IN-SILICO DESIGN, SYNTHESIS AND PHARMACOLOGICAL EVALUATION OF NOVEL PROTEIN TYROSINE KINASE INHIBITORS

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Abstract

Background: *Cancer is continuing to be a major health problem in developing as well as undeveloped countries. It is a leading cause of mortality worldwide accounting for most of deaths.*

Origin: *Epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein and mutations that leadto EGFR overexpression or over activity have been associated with a variety of human cancers.* ***Purpose:*** *Synthesis of EGFR inhibitors by inhibiting EGFR kinase activity which competes with its cognate ligands and it may potentially constitute a new class of effective drugs in clinical use or cancer therapy.*

Experimental work: *In the present investigation, we have performed similarity/sub-structure based search of eMolecule database and find out promising benzothiazole derivatives as EGFR inhibitors by several screening criteria including molecular docking and pharmacokinetics features. The best docked pose of each molecule was considered for binding interactions followed by molecular dynamics and binding energy calculation.*

Result and Discussion:*Molecular docking clearly showed that final proposed derivatives potential to form a number of binding interactions. Molecular dynamics simulation trajectories undoubtedly indicated that the EGFR protein become stable when proposed derivatives bind to the receptor cavity. Strong binding affinity was found for all molecules towards the EGFR which substantiated by the binding energy calculation using MM-PBSA approach.*

Conclusion: *In outlook of this, proposed promising benzothiazole derivatives were successfully synthesized and characterized by IR, ^{1}H NMR and Mass spectroscopy techniques. Subsequently, they were screened against MCF-7 cancer cell lines in search of their anticancer potential and H9 and H12 compounds have shown great potential for anticancer therapy.*

Keywords: *Molecular docking, Tyrosine kinase, Molecular dynamic, Pharmacokinetics, EGFR.*

INTRODUCTION

Cancer is continuing to be a major health problem in developing as well as undeveloped countries. It is a leading cause of mortality worldwide accounting for most of deaths. Among all types of cancer, lung, breast, colorectal, stomach, and prostate cancers are the underlying causes for the majority of cancer deaths[1]. In this study, we illustrate the epidermal growth factor receptor (EGFR) which is a transmembrane glycoprotein belonging to the human epidermal receptor (HER) family. It plays a vital role in signal transduction pathways and regulating key cellular functions such as cell proliferation, survival, adhesion, migration and differentiation. Mutations that lead to EGFR overexpression or over activity have been associated with a variety of human cancers, including lung, colon and breast cancers[2]. The binding of a ligand to EGFR induces conformational changes within the receptor which increase its intrinsic catalytic activity of a tyrosine kinase and result in autophosphorylation, which is necessary for

biological activity. Therefore, inhibitors of EGFR-inhibiting EGFR kinase activity by competing with its cognate ligands-may potentially constitute a new class of effective drugs in cancer therapy[3].
Nitrogen containing heterocycles comprising of triazoles, benzothiazoles, benzimidazoles, indoles, etc. constitute an important scaffold in biological science and medicinal chemistry, and has fascinating applications in drug discovery and development[4].
Benzothiazole derivatives become a major area of emphasis for the organic chemists due to varied spectrum of pharmacological profile for instance, antimicrobial[5], antimalarial[6], anticonvulsant[7], anthelmintic[8], analgesic[9] , antidiabetic[10] and anticancer[11].
In continuation of our research on the synthesis and biological evaluation of benzothiazoles, here we reported the click synthesis and antiproliferative evaluation of new series of benzothiazole scaffold which could generate active pharmaceutical ingredients (API) dotted with relevant chemotherapeutic activities comparable to the clinically approved standard drugs[12] .

MATERIAL AND METHODS

Molecular Modeling

Protein Structure Preparation

The X-ray crystal structure of EGFR kinase domain (PDB ID: 2J5F) in complex with small molecule 6-acrylamido-4-anilinoquinazoline, an irreversible kinase inhibitor was retrieved from the RCSB Protein Data Bank (http://www.rcsb.org/pdb) for molecular docking study analysis[13]. Refinement of crude PDB structure of protein was performed. By using AutoDock Tool 4.2, protein and grid preparation was performed[14].

Ligand Structure Preparation

Similarity/sub-structure based search of eMolecule database has been developed and used for further study against the target. The proposed promising molecules of benzothiazole derivatives are shown in figure 1.

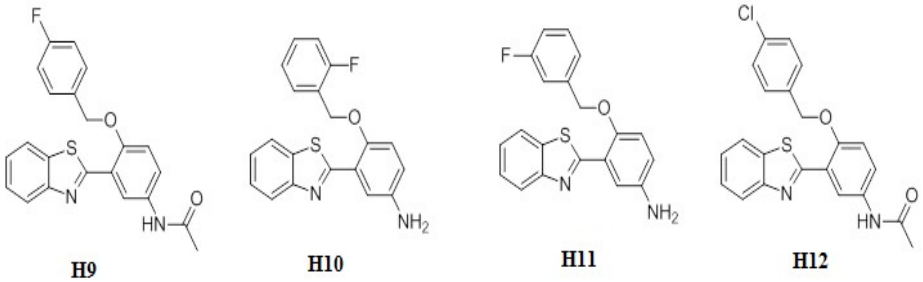


Fig. 1: Proposed promising molecules of benzothiazole derivatives

Docking Protocol

Using the gradient optimization algorithm and an empirical scoring function the molecular docking was performed to generate the best binding affinity or fitness of protein-ligand binding poses between tyrosine kinase protein and each benzothiazole derivatives[15]. Molecular docking study for all compounds of benzothiazole derivatives was performed with AutoDdockVina tool.
Graphical representation of in- silico approach for the identification of hit molecules is shown in figure 2.

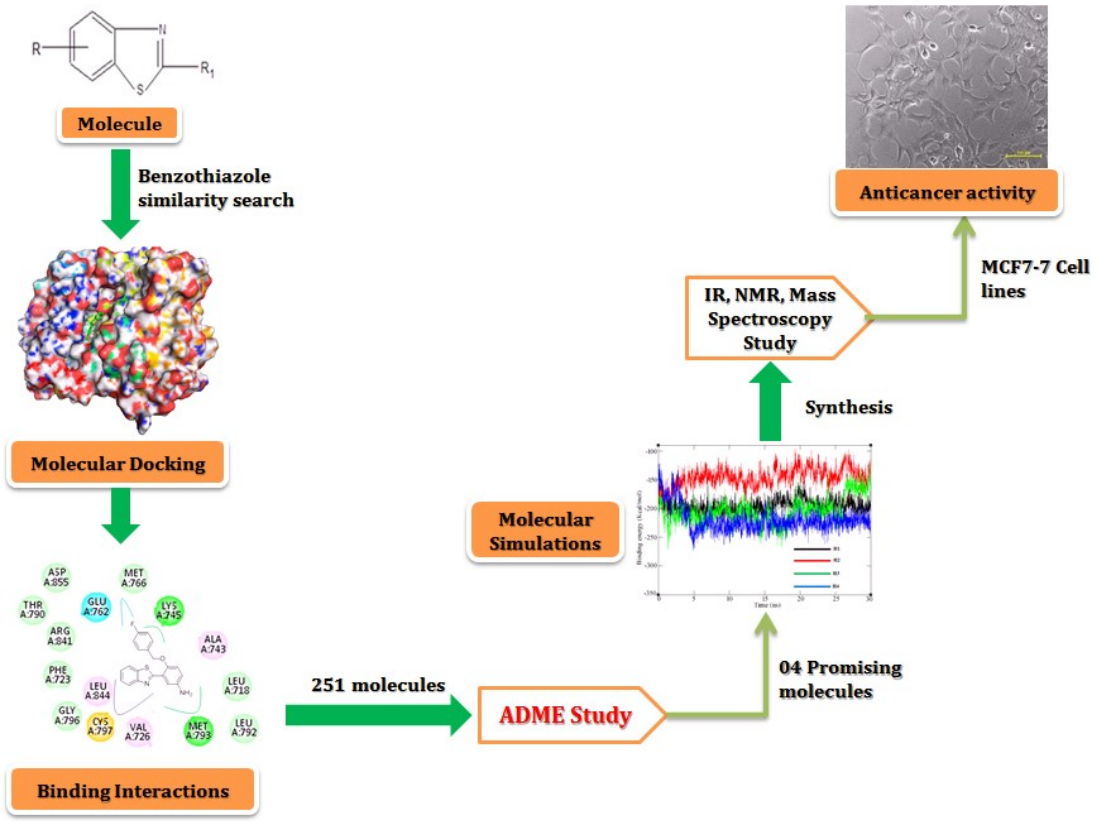


Fig. 2: Graphical representation of in- silico approach for the identification of hit molecules

In-silico Pharmacokinetic Study

Several important parameters such as drug-likeness properties, nature of lipophillicity, solubility and other physicochemical and pharmacokinetics profiles were computationally predicted for all best docked compounds whose binding affinity observed around -9.0 Kcal/mol in docking study. Swiss ADME a web server based prediction tool which was used to predict the information on absorption, distribution, metabolism, excretion (ADME) and pharmacokinetic properties[16]. Another standalone open source tool, OSIRIS Property Explorer (available at www.organic-chemistry.org/prog/peo/) was used for theoretically predicating toxicity risk assessment[17].

Molecular Dynamic Simulation Study

Molecular dynamic (MD) simulation study is carried out to understand the dynamic behavior of protein-ligand complex. In the current study, the best docked complexes between proposed molecules and EGFR were considered for MD simulation study using Gromacs 5.1.1[18]. For each docked complex 30ns of time span MD simulation was performed with a time step of 2 fs at constant pressure (1 atm) and temperature (300 K). The root mean-square deviation (RMSD), root mean-square fluctuation (RMSF) and radius of gyration were used to analyze the performance of each system.

Synthesis of Selected Benzothiazoles

All the chemicals used were procured from commercial sources such as Sigma-Aldrich, Merck and Loba Chemie and were purified prior to use. The melting points of synthesized compounds were taken on Veego VMP-D digital melting point apparatus by open capillary. Merck pre-coated silica gel F_{254} TLC plates was used for monitoring of reaction. TLC plates were visualized using iodine in a chamber or observed under UV light. Fourier transform infrared (FT-IR) spectra were recorded in anhydrous potassium bromide (KBr) disk on "Jasco FTIR 4100" and are reported in cm^{-1}. Proton nuclear magnetic

resonance (^{1}H-NMR) spectra were recorded in DMSO-D_6 using "BrukerAvance (400 MHz) with tetra methyl silane (TMS) as an internal standard. General synthetic scheme of benzothiazole derivatives (H9 & H12) is shown in figure 3.

Synthetic scheme:

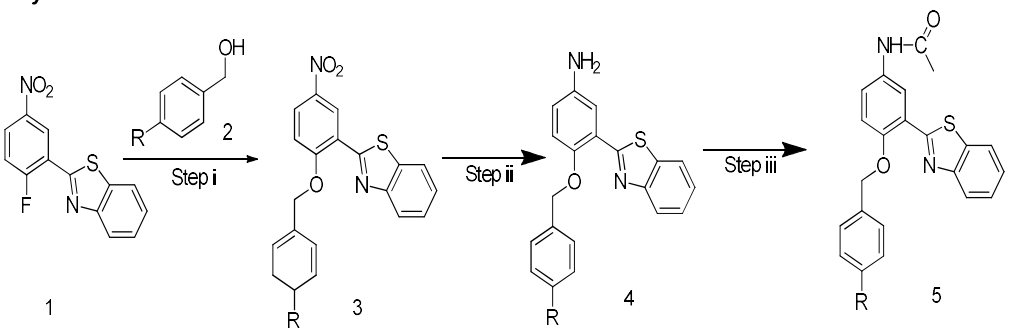


Fig. 3: General synthetic scheme of benzothiazole derivatives (H9 & H12)

Step i involves reflux at 100°C for 5 Hrs. Step ii involves in methanol (10 ml) & Pd/C was added, reaction mixture was stirred at RT for 4 Hrs under Hydrogen atmosphere. Step III involves in DCM, TEA and Acetyl chloride was added, reaction mixture was stirred at RT for 4 Hrs.

Anticancer Activity

Selected synthesized benzothiazole molecules were screened for anticancer activity against MCF-7 cell lines and found to be with potential good results. The IC50 concentration MCF-7 cells were determined by MTT assay. The inhibition activity of Compounds H9 & H12 on MCF-7 cells were plated and treated with different concentration such as 0.1, 10, 25, 50, 100 µg/ml. The IC50 value was determined based on cell viability rates[19].

RESULTS AND DISCUSSION

Substructure Search and Molecular Docking

All designed molecules were successfully docked and to screen these molecules the cut-off value of binding energy was considered as -9.0 Kcal/mol. Total 251 compounds were found to be satisfied the above condition. Pharmacokinetic profiling was done for all 251 molecules and found that 04 molecules showed good absorption and distribution. Among these, four molecules have taken for study and further analysis. The 2D- representation of the concluded four promising molecules is given in Figure 1.

In-silico Pharmacokinetic Study:

The results of in silico ADMET analyses of most potent four compounds (**H9**, **H10**, **H11** and **H12**) were evaluated for drug-likeness and physicochemical properties, water solubility (Log S scale) and pharmacokinetics profiles (Gastrointestinal absorption) etc., and their results are represented in Table 1. The 'Lipinski's rule of five' which was crucially measured as drug-likeness profiles for all compounds showed non-violation of the rule. OSIRIS Property Explorer predicted toxicity risks assessment profiles suggest that no indication of tumorigenicity, mutagenicity, irritating effect and reproductive toxicity effect was found for the selected four compounds. The above findings explained that all four compounds have potential drug-likeness properties for exhibiting numerous numbers of pharmacological implications.

Table 1: Pharmacokinetics & Drug-Likeness Properties Of Molecules

Parameters	**H9**	**H10**	**H11**	**H12**
Molecular weight	392	350	350	408
HB acceptor	3	1	1	3

HB donor	1	1	1	1
LogP	4.26	5.84	5.65	4.34
$^{1}N_{rot}$	6	4	4	6
^{2}MR	100.4	110.3	110.2	104.4
Polar surface area	78.10	74.86	76.14	78.60
Log S	Soluble	Soluble	Soluble	Soluble
GI absorption	High	High	High	High
log Kp (cm/s)	-4.84	-4.14	-4.36	-4.98
Bioavailability score	0.65	0.65	0.65	0.65
Mutagenicity risk	Low	Low	Low	Low
Irritating effect and	Low	Low	Low	Low
Reproductive Toxicity effect	Low	Low	Low	Low
Tumorigenicity risk	Low	Low	Low	Low

[1]Number of rotatable bonds; [2]Molecular refractivity

Molecular Docking

The binding interactions between designed molecules and catalytic amino residues of EGFR protein were explored from the best docked pose. Based on obtained high negative binding energy of each molecule, explained that all molecules are capable enough to bind perfectly at the receptor cavity of the EGFR. The Binding interactions of proposed molecules with target is shown in figure 4.

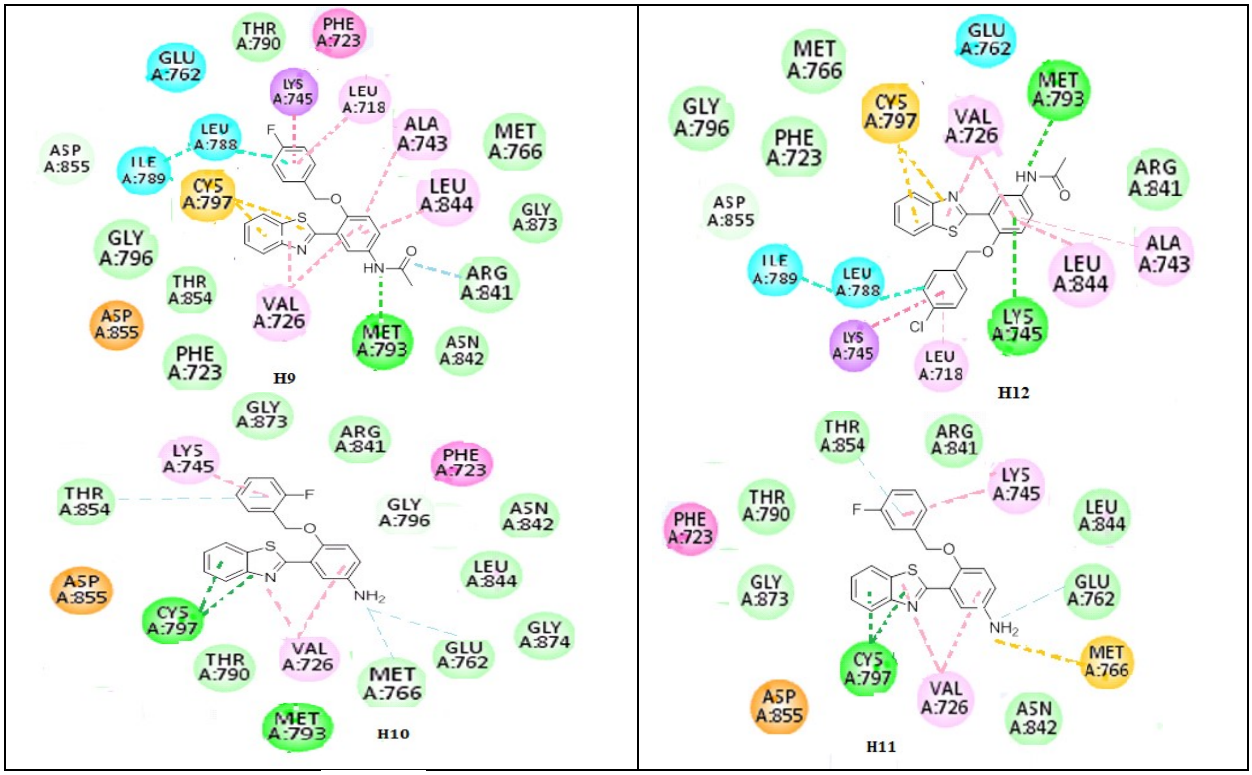


Fig. 4: Binding interactions of proposed molecules

The 2D diagram of binding interaction showed a number of hydrogen and non-hydrogen bonding interactions among the designed benzothiazole molecules and catalytic amino acid residues of EGFR. It was observed that Glu762, Lys745, Met793 and Cys793 was found to be important amino acid residue to interact with molecules via hydrogen bond interaction. Above findings unquestionably revealed that all proposed molecules are capable enough to form a number of crucial interactions with the catalytic amino acid residues of EGFR and favor the potentiality of the molecules as anticancer agents. The Binding mode of proposed benzothiazole compounds in 3D orientation is shown in figure 5.

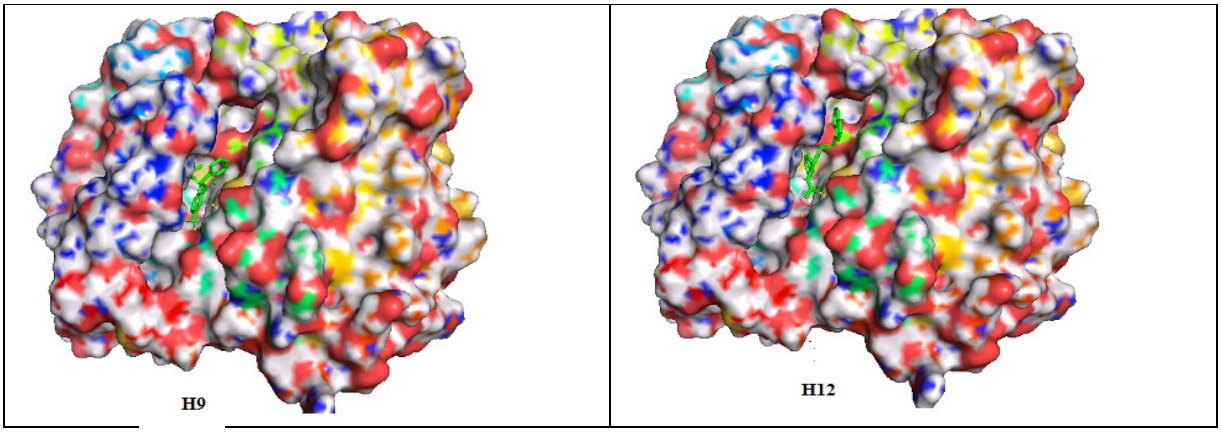
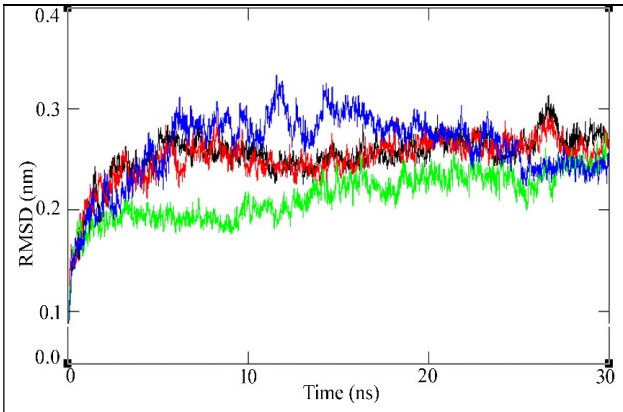


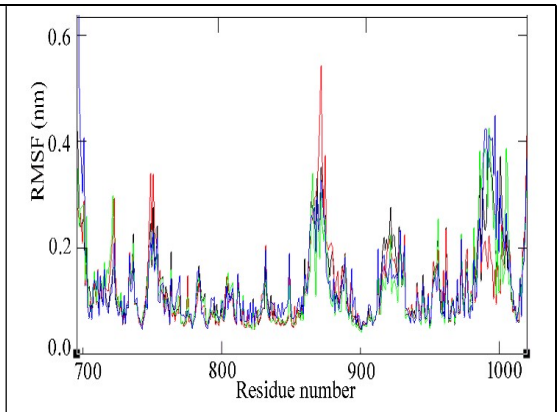
Fig. 5: Binding mode of proposed benzothiazole compounds in 3D orientation

Bioactivity Prediction and Quality Assessment

In order to assess the quality of the proposed molecules several paramaters included inhibitory constant (K_i) based on binding energy derived from AutodockVina, (LE), LE scale (LE_{scale}), fit quality (FQ) and LE-dependent lipophilicity (LELP). Values of all parameters are given in Table 2. The predicted inhibitory activity of all designed benzothiazole derivatives were found to be in the range of 0.249 to 0.091 µM. Therefore low K_i value of the molecules clearly explained that all compounds derived from the sub-structure searching approach have capability to being lead molecules with high potency[20,21].



RMSD vs. time of protein-ligand complexes of proposed molecules



RMSF vs. residue number of complexes with proposed molecules

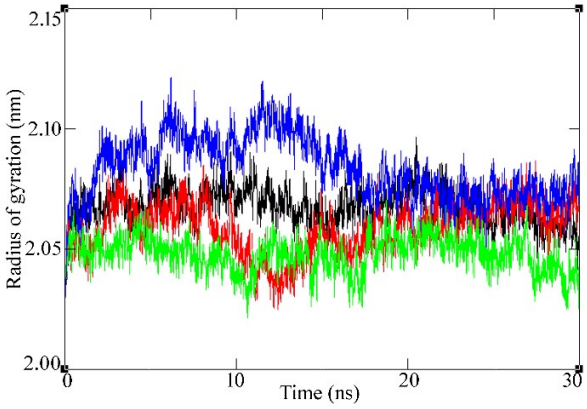


Fig. 6: Molecular dynamic silmulation study

Another parameter, the ligand efficiency (LE) can be calculated as the ratio between negative binding energy and number of heavy atoms (NHA) in a molecule[22]. LE cannot be estimated without molecular size hence they have reported LE scaling (LE Scale) which enable a size-independent comparison of ligands with the help of an exponential function to the maximal LE values for a given NHA count. The fit quality (FQ) is the ration between LE and the LE_Scale[23]. It is illustrated that FQ values near one suggest close to ideal ligand binding. Finally, the ligand-efficiency-dependent lipophilicity (LELP) reported by Keseru and Makara was derived with regards to lipophillicity[24].

Molecular Dynamic Simulation

The 30ns time span of molecular dynamics study was performed to check stability of best docked complex between proposed molecules and EGFR protein. To analyze the trajectories of the MD simulation the RMSD, RMSF and Rg were calculated and explored. The RMSD of all protein backbone from the complex were calculated. The RMSD trajectories clearly showed that complex with all designed molecule were equilibrated in the range of RMSD value 0.0005 to 0.334 nm. Average, maximum and minimum RMSD values of each complex are given in Table 3.These observations emphatically explained that there was no much fluctuation of the backbone of the EFGR bound with proposed promising molecules.

Table 2: Bioactivity & Efficiency Parameters Of Molecules

Molecule	[1]BE	[2]K_i	[3]LE	[4]LE_Scale	[5]FQ	[6]LELP
H9	-9.010	0.237	0.340	0.362	0.924	8.642
H10	-9.260	0.221	0.338	0.354	0.922	8.584
H11	-9.840	0.149	0.322	0.346	0.917	10.412
H12	-9.120	0.085	0.312	0.316	0.943	11.532

[1]Binding energy; [2]Predicted inhibition constant; [3]Ligand efficiency; [4]Ligand efficiency scale; [5]Fit quality; [6] ligand-efficiency-dependent lipophilicity

In the process of MD simulation the fluctuation of individual amino acids can explain the stability of protein-ligand complex. Fluctuations of amino acid residues were explored using the RMSF parameters and plotted. To achieve the relative fluctuation the maximum, minimum and average RMSF were recorded and present in Table 3. The observations clearly explained that amino acid residues not much deviated from the original position.

The rigidity of protein-ligand complexes can be explained using the radius of gyration (Rg) parameters obtained from MD simulation trajectories. The Rg values were recorded from the trajectories and plotted against time. The average, minimum and maximum Rg values were calculated and presented in Table 3. From the analysis of trajectories obtained from MD simulation study exhibited that all proposed molecules can form stable complex with EGFR.

Table 3: Maximum, Minimum And Average Rmsd, Rmsf And Rg Values

Complex		H9	H10	H11	H12
RMSD (nm)	[1]Max.	0.284	0.336	0.342	0.239
	[2]Min.	0.0005	0.0005	0.0005	0.0005
	Average	0.274	0.314	0.304	0.226
RMSF (nm)	[1]Max.	0.446	0.469	0.421	0.484
	[2]Min.	0.042	0.045	0.045	0.043
	Average	0.102	0.126	0.139	0.113
Rg (nm)	[1]Max.	2.014	2.180	2.144	2.023
	[2]Min.	2.009	2.104	2.107	2.012
	Average	2.012	2.016	2.012	2.014

[1]Maximum; [2]Minimum

Synthesis of Benzothiazoles

Based on molecular modeling study of benzothiazole derivatives which has shown better interactions with protein and their pharmacokinetic profiles, those derivatives only proceed to synthesize and for screening against MCF-7 cell lines. Among these, H9 and H12 have shown some significant results based on molecular modeling and so these molecules only has been taken in consideration for further study. These molecules successfully synthesized by three steps and were purified by column chromatography. Their structures were characterized by FTIR, ^{1}H NMR and Mass spectroscopy.

Spectral data

N-(3-(benzo[d]thiazol-2-yl)-4-((4-fluorobenzyl)oxy)phenyl)acetamide (H9)

Yellow crystals, % yield: 68%, MP: 292^{0}C, FTIR: 3062 (ArC-H), 1652 (C=O), 1592 (C=N), 1519 (C=C), **755** (C-F), ^{1}H NMR: δ 2.64 (3H, s), 5.40 (2H, s), 6.82-6.92 (2H, 6.89 (dd, J = 8.7, 1.5 Hz), 6.91 (dd, J = 8.6, 0.4 Hz)), 7.02-7.22 (4H, 7.06 (dd, J = 8.1, 7.5, 1.8 Hz), 7.16 (dd, J = 7.6, 7.2, 1.5 Hz), 7.09 (ddd, J = 8.1, 1.2, 0.5 Hz), 7.08 (ddd, J = 8.0, 7.5, 1.2 Hz)), 7.10 (1H, ddd, J = 8.0, 1.8, 0.5 Hz), 7.42-7.49 (3H, 7.43 (ddd, J = 7.6, 1.6, 0.5 Hz), 7.47 (dd, J = 1.5, 0.4 Hz), 7.44 (ddd, J = 7.2, 6.9, 1.6 Hz)), 7.89 (1H, ddd, J = 6.9, 1.5, 0.5 Hz), EI-MS: m/z = 392.44.

N-(3-(benzo[d]thiazol-2-yl)-4-((4-chlororobenzyl)oxy)phenyl)acetamide (H12)

Pale Yellow crystals, % yield: 73%, MP: 276^{0}C, FTIR: 30632 (ArC-H), 1624 (C=O), 1597 (C=N), 1523 (C=C), 762(C-Cl), ^{1}H NMR: δ 2.49 (3H, s), 5.37 (2H, s), 6.79-6.89 (2H, 6.82 (dd, J = 8.7, 1.5 Hz), 6.89 (dd, J = 8.6, 0.4 Hz)), 7.09-7.18 (4H, 7.10 (dd, J = 8.1, 7.5, 1.8 Hz), 7.12 (dd, J = 7.6, 7.2, 1.5 Hz), 7.09 (ddd, J = 8.1, 1.2, 0.5 Hz), 7.07 (ddd, J = 8.0, 7.5, 1.2 Hz)), 7.08 (1H, ddd, J = 8.0, 1.8, 0.5 Hz), 7.43-7.52 (3H, 7.44 (ddd, J = 7.6, 1.6, 0.5 Hz), 7.45 (dd, J = 1.5, 0.4 Hz), 7.46 (ddd, J = 7.2, 6.9, 1.6 Hz)), 7.47 (1H, ddd, J = 6.9, 1.5, 0.5 Hz), EI-MS: m/z = 408 .38

Anticancer Activity

Selected synthesizes benzothiazole molecules (H9 & H12) were screened for anticancer activity against MCF-7 cell lines and found to be with potential good results. The IC50 concentration MCF-7 cells were determined by MTT assay. The inhibition activity of Compound H9 & H12 on MCF-7 cells were plated and treated with different concentration such as 0.1 1, 10, 50, 100 µg/ml . The IC50 value was determined based on cell viability rates. The % cell viability rates verses different concentrations is shown in figure 7.

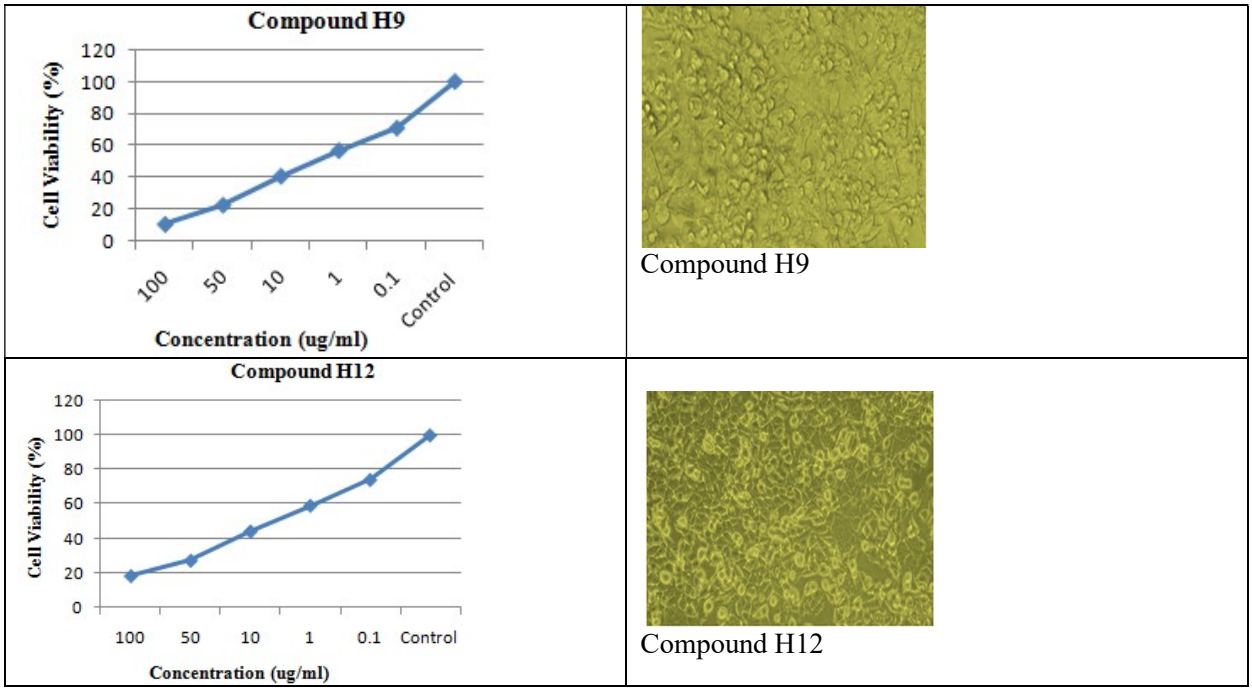


Fig. 7: The % cell viability rates verses different concentrations and Microscopic image of MCF-7 cells treated with compound

CONCLUSION

Similarity/sub-structure search of eMolecule database was performed to get important benzothiazole derivatives as promising inhibitors for the EGFR. All molecules were docked followed by pharmacokinetics and synthetic accessibility checked. Finally four molecules were found to be important as promising EGFR inhibitors. The binding interactions were explored and found that a number of hydrogen and non-hydrogen bonds formed between proposed molecules and catalytic amino acid residues of EGFR. The inhibition constant and lead like parameters such as LE, LE_Scale and FQ were calculated. The low inhibition constant definitely indicated that proposed molecules possess high potential to being potential EGFR inhibitors. A 30ns time span of MD simulations was performed. The RMSD, RMSF and Rg values and MD trajectories of all complexes showed that each proposed compound formed a strong complex with the EFGR. The compounds with higher selectivity and great potential H9 and H12 have been synthesized and confirmed their structures by spectroscopy techniques. Subsequently screening of these characterized compounds against MCF-7 cancel cell lines shows a high inhibition activity and therefore, it can be concluded that proposed molecules may be potential and safer chemical agents for therapeutic application in cancer therapy.

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CONFLICT OF INTEREST

There is no conflict of interest.

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