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Design, Synthesis and *Invitro* Anticancer Activity of Novel Benzimidazole Derivatives

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Abstract: ***Benzimidazole and its amine derivatives having promising activities like anticancer, antibacterial, antiparasitic, antihistamine, antiulcerative, antineoplastic, antifungal, antitubercular, anti HIV etc. In this view of these observation it was thought of interest to undertake the design and synthesis of compounds having Benzimidazole moiety and evaluate their anticancer activity. To select and prepare targets of interest and carry out the docking studies of analogues using Schrodinger software to obtained the docking scores, Five different Benzimidazole analogues were synthesized by both conventional and microwave menthod, the purity of the compounds was checked by TLC, the structure of compounds were confirmed by IR, NMR, UV and Mass spectra. preliminary pharmacological screening of the syntheisized analogues were performed, which showed promising glide score was screened for anticancer activity against HEPG$_2$ cell line.***

Keywords: MTT Assay, HEPG2 Cell, Schrodinger, JAK/STAT Pathway, 5 –Fluorouracil

1. Introduction

Heterocyclic chemistry is vastly expending because of the enormous research work being done in this area. The majority of known molecule are heterocycles and heterocycles dominate the important field of biochemistry, medicinal chemistry, photographic science, dye stuff, polymers, adhesive and molecular engineering.[1-3] Medicinal chemistry is the discipline concerned with determining the influence of chemical structure on biological activity. As such, it is necessary for medicinal chemist to understand mechanism and pharmaco properties of the molecules, such as, which organic functional groups within the molecule on its acid base properties, water solubility, partition coefficient, crystal structure, stereochemistry, ect. All these properties influence the absorption, distribution, metabolism, excretion, and toxicity of the molecules. To design better medicinal agent, the medicinal chemist need to understand the relative contributions that each functional group makes to the overall physicochemical properties of the molecules. This type of modification of the molecule in systematic fusion and determination of biological activity is structural activity relationship.[4]Molecular modelling become a valuable and essential tool to medicinal chemists in the drug design process. Molecular modelling describes the generation, manipulation or representation of three-dimensional structures of molecules and associated physicochemical properties. It involves a range of computerizedtechniques based on theoretical chemistry methods and experimental data to predict molecular and biological properties. Cancer of the liver is one of the most common malignancies disease occurs more frequently in males than females. There is a strong association between chronic hepatitis B infection and the development of hepatocellular carcinoma. People with cirrhosis also have an increased risk of liver cancer. Other possible hepatocarcinogens include aflatoxin, nitrosamines, oraloestrogen compounds, and numerous other chemicals. Liver cancer is the growth and spread of unhealthy cells in liver[12-14].

MTT ASSAY

This is a colorimetric assay that measures the reduction of yellow 3-(4, 5- dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilised with an organic solvent (eg. isopropanol) and the released, solubilised formazan reagent is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells.[20-23]

$$\% \text{ Cytotoxicity} = \frac{\text{Mean absorbance of sample} \times 100}{\text{Mean absorbance of control}}$$

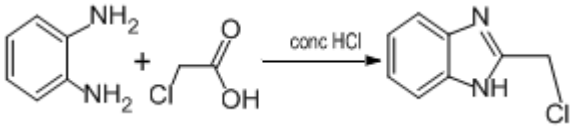
Jak/stat pathway: Signal transducers and activators of transcription (STATs) comprise a family of transcription factors that are activated by a variety of cytokines, hormones, and growth factors. Their activation occurs through tyrosine phosphorylation by Janus kinases (JAKs). Activated STATs stimulate thetranscription of suppressors of cytokine signaling (SOCS) genes. SOCS proteins, in turn, bind phosphorylated JAKs and their receptors to inhibit this pathway, thereby preventing over activation of cytokine-stimulated cells. Thus, SOCS are part of the negative feedback loop in the JAK/ STAT circuitry. Two other families of STAT inhibitors that are described in the literature include the protein inhibitors of activated STATs and the SH2-containing proteins. JAK stimulation of STATs activates cell proliferation, migration, differentiation, and apoptosis, and deregulation of inhibitors leads to human diseases, including cancer. Inactivation of SOCS-1 and SSI-1, a JAK-binding protein, in HCC have been reported as has the ubiquitous activation of the JAK/STAT pathway.[39]

2. Material and Methods

Analytical and instrumental techniques: The melting points of the synthesized compounds were determined by Thiel's melting point apparatus and electrical melting point apparatus all the compounds gave sharp melting points. Purity of the compounds were ascertained by thin layer chromatography using silica gel as stationary phase and appropriate mixture of the following solvents as mobile phase Hexane, Chloroform, Ethyl acetate the spots resolved were visualized using iodine chamber. The IR spectra of the synthesized compounds were recorded on IR affinity-1FTIR spectrophotometer Shimadzu in range of 400 – 4000. The NMR spectra was recorded by NMR 400MHZ spectrometer Brucker. The Mass spectra was recorded by jeol G.C Mass spectrometer technique electronionization IIT Chennai. The docking study of the compounds using software Schrodinger suit maestro V 9.3.

Synthesis of amine derivatives of benzimidazole[85-86]

Step 1: Synthesis of 2- (Chloromethyl)-1 H benzimidazole
Orthophenylene diamine (0.1mole) and monochloro acetic acid (0.1mole) acid reflux in Mandle for 4hr. with 6N HCl the reaction mixture was cooled and basify with sodium hydroxide solution. Yellow colored precipitate was obtained and recrystallized from ethanol.



Step 2: Synthesis of amine derivatives of benzimidazole

Conversional method: 2-Chloromethyl benzimidazole (0.1 mole) and different amines (0.1mole) were refluxed in mandle for 48hrs with 50ml alcohol. The hot mixture was poured into crushed ice with vigorous stirring yellow colored solid precipitated and recrystallized from chloroform.

Microwave method: 2 –Chloromethyl benzimidazole (0.1 mole) and different amines (0.1 mole) with 10ml alcohol refluxed in microwave for (20-27) minutes at 420 W, the hot mixture was poured into crushed ice and vigorous stirring yellow colored precipitate was occurred.

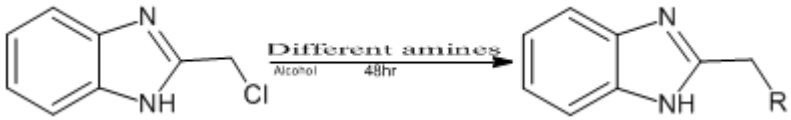
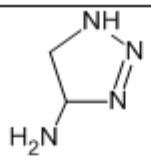
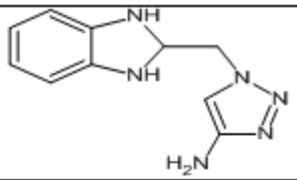
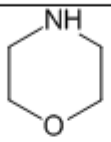
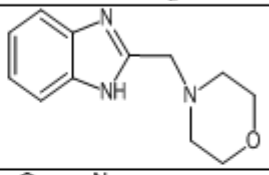
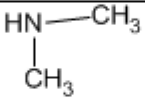
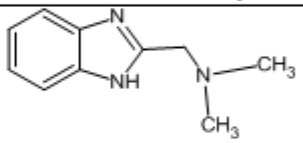
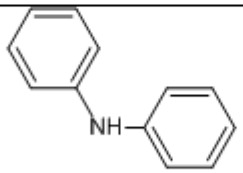
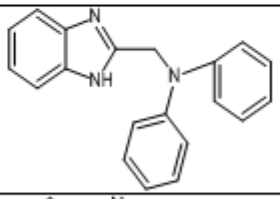
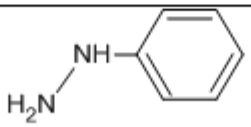
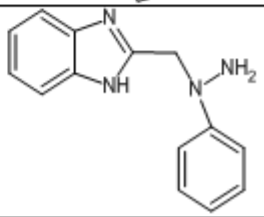


Table 1: Derivatives of benzimidazole

Slno	Compound name	Amines	Structure
1.	BZ1		
2.	BZ2		
3.	BZ3		
4.	BZ4		
5.	BZ5		

Determination of antiproliferative activity in $HEPG_2$ cell line.

Reagent preparation: Prepare a 12 mM MTT stock solution by adding 1 mL of sterile PBS to one 5 mg vial of MTT (Component A). Mix by vortexing or sonication until dissolved. Occasionally there may be some particulate material that will not dissolve; this can be removed by filtration or centrifugation. Each 5 mg vial of MTT provides sufficient reagent for 100 tests, using 10 µL of the stock solution per well. Once prepared, the MTT solution can be stored for four weeks at 4°C protected from light. Add 10 mL of 0.01 M HCl to one tube containing 1 gm of SDS (Component B). Mix the solution gently by inversion or sonication until the SDS dissolves. Once prepared, the solution should be used promptly. Each tube makes sufficient solution for 100 tests, using 100 µL per well. For adherent cells, remove the medium and replace it with 100 µL of fresh culture medium. For non-adherent cells, centrifuge the microplate, pellet the cells, carefully remove as much medium as possible and replace it with 100 µL of fresh medium. Add 10 µL of the 12 mM MTT stock solution (prepared in step 1.1) to each well. Include a negative control of 10 µL of the MTT stock solution added to 100 µL of medium alone. Incubate at 37°C for 4 hours. At high cell densities (>100, 000 cells per well) the incubation time can be shortened to 2 hours. Add 100 µL of the SDS-HCl solution (prepared in step 1.2) to each well and mix thoroughly using the pipette. Incubate the microplate at 37°C for 4– hours in a humidified chamber. Longer incubations will decrease the sensitivity of the assay. Mix each sample again using a pipette and read absorbance at 570 nm. A graph can be drawn by taking the percentage of cytotoxicity in the X-axis and the concentration of the sample in the Y axis and the IC_{50} can be calculated

3. Result and Discussion

1) Synthetic methodology: Analogues designed via insilico methods were chosen for comparative wet lab synthesis by using convensional and microwave methods.

Table 2: Comparative study of microwave and convensional methods

S. No	Compounds	Microwave methods		Convensional methods	
		Reaction time	% yields	Reaction time	% yields
1	BZI	420W for 20min	83%	100°C for 48 hrs	50%
2	BZ2	420W for 27min	79%	100°C for 48 hrs	52%
3	BZ3	420W for 25min	82%	100°C for 48 hrs	60%
4	BZ4	420W for 23min	75%	100°C for 48 hrs	58%
5	BZ5	420W for 23min	81%	100°C for 48 hrs	62%

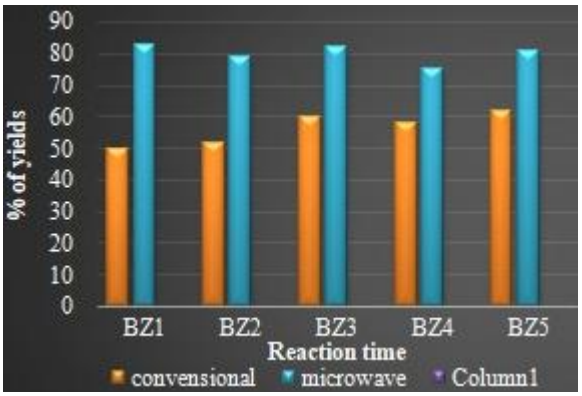


Figure 1: Convensional vs microwave

2) Docking scores: Derivatives(BZ1, BZ2, BZ3, BZ4, BZ5) and natural ligand are docking with receptor (2B7A) and the docking scores obtained are -7.358, -7.270, -6.824, -6.358, -6.595, -6.231 respectively.
3) Anticancer activity: BZ1 and BZ2 are having high docking score compared to natural ligand so BZ1 and BZ2 compound only using to MTT assay *invitro* cytotoxicity studies and IC50 value of BZ1, BZ2 &standard 115 µg/ml, 120 µg/ml &47µg/ml respectively.

Table 3: Anticancer activity of benzimidazole derivatives

Sl no	Concentration in µg/ml	% of inhibition		
		BZ1	BZ2	Standard
1	0	0	0	0
2	10	18.97	19.25	37.23
3	50	27.85	24.65	64.18
4	100	44.23	45.02	89.54
5	150	67.65	65.24	98.34
6	200	85.34	76.24	100
7	250	92.23	88.78	100

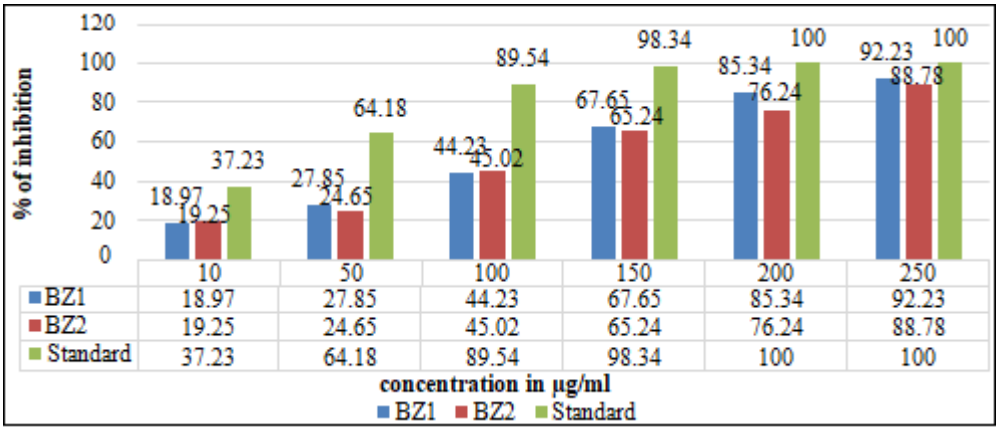


Figure 2: Column chart of anticancer activity

4. Discussion

1) *Insilico* molecular analyses of different benzimidazole derivatives were done all these compounds obeyed "Lipinski rule of five". These analogues were taken for computing molecular descriptors and then synthesis. The designed analogues were synthesized by both conventional and microwave methods. The purity of the synthesized compounds were ascertained routinely by TLC Ethyl acetate & chloroform (2:3), and melting point determinations.
2) Docking studies were carried out using protein janus kinase inhibitor of the five analogues docked, compounds exhibited highest glide score. Compounds BZ1 and BZ2 showed a maximum G score were taken out for wet laboratory validations and screened anticancer activity.
3) A substituent in the 2nd position of benzimidazole seems to enhance the biological activity.
4) From cytotoxic screening it was observed that compounds BZ1 and BZ2 exhibited activity by optimal percentage inhibition on HEPG2 cell line. The result cytotoxic activity in vitro was expressed as an IC_{50} value. The result cytotoxic activity in vitro was expressed as an IC_{50} value. The compound BZ1, BZ2 showed better activity (IC50 of 115µg/ml, 120µg/ml) respectively compared with standard drug 5-fluro uracil (47µg/ml).

5. Conclusion

1) The present investigation was designed and extensive interest has been shown in 2- methyl benzimidazole containing compounds in search of potential drugs.
2) The objective of the present work was to perform the *insilico* screening using molinspiration software, synthesis, characterization and biological activity studies of newly synthesized derivatives.
3) Molecular docking experiments were carried out to benzimidazole derivatives.
4) The compounds which obeyed Lipinski's rule of five and showed good ADME/T profile were taken for wet lab synthesis.
5) Five different benzimidazole analogues were synthesized by both conventional and microwave method.
6) The newly synthesized compounds were found to be good yield in microwave than conventional method for synthesis.
7) The synthesized compounds were ascertained by consistency in the M.P. Purity of the compounds was checked by TLC.
8) The structures of the newly synthesized compounds were confirmed by IR, NMR, UV and Mass spectra.
9) Preliminary pharmacological screenings of the synthesized analogues were performed. The analogue which showed promising glide score was screened for anticancer activity. The compound was found to be active against HEPG2 cell line.

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