benzo[d]imidazol-1-yl)-1-phenylpropan-1-one
Cyanomethyl benzimidazole (CMB) 	 CMB1 = R_1, R_2, R4, R_5 = H, R_3 = CH_3 CMB2 = R_1, R_3, R_5 = H, R_2, R_4= NO_2	CMB1: 2-(1-(4- methyl phenyl) carboxyl)-H-1,3-Benzodiazol-2-yl)acetonitrile CMB2: 2-(1-(3,5-dinitrobenzoyl)-1H-benzo[d]imidazol-2-yl)acetonitrile
	 CMB3	CMB3: 2-(1-(1-oxo-1-phenylpropan-2-yl)-1Hbenzo[d]imidazol-2-yl)acetonitrile

to patient noncompliance. Anxiety disorders are the most common mental illnesses and they are a major source of impairment. Despite this, during the last 5-10 years, there has been significantly less recent research on innovative drug therapies for anxiety disorders.

The trajectory of present and future research in analgesic and anti-anxiety treatments implies that further investigation of these pathways is needed with larger-scale studies of potential medicines with favorable findings from smaller trials. Therefore in this study, our previously synthesized benzimidazole analogues were checked for their potential as analgesic and anxiolytic compounds following tail immersion and open field test.

Analgesic activity

The results of tail immersion test and PMAP were presented in table 2 and fig. 1 respectively. According to the study, parent BI itself provided relief in pain at 30min that continued up to 180min. Its corresponding PMAP was 1.08 at 30min which increased up to 2.99 till 180min. The outcomes of its synthetic analogues were variable where analgesic effect of BI1 was slightly reduced. The anti-nociception property was retained in both BI2 and BI3 as seen by early onset and longer duration of action.

In BI2 and BI3, the analgesic effects reduced after achieving the maximum relief at first hour with PMAP values of 4.1 & 2.9 respectively more than their parent.

The second parent, CMB lacked anti-nociceptive potential as obvious by un-noticeable analgesic effects during the study. However its analogues CMB1-CMB3 exhibited this potential at different levels. Among these, CMB2 showed greatest strength of analgesia with early onset at 30min and maximum effect at 150min with PMAP values 3 and 10.7 respectively. CMB1 and CMB3 produced maximum relief from pain at 90min and 60min of the study with PMAP of 3.5 and 5.1 respectively.

Table 2: Tail immersion activity of benzimidazole and its analogues

Compound (50mg/Kg)	Control	TFLD (mean ± SEM)					
		30min.	60min.	90min.	120min.	150min.	180min.
BI	10% DMSO	1.06±0.35	1.97±0.48	2.03±0.48	2.25±0.52	2.30±0.53	2.29±0.52
BI1	10% DMSO	0.82±0.14	1.37±0.08	1.36±0.06*	0.93±0.14*	0.65±0.12	0.50±0.07
BI2	10% DMSO	1.83±0.13	2.47±0.18*	2.42±0.18**	1.57±0.10	1.50±0.07*	1.50±0.07
BI3	10% DMSO	2.01±0.31**	2.3±0.40	2.12±0.26	1.62±0.22*	1.31±0.13*	1.03±0.11
CMB	10% DMSO	0.58±0.05	0.62±0.06	0.66±0.06	0.68±0.04	0.67±0.03	0.55±0.05
CMB1	WFI	2.73±0.16	3.80±0.24	3.60±0.26	3.01±0.23	2.10±0.25**	2.0±0.25**
CMB2	WFI	2.50±0.16	4.40±0.32	5.00±0.49**	6.62±1.26	6.89±0.69**	2.58±0.4
CMB3	WFI	2.42±0.14	3.55±0.25	3.25±0.36**	2.35±0.12	1.38±0.15*	1.00±0.05

TFLD7 = Post-drug TFL – Pre-drug TFL, n/group = 08, SEM = Standard Error Mean
Significant, or highly significant difference by student's t-test *p<0.05, **p<0.01 as compared to control

Table 3: Open field activity of Benzimidazole and its analogues

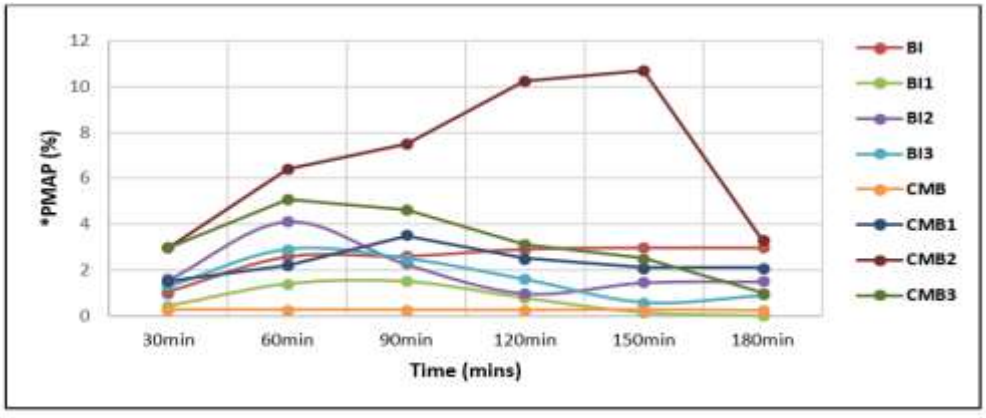
Compound (50mg/Kg)	Control	Number of square crossing by control group	Number of square crossing by test compounds
BI	10% DMSO	85.25± 3.52	106.87± 5.71
BI1	10% DMSO	135.5± 7.5	104.6± 5.5
BI2	10% DMSO	85.20± 1.21	116.3± 3.56**
BI3	10% DMSO	52.2± 2.92	109.3± 5.13**
CMB	10% DMSO	85.25 ±3.52	89.37± 4.38
CMB1	WFI	104.3± 3.57	132.2± 4.49*
CMB2	WFI	96.04± 6.67	124.62± 4.38**
CMB3	WFI	99.40± 2.91	103.25± 2.95**

n/group = 08, SEM = Standard Error Mean
Significant or highly significant difference by student's t-test *p<0.05, **p<0.01 as compared to control

Table 4: Binding energies as computed by AutoDock Vina

Protein (PDB CODE)	Binding Affinity (kcal/mol)								
	*Standard	BI	BI1	BI2	BI3	CMB	CMB1	CMB2	CMB3
1EQG	-7.8	-5.6	-7.6	-7.9	-9.0	-6.7	-5.7	-5.6	-6.7
4COX	-8.4	-5.3	-7.4	-7.7	-8.1	-6.5	-7.5	-6.6	-7.1
5C1M	-9.5	-4.6	-8.2	-8.4	-7.6	-6.0	-8.0	-8.9	-8.0
6X3X	-8.1	-6.0	-8.1	-10.0	-9.1	-7.3	-8.7	-9.6	-9.5

*Standards are Ibuprofen (IBP), Indomethacin (IMN), Morphinan agonist (BU72) and Diazepam (DZP) for proteins 1EQG, 4COX, 5C1M and 6X3X respectively



*PMAP = Latency after administration – Latency before administration x 100
60 – Latency before administration

Fig. 1: Proximal maximum analgesia percentages of benzimidazoles and its analogues

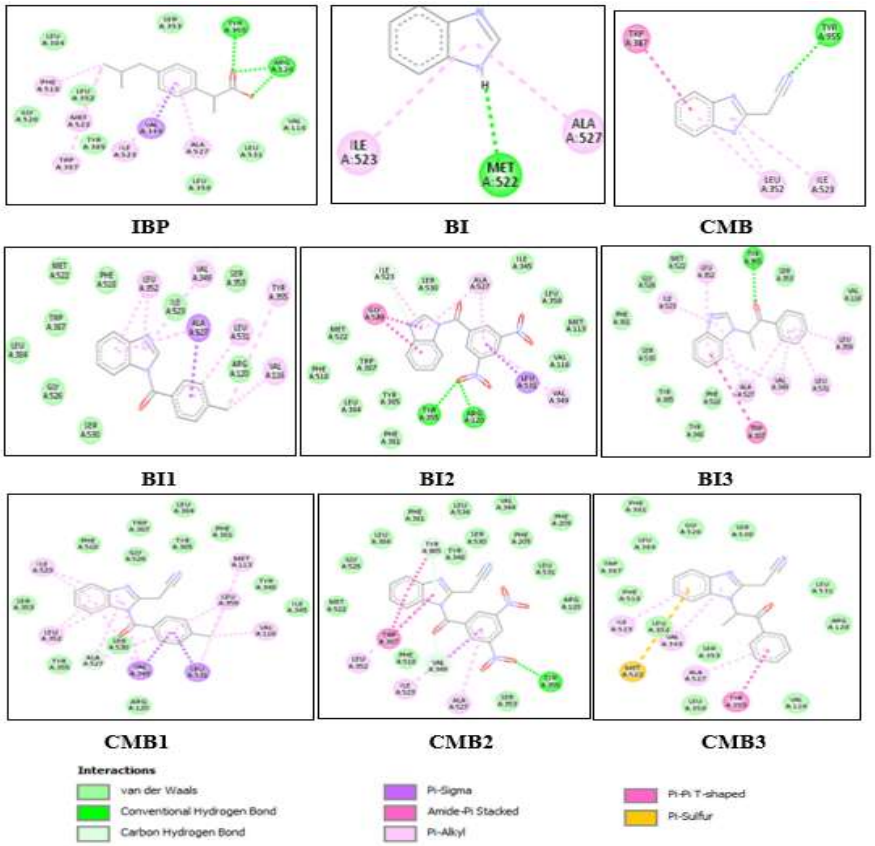


Fig. 2: Interaction of 1EQG with benzimidazole derivatives

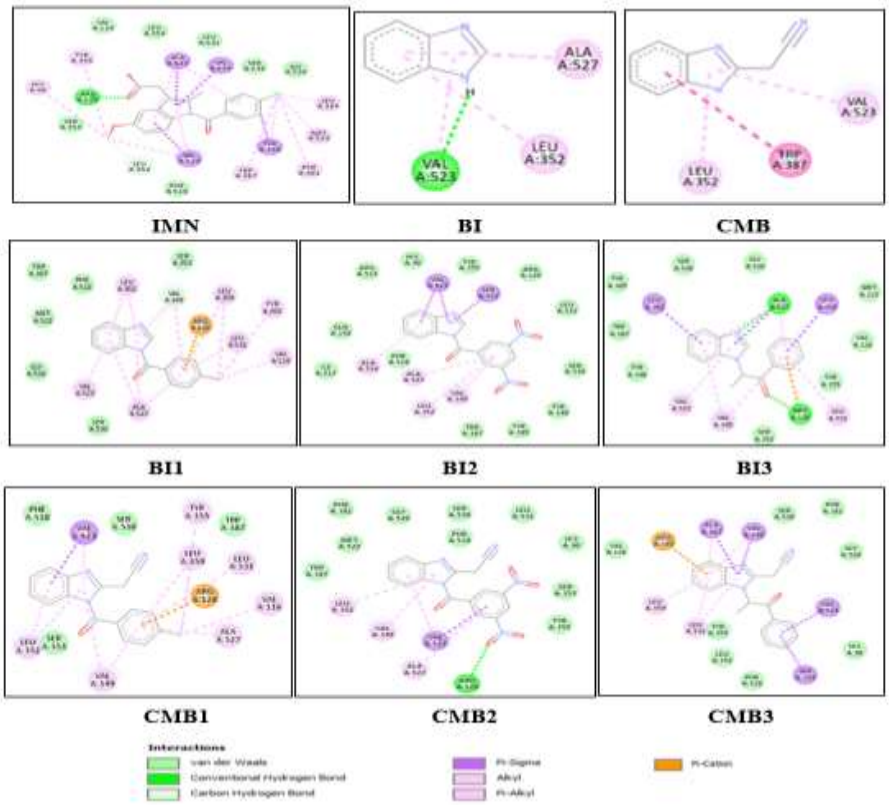


Fig. 3: Interaction of 4COX with benzimidazole derivatives

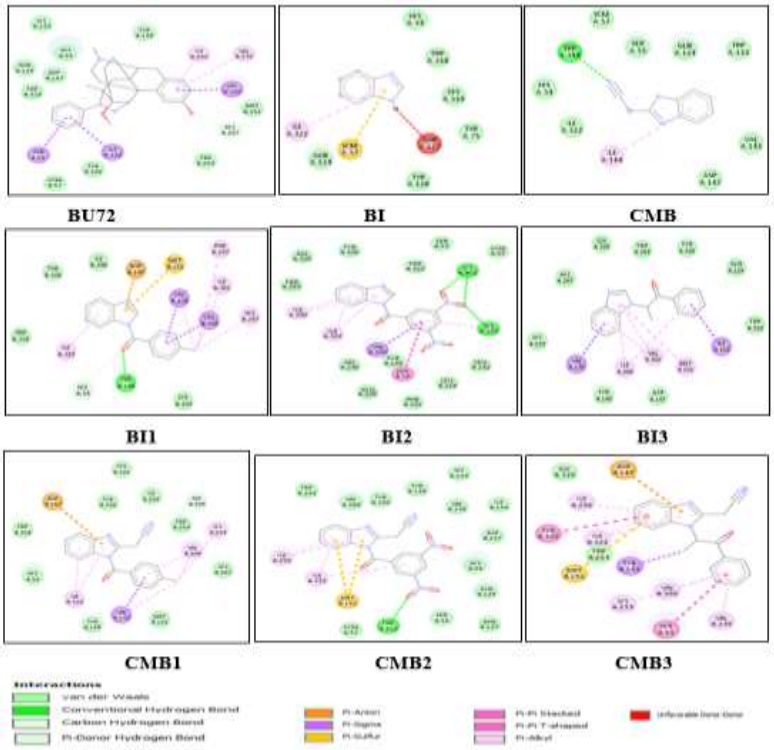


Fig. 4: Interaction of 5C1M with Benzimidazole derivatives

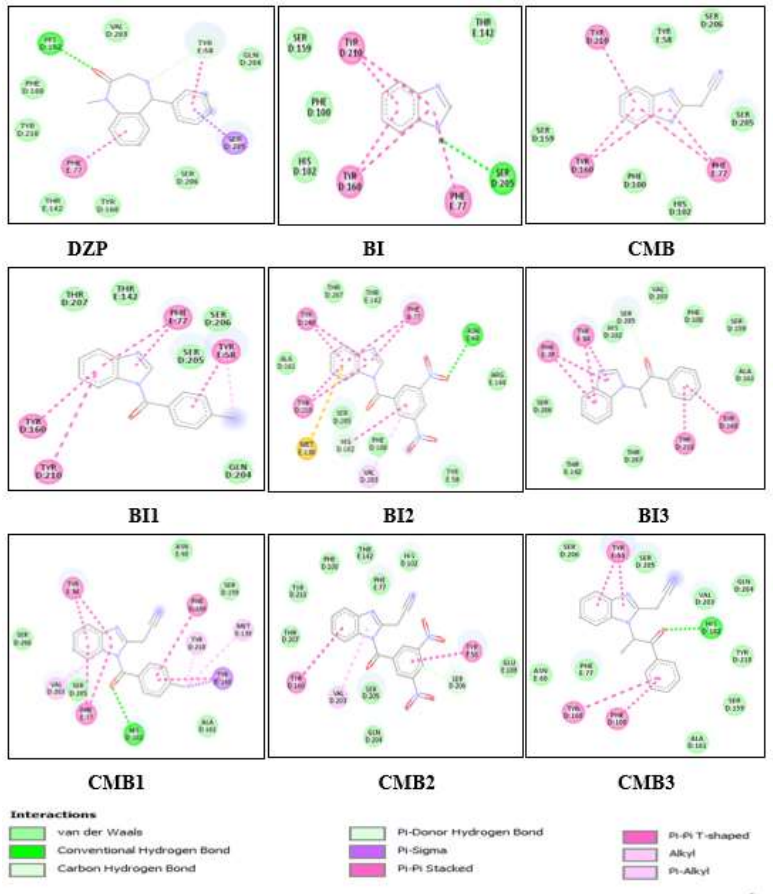


Fig. 5: Interaction of 6X3X with Benzimidazole derivatives

Anxiolytic activity

The findings of the OFT were reported in table 3. According to the study, BI showed more number of square crossings (106.87±5.71) as compared to control in a time period of 5minutes. Its two derivative BI2 and BI3 also crossed more squares (116.3±3.56** and 109.3± 5.13**) in a same time and the results were highly significant. Insignificant increase was noticed in CMB treated mice but its analogues CMB1-CMB3 displayed significant hyperactivity as observed by increase square crossing in 5 minutes. Their number of score crossings in OFT were monitored as 132.2±4.49*, 124.62±4.38** and 103.25±2.95** respectively.

Molecular docking

The binding energies as computed by AutoDock Vina were shown in table 4 for the proteins 1EQG, 4COX, 5C1M and 6X3X respectively. Their two-dimensional (2D) interaction plots as pictured by Discovery Studio were presented in fig. 2-5.

It was seen form the table 4 that binding affinities of all the BI derivatives were more as compare to the parent for all the tested proteins. The same was observed in CMB derivatives except for protein 1EQG. Among the analogues, compound BI3 has shown better binding affinity against 1EQG with minimum ΔG value of -9.0 kcal/mol as compare to the -7.8 kcal/mol of standard IBP. Similarly, the lower ΔG values -10.2, -9.1, -8.7, -9.6 and -9.5 kcal/mol in derivatives BI2, BI3, CMB1, CMB2 and CMB3 respectively also indicated improved interactions within the binding pocket of 6X3X as compare to -8.1 kcal/mol of standard DZP. In case of 4COX and 5C1M, none of the analogue gave better binding affinity when compared to the standards IMN and BU72 respectively.

DISCUSSION

Analgesics are the compounds primarily used to control pain. These might influence CNS and/or peripheral nervous system in diverse manner. As mentioned earlier, benzimidazole had shown some analgesic activity which was reduced by the introduction of 4-methyl benzoyl group as in case of BI1. The same can be explained for BI2 and BI3 where the attachment of 3,5-dinitro benzoyl and propiophenone in the parent molecule resulted in less impressive anti-nociceptive profile respectively. For cyanomethyl benzimidazole and its derivatives, we could say that the addition of substituted benzoyl and propiophenyl moieties in CMB1-CMB3 were responsible to induce analgesic property to primarily inactive CMB nucleus.

Change in locomotion and exploration indicates the state of mind and so comes under anxiety behavior. According to the study, benzimidazole treated mice showed less fear. This effect retained as highly significant in its derivatives BI2 and BI3 by the introduction of 3,5-dinitro benzoyl and propiophenone respectively. Despite this, presence of *para*-methyl benzoyl moiety in BI1 produced anxiety. The ability of cyanomethyl benzimidazole to reduce fear was increased in all its derivatives CMB1-CMB3 having 4-methyl, 3,5-dinitro benzoyl and propiophenone respectively.

Molecule docking has grown to be a significant step in the drug discovery process in which small molecules are docked into macromolecular structures in order to score their complimentary values at the binding sites. It is a busy field of research as the most appealing tool in structure-based drug design, lead optimization and biochemical routes. Correct posture and affinity prediction are two essential components of an effective docking experiment (Pinzi and Rastelli, 2019; Saikia and Bordoloi, 2019; Stanzione *et al.*, 2021). In this study, we performed molecular docking to predict the binding pattern of the compounds for analgesic and anxiolytic activities. For this, first we docked our compounds to evaluate their role in pain management via COX (I and II) enzymes and mu opioid receptors. Interesting to note that the ΔG values for CMB in 1EQG, 4COX and 5CQ1M were better than the BI, but the molecule did not show analgesia during *in-vivo* study. Nevertheless, all BI analogues had shown potential to bind and interact with COX-I, COX-II and μOR, where this finding was further endorsed by their respective analgesic profiles. In CMB, *in-silico* study suggested that the analgesia in structures CMB1-CMB3 was associated with interaction at COX-II (4COX) and μOR (5C1M). While the high analgesic action of CMB2 might be correlated to its high binding affinity and interaction at μOR as compare to the parent CMB. It was also noticed that all structures showed possible binding interaction with $GABA_A$ receptor and hence possessed anxiolytic like potential except CMB and BI1.

If we talk about the structural moieties, in case of 1EQG (fig. 2), IBP formed hydrogen bonds via two oxygen atoms of its carboxylic acid with Tyr355 and Arg120. Instead, in parents BI and CMB, the -NH of imidazole ring and the nitrogen atom of cyano group were forming hydrogen bonds with Met522 and Tyr355 respectively. In BI2, interesting observation was seen where nitro group of substituted benzene ring formed conventional hydrogen bond with Tyr355 and Arg120 (same as IBP). The carbonyl oxygen atom of substituted propiophenone accepted hydrogen atom from Tyr355 in BI3. Same amino acid residue was stabilized by a hydrogen bond formed with the nitro group of substituted benzene ring group in CMB2. In case of 4COX (fig. 3), IMN produced one hydrogen bond with Arg120 via oxygen atoms of the carboxylic acid. Same amino acid residue also showed interaction with the carbonyl group of propiophenone and nitro group of benzene ring in BI3 and CMB2

respectively. Talking about interactions for 5C1M (fig. 4), the carbonyl group of benzoyl ring produced conventional hydrogen bond by accepting one hydrogen atom from Tyr148 in BI1. In BI2, nitro group present on the benzene ring bound to Lys303 and Lys203 to form two hydrogen bonds. It was also observed that the cyano group in CMB and nitro group of benzene ring in CMB2 bound to Trp318 respectively. In case of 6X3X (fig. 5), DZP formed one hydrogen bond with His102 via oxygen atom of benzodiazepine nucleus. Ser205 was stabilized by hydrogen bond with -NH of imidazole ring in BI whereas in its derivative BI2, Asn60 established hydrogen bond with the substituent nitro group of the benzene ring. Interestingly, in both CMB1 and CMB3, the carbonyl oxygen atoms were used to make hydrogen bonds with amino acid His102. Based on the above findings, one could say that the structures of our synthetic derivatives comprised of three basic parts a) benzimidazole core moiety, b) a carbonyl bridge and c) substituted benzene ring. Among these, the substituted benzene ring showed more interaction with the target proteins especially benzene ring with the nitro substitution had major role in binding at the pocket sites.

CONCLUSION

The study concluded that the substitution of different groups in benzimidazole affects its activity and interaction at the target site. The study revealed anxiolytic like potential in most of the synthetic analogues, moreover all the cyanomethyl benzimidazole derivatives showed good analgesic response and could be offered for further development in this regard.

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