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1H-Benzimidazole-5-carboxamide derivatives as a new scaffold of potent anticancer agents: Design, Synthesis, Pharmacological evaluation and Molecular docking studies

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ABSTRACT:

Introduction: Now days, cancer is one of the most dangerous disease and is a widest topic for research as a challenging task is to develop new entities with selectivity towards cancerous cells. Compounds containing a benzimidazole moiety are an important member of the nitrogen containing heterocyclic ring system since it confers surprisingly good anticancer properties.

Methodology: A series of 1H-Benzimidazole-5-carboxamide compounds have been designed and synthesized as per synthetic scheme. Newly synthesized compound(s) were characterized by their infrared, proton nuclear magnetic resonance and mass spectral analysis. In order to evaluate their biological activity, these compounds were tested for their anticancer potential. Furthermore, the experimental results were supported by molecular docking study.

Result and Discussion: The results revealed that all newly synthesized compounds were exhibited moderate to potent anticancer activity. In case of potent anticancer activity, compound no. 5 and 3 gives prominent activity with comparison of all synthesized compounds.

Conclusion: In conclusion, we have designed, synthesized and characterized an interesting and biologically important series of Benzimidazole bridged analogues of Benzimidazole derivatives derivatives. Most of the synthesized compounds were found more active as comparison with each other as manifested by theoretical as well as experimental results.

KEY- WORDS: Benzimidazole, Anticancer**,** Benzimidazole derivatives, molecular docking.

INTRODUCTION: [1-18]

Cancer is one of the most dangerous diseases and is a widest topic for research as a challenging task is to develop new entities with selectivity towards cancerous cells.[1-2] Heterocyclic compounds are of great importance in medicinal chemistry as they possess an wide range of therapeutic application. Since long time, multiple studies have demonstrated those bioactivities of benzimidazole and its derivatives as potential anticancer therapeutics [3-4].

Benzimidazole is an organic heterocyclic compound having two fused ring system whose benzene ring fused with five-membered ring contains two nitrogen atoms. In current research; we are working on the synthesis of various benzimidazole derivatives of biological interest, which have been reported in a wide spectrum of biological and pharmacological activities. The benzimidazole ring is the essential feature of many biologically active compounds[5-14].

In the field of Molecular modeling, docking is a computational method which estimates the preferred orientation of the ligand relative to the receptor as well as the confirmation of the ligand and receptor when bound to each other [15-18].

The present research work deals with the synthesis of the title parent compounds starting from substituted 2-p-tolyl-1H-benzo[d]imidazole-5-carboxylate, followed by their molecular docking study and anticancer evaluation.

METHODS AND MATERIAL:

Chemicals and Reagents

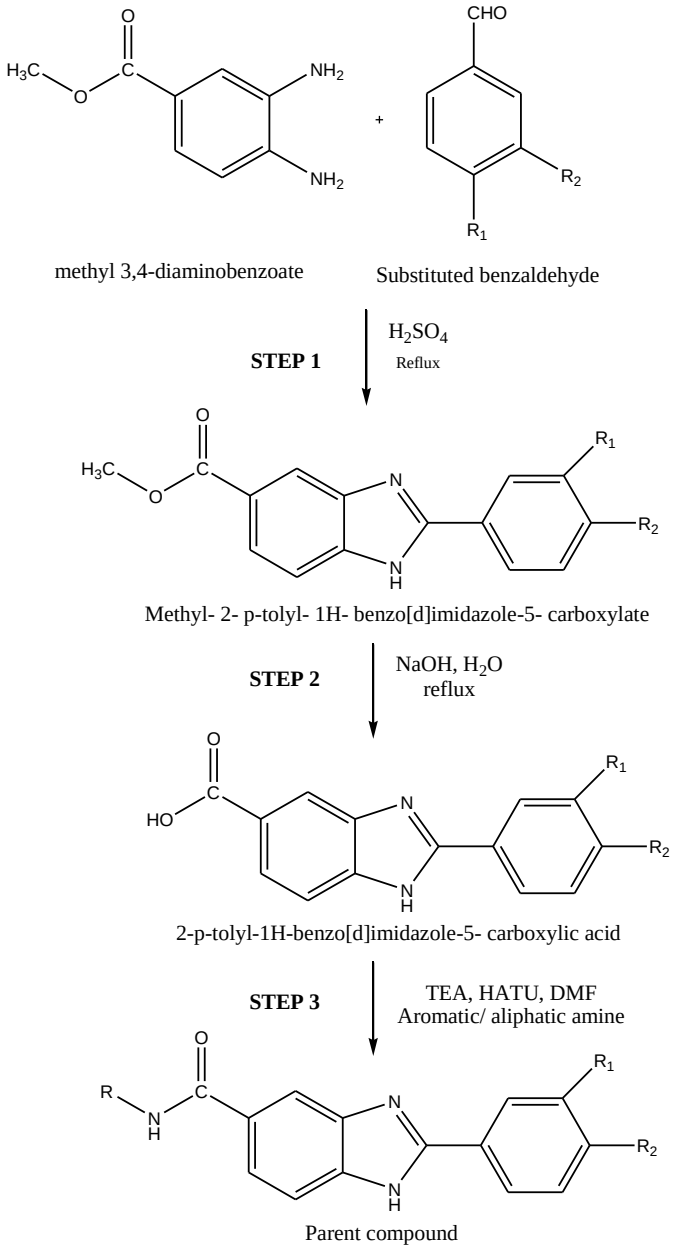
All of the chemicals were commercially available and procured from Aldrich, Merck and Vijay chemicals. All reactants and solvents were analytically pure and were used without further purification. The starting compounds, methyl 3,4- diamino benzoate (I), 3-methylbenzaldehyde (IIa) and 3,4- dimethoxy Benzaldehyde (IIb) were purchased from Vijay chemicals, Mehsana.

Instrumentation

Thin layer chromatography (TLC) was performed on silica gel plates (silica gel GF254) visualized at 254 nm. Infrared (IR) spectra were measured on Bruker, Alpha- II. Nuclear Magnetic resonance (NMR) spectra were measured on a Bruker biospin AV400 instrument at 400 MHz for ^{1}H NMR spectra. The high resolution mass spectra were acquired with a Q-TOF mass spectrometer (Impact II, Bruker).

Chemistry

Benzimidazole derivatives were synthesized according to **Scheme 1**.According to designed scheme, six derivatives were prepared. All the intermediate and target molecules were confirmed by TLC, IR, NMR and Mass Spectra.



Scheme 1: Synthetic Scheme of Target compounds N1-N6

General procedure for synthesis of newer compounds:

Synthesis of Methyl-2-p-tolyl-1H-benzo[d]imidazole-5-carboxylate & Methyl-2-(3,4-dimethoxyphenyl)-1H-benzo[d]imidazole-5-carboxylate (Intermediates of STEP- 1):

Assemble a round bottom flask on water bath. Take methyl-3,4-diamino benzoate (2 gm, 1 eq.) and various substituted aldehyde (1 eq.) in IPA as a solvent followed by addition of sulphuric acid (1.5 eq.) on reflux condition. The reaction takes 2 h for completion at 100℃ temperature. Progression of reaction was monitored by TLC. After completion of reaction, reaction mixture was poured into ice cold water and treated it with base and the precipitate fall out. Recrystallization was done with hot methanol.

Synthesis of 2-p-tolyl-1H-benzo[d]imidazole-5-carboxylic acid & 2-(3,4-dimethoxyphenyl)-1H-benzo[d]imidazole-5-carboxylic acid (Intermediates of STEP- 2):

Assemble a round bottom flask on a water bath. Hydrolysis of ester is done by addition of NaOH base solution in step-1 product under reflux condition for 2 h at 100℃ to afford the desire carboxylic acid derivative. Progression of reaction was monitored by TLC. After completion of

reaction, HCl is added drop wise in reaction mixture. The precipitates fall out by acid base workup.

Synthesis of substituted benzimidazole (7):

Assemble 100 ml two neck round bottom flask on magnetic stirrer, take step 2 (1 eq.) in N, N-Dimethyl formamide was cooled to 0˚C and added HATU (2 eq.) and stirred at 0˚C for 30 min. Reaction mixture was allowed to warm at room temperature followed by addition of triethylamine (3 eq.) and substituted amine (1.2 eq.). Stir the reaction mixture for 3 h at room temperature. Progression of reaction was monitored by TLC. After completion of reaction, reaction mixture was poured into ice cold water and precipitate fall out. Recrystallization was done with hot methanol.

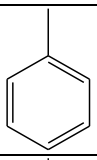
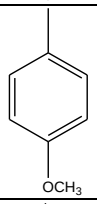
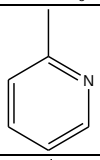
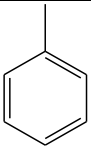
Physical and Spectral characterization of newer compounds:

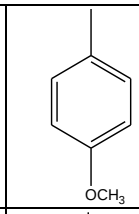
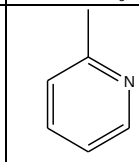
Table 1: Physical characterization data of Intermediates

Intermediates	Molecular Formula	IUPAC name	Mol. Wt. (g/mol)	Melting Point (^{o}C)	Yield %	*Rf
Step 1 Intermediate- I	$C_{16}H_{14}N_2O_2$	Methyl-2-p-tolyl-1H-benzo[d]imidazole-5-carboxylate	266.11	272- 275	79.26	0.53
Step 1 Intermediate- II	$C_{17}H_{16}N_2O_4$	Methyl-2-(3,4-dimethoxyphenyl)-1H-benzo[d]imidazole-5-carboxylate	312.11	265- 267	82.35	0.57
Step 2 Intermediate- I	$C_{15}H_{12}N_2O_2$	2-p-tolyl-1H-benzo[d]imidazole-5-carboxylic acid	252.09	269- 271	65.62	0.10
Step 2 Intermediate- II	$C_{16}H_{14}N_2O_4$	2-(3,4-dimethoxyphenyl)-1H-benzo[d]imidazole-5-carboxylic acid	298.10	266- 268	78.05	0.20

Mobile phase combination used for TLC:*Hexane: Ethyl acetate (8:2)

Table 2: Physical characterization data of synthesized compounds

Compound	R	R1	R2	Molecular formula	IUPAC name	Mol. Wt. (g/mol)	Melting Point (^{o}C)	Yield %	*Rf
N1		-H	$-CH_3$	$C_{21}H_{17}N_3O$	N-phenyl-2-p-tolyl-1H-benzo[d]imidazole-5-carboxamide	327.14	253-255	69.06	0.71
N2		-H	$-CH_3$	$C_{22}H_{19}N_3O_2$	N-(4-methoxyphenyl)-2-p-tolyl-1H-benzo[d]imidazole-5-carboxamide	357.15	267-269	73.80	0.69
N3		-H	$-CH_3$	$C_{20}H_{16}N_4O$	N-(pyridine-2-yl)-2-p-tolyl-1H-benzo[d]imidazole-5-carboxamide	328.13	271-273	61.85	0.63
N4		$-OCH_3$	$-OCH_3$	$C_{22}H_{19}N_3O_3$	2-(3,4-dimethoxyphenyl)-N-phenyl-2-p-tolyl-1H-benzo[d]imidazole-5-carboxamide	373.14	247-249	76.12	0.74

N5		$-OCH_3$	$-OCH_3$	$C_{23}H_{21}N_3O_4$	2-(3,4-dimethoxyphenyl)-N-(4-methoxyphenyl)-1H-benzo[d]imidazole-5-carboxamide	403.15	263-265	79.14	0.78
N6		$-OCH_3$	$-OCH_3$	$C_{21}H_{18}N_4O_3$	2-(3,4-dimethoxyphenyl)-N-(pyridine-2-yl)-1H-benzo[d]imidazole-5-carboxamide	374.14	276-278	66.23	0.61

Mobile phase combination used for TLC:*Chloroform: methanol (9.5:0.5)

N-phenyl-2-p-tolyl-1H-benzo[d]imidazole-5-carboxamide
IR in cm^{-1} (KBr**)** 3038, 3079 (Aromatic CH), 1681 (C=O Amide), 1108 (C=O), 3156 (2^o NH), 1376 (C-N);
^1H NMR in δ ppm (DMSO, 400 MHz) 2.78 ($-CH_3$), 7.31 (Aromatic- H), 9.11 (NH_2^+ Aromatic), 12.73 (NH^+ Aromatic);
Mass M^+ peak (m/z) 328.30 $(M+H)^+$

N-(4-methoxyphenyl)-2-p-tolyl-1H-benzo[d]imidazole-5-carboxamide
IR in cm^{-1} (KBr**)** 3053 (Aromatic CH), 2917 (Aliphatic CH), 1655 (C=O Amide), 1129 (C=O), 3335 (2^o NH), 1333 (C≡N);
Mass M^+ peak (m/z) 358.32 $(M+H)^+$

N-(pyridine-2-yl)-2-p-tolyl-1H-benzo[d]imidazole-5-carboxamide
IR in cm^{-1} (KBr**)** 3074, 3014 (Aromatic CH), 2826 (Aliphatic CH), 1691 (C=O Amide), 1055 (C=O), 3162 (2^o NH), 1416 (C≡N);
Mass M^+ peak (m/z) 348.4 $(M+H)^+$

2-(3,4-dimethoxyphenyl)-N-phenyl-2-p-tolyl-1H-benzo[d]imidazole-5-carboxamide
IR in cm^{-1} (KBr**)** 3065 (Aromatic CH), 3014 (Aliphatic CH), 1694 (C=O Amide), 1110 (C=O), 3483 (2^o NH), 3483 (C≡N);
^1H NMR in δ ppm (DMSO, 400 MHz) 3.33 ($-CH_3$), 7.31 (Aromatic- H), 9.11 (NH_2^+ Aromatic), 12.73 (NH^+ Aromatic);
Mass M^+ peak (m/z) 374.05 $(M+H)^+$

2-(3, 4-dimethoxyphenyl)-N-(4-methoxyphenyl)-1H-benzo[d]imidazole-5-carboxamide
IR in cm^{-1} (KBr**)** 3113 (Aromatic CH), 2934 (Aliphatic CH), 1670 (C=O Amide), 1150 (C=O), 3424 (2^o NH), 1277 (C≡N);
Mass M^+ peak (m/z) 404.45 $(M+H)^+$

2-(3, 4-dimethoxyphenyl)-N-(pyridine-2-yl)-1H-benzo[d]imidazole-5-carboxamide
IR in cm^{-1} (KBr**)** 3138 (Aromatic CH), 3101 (Aliphatic CH), 1670 (C=O Amide), 1085 (C=O), 3434 (2^o NH), 1170 (C≡N);
Mass M^+ peak (m/z) 375.35 $(M+H)^+$

Molecular Docking study
Software Used: AutoDock Vina 4.2.6
Procedure: Protein-Ligand Docking

Steps for AutoDock Vina software
Firstly, choose the protein target and ligand molecule.
For protein target: use Protein Data Bank.
Process: go to PDB site------Search required PDB------click on download file click on PDF format ----------protein

was downloading in downloads.
For ligand target: Synthetically ligand molecule.
Process: go to PubChem------select download------select 3D conformers save it as SDF file format ----------Ligand
Was download in downloads. Both Protein and Ligand was saved in Docking Folder.

Carry out protein and ligand preparation. For Protein Preparation:
Go to AutoDock Vina tools--copy protein and paste in AutoDock Vina tools.
Step 1: Go to edit option --Delete water molecules.
Step 2: Go to Edit Option -------- Go to Hydrogen--------Add hydrogen------select on polar only ----------click ok.
Step 3: Go to Edit-------- select charges-------- Add Kollaman charge click ok (Protein was prepared).
For PDBQT format: go to Grid ----- select Macromolecule----- click on choose----- select protein option-----
select Molecules----- click ok ----- one tab is open--- save as PDBQT format ok.
For Ligand Preparation:
Step 1: Convert SDF format to Ligand PDB format
go to PyMol tool----- copy and paste ligand to PyMol tool---- go to File---- Export Molecule ---------- save as ligand
in Docking Folder.
Step 2: Convert PDB format to PDBQT format.
Copy and paste Ligand to AutoDock Vina Tool---- go to Ligand---- select input----select Choose ---------- select Ligand---- select molecule for AutoDock 4 ----click ok
Step 3: Save as PDBQT format.
Go to ligand---- select Output----save as PDBQT format Save in Docking Folder.
Select the docking site in protein.
Step 1: Preparation of Grid: copy and paste, protein and ligand in AutoDock Vina tool--- go to Grid----------Select Macromolecule----Click Choose-----Select Ligand and Protein-----Select Molecule-----Click No-----
Click ok.
Step 2: For Grid Formation: go to Grid----- select Grid Box---- select Grid Option Tab open---- Click on file----
choose output Grid dimension file----Tab is open -----select Docking folder save as grid.txt format in
Docking folder.
Docking process using AutoDock Vina Tool
Installed AutoDock Vina tool----go to search--- type cmd (command prompt) ------- go to command prompt box----- "C:\Program Files (x86)\The Scripps Research Institute\Vina\vina.exe" --receptor 4dbn1.pdbqt -- ligand C36.pdbqt --config config.txt --log log.txt --out output.pdbqt -----click on enter log file and output .pdbqt file are generated in Docking folder.
Different ligand poses are generated.
Go to PyMol----copy protein----- copy output .pdbqt copy and paste.
ANTICANCER ACTIVITY:
All the synthesized compounds were screened for the *In vitro* cytotoxic activity by MTT assay method.

Determination of anticancer activity by MTT assays Method:
Composition of Media:
- Appropriate media (MEM media)
- Fetal Bovine Serum (5 ml)
- Antibiotic- Antimycotic solution (0.5 ml)
- Sodium Pyruvate (0.5 ml)
- HEPES [(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)] (1.25 ml)
- MEM- non-essential amino acid solution (100X) (0.5 ml)

Glassware used:
- 50 ml sterile tubes
- 0.22 µ Filter
- Tips and micropipettes

Cell-line used:
MCF-7 cell line

Preparation of test solutions:
For anticancer study, each weighed test drugs were separately dissolved in distilled DMSO and volume was made up with DMEM supplemented with 2% inactivated FBS to obtain a stock solution 1 mg/ml concentration and sterilized by filtration. Serial two fold dilutions were prepared from stock solution for carrying out anticancer studies.

Standard drug used:
Doxorubicin

Preparation of media for MTT assay:
Pour 20-30 ml of commercially available MEM media in 50 ml tube. Add 5 ml of Fetal Bovine Serum (final concentration 10%). Add 0.5 ml of Antibiotic- Antimycotic solution (100X) (10,000 units/mL ofpenicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Gibco Amphotericin B). Add 1.25 ml of 1M HEPES [(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)] (final concentration 1.25 mM). Add 0.5 ml MEM- non-essential amino acid solution (100X). Add 0.5 ml Sodium pyruvate solution. Make volume up to 50 ml by adding the appropriate media. Mix by inverting the tube and filter using 0.22 µ filters. Store it at 2-8°C (up to 4-6 weeks).

Procedure of MTT [3-(4, 5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] assay:
Day 1:
Take a culture flask with 80-90% confluent cells. Remove the media and wash the cells with 1ml Phosphate buffered saline (PBS) twice. Remove PBS and trypsinized the cells by adding 1ml Trypsin-EDTA solution. Incubate the flask at 37°C in CO_2 incubator for 7-8 minutes at 5% CO_2. Observe the rounding of cells and mechanically tap the flask by hands for the complete detachment of cells. Transfer 1 ml of cell suspension three 1.5 ml micro centrifuge tubes. Centrifuge the cells at 500 g for 10 minutes at 25°C. Remove media carefully without disturbing the pellet. Add 1 ml 1X PBS in each vial and mix gently to remove cell clumps. Take 10µl of cell suspension and proceed for cell counting. Centrifuge the cells at 200g for 10 minutes at room temperature. Remove the supernatant without disturbing the pellet. Add 1ml media and mix it gently. Add 5000 to 1000 cells in each well of 96-well plate according to cell count (keep inverting the tube while adding the cells). For example, if there is 106 cells/ ml, then to add 10000 or 104 cell in each well take 10µl cell suspension. Make up the volume of each well up to 100µl by adding complete media. Incubate at 37 °C for 24 hours.

Day 2: Drug Treatment
After 24 hours, remove media from each well. Add drug at different concentration (10 to 60 µM)

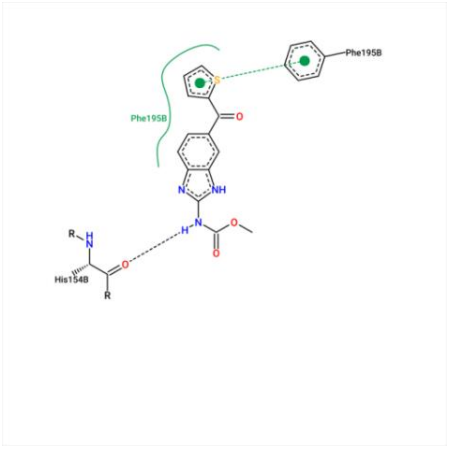
in triplicate and make up the volume of each well up to 300 µl with media. Keep one set of the three wells as untreated that will serve as controls. Doxorubicin was used as a standard drug. Incubate it at 37°C in CO_2 incubator for 24 hours.

Day 3: MTT assay

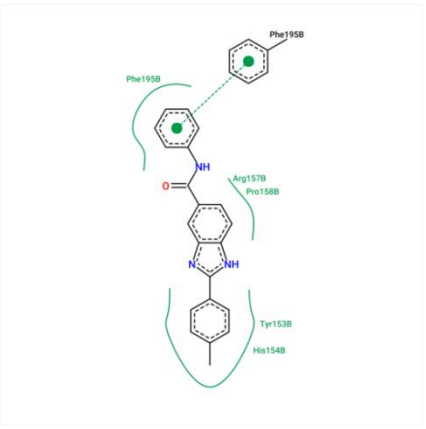
Prepare fresh solution of MTT (5 mg/ml) in PBS and filtering it using 0.22µ filterand store in dark until use (final concentration of MTT should be in the range of 0.2 to 0.5 mg/ml). Add 25 µl of freshly prepared MTT solution in each well. Incubate it at 37°C for 2-3 hours. After Removal of media, add 100 µl DMSO and mix gently by pipetting (avoid bubble formation). Incubate at 37°C overnight. Read absorbance at 570 nm.

RESULT AND DISCUSSION:

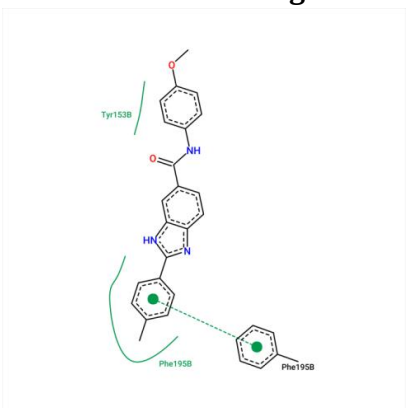
Docking score



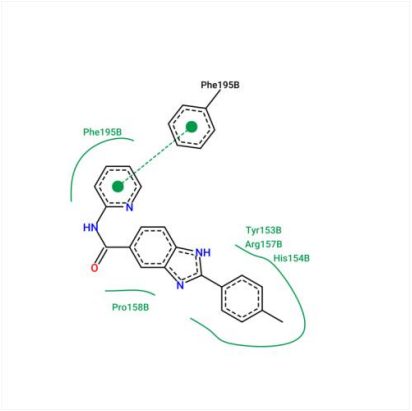
Standard drug



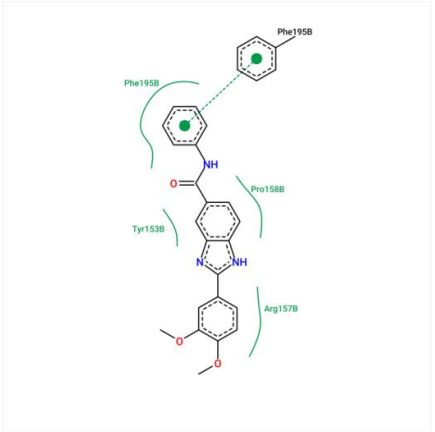
N1

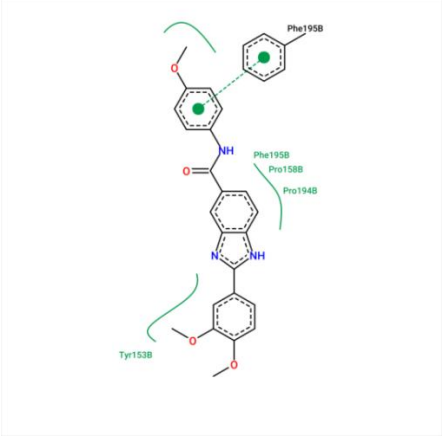


N2

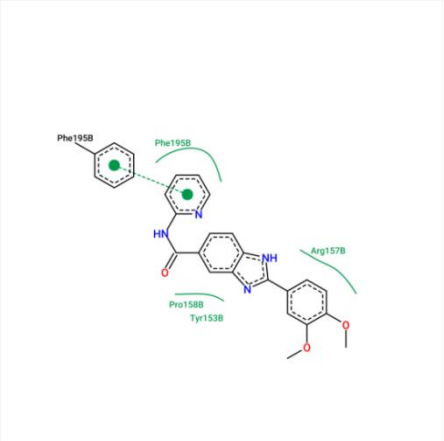


N3





N4 **N5**



N6

Figure 1: 2D interaction image Molecular

docking study of derivatives

Table 5: Docking score of benzimidazole derivatives

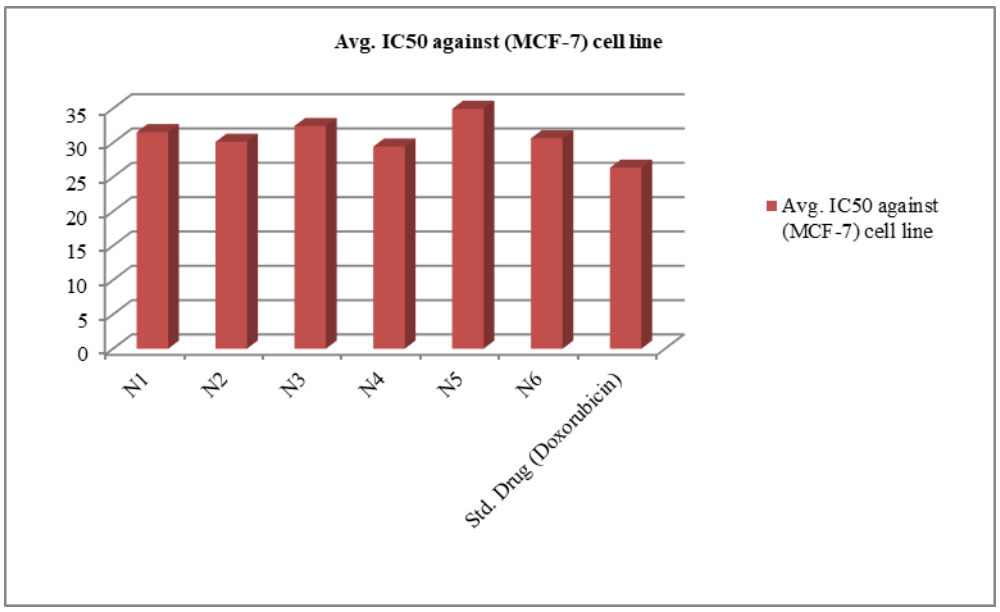
Compounds	Docking Binding energy (kcal/mol)
Doxorubicin	-8.7
N1	-10.6
N2	-10.3
N3	-10.2
N4	-10.3
N5	-9.8
N6	-10.0

From the docking study, we conclude that all Benzimidazole derivatives may not give potent activity as per docking binding energy score. But compound(s) having docking binding energy score -9.8, -10.2 and -10.3 may give anti cancer activity but they are not potent as compare to Standard compound (Doxorubicin).

Anticancer activity

Table 6: Comparison of IC 50 of synthesized Compounds with standard drug

Compounds	Avg. IC50 against (MCF-7) cell line
N1	31.54
N2	30.14
N3	32.44
N4	29.45
N5	34.94
N6	30.66
Std. Drug (Doxorubicin)	26.36



Graph 1: Comparison of avg. IC50 of all compounds with standard drug

CONCLUSION

In this study, we introduced the synthesis and successfully characterization with TLC, IR, NMR and Mass Spectra and screened for their chemotherapeutics anti cancer activity. In the series, the compound N3 and N6 bearing good anticancer activity compared to remaining synthesized compounds. Moreover, these *In-vitro* evaluations in different biological models and detailed studies support Molecular docking and their interaction results.

CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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