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Synthesis, anticancer activity and docking of some substituted benzothiazoles as tyrosine kinase inhibitors

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ABSTRACT

Protein tyrosine kinases occupy a central position in the control of cellular proliferation and its inactivation might lead to the discovery of a new generation anticancer compounds. Substituted benzothiazoles have been found to mimic the ATP-competitive binding of genistein and quercetin to tyrosine kinase. A series of novel 2-phenyl-1,3-benzothiazoles were synthesized and characterised by IR, ^{1}H NMR and mass spectroscopy. All the compounds were tested for their anticancer activity against MCF-7 breast cancer cell line with the MTT assay. Most of the compounds showed moderate to good anti-breast cancer activity. Anticancer activity varied with substitution on the benzothiazole nucleus with halogens and at 4 position, substitution of the 2-phenyl moiety with methyl and methoxy groups was also explored. Among the compounds tested with MTT assay, mono fluoro substitution on benzothiazole nucleus and 4′-methyl variations at 2-phenyl position demonstrated highest percent growth inhibition of MCF-7 cells. Docking studies of the synthesised compounds was done on EGFR using GRIP batch docking method to study their observed activity.

1. Introduction

Cancer is a group of diseases in which cells can be aggressive (grow and divide without respect to normal limits), invasive (invade and destroy adjacent tissues) and/or metastatic (spread to other locations in body). These three malignant properties of cancer differentiate them from benign tumors, which are self-limited in their growth and do not invade or metastasize. Cancer may affect people at all ages, even fetuses, but risk for the more common varieties tends to increase with age [1]. Cancer causes about 13% of all deaths [2]. According to the American Cancer Society, 7.6 million people died from cancer in the world during 2007 [3]. Nearly all cancers are caused by abnormalities in the genetic material of the transformed cells [4]. With the current chemotherapy, lack of selectivity of chemotherapeutic agents against cancerous cells is a significant problem.

Receptor tyrosine kinases (RTKs) are high affinity cell surface receptors that bind polypeptide growth factors, cytokines and hormones. They have been shown to be key regulators of normal cellular processes and additionally play a critical role in the development and progression of many types of cancer [5]. Protein tyrosine kinases occupy a central position in the control of cellular proliferation. Several transforming oncogens (e.g., *src* – a gene obtained from Rous sarcoma virus, *abl* – a gene obtained from abelson murine leukemia virus) are known to possess tyrosine kinase activity; and it is well recognized that the response of many cells to growth factors is initiated by activation of RTKs. Over expression of certain RTKs show association with promotion and maintenance of malignancies. For example, the epidermal growth factor (EGF) receptor tyrosine kinases of the erbB family (which includes erbB1–erbB4) is frequently expressed at high levels in certain carcinomas and shows an inverse correlation with survival (particularly breast, colon and bladder cancers) [6]. Thus, inactivation of the specific tyrosine kinases that are responsible for the malignant phenotype of certain cancers represent a potential approach for design of antiproliferative drugs [7].

The isoflavone, genistein [8] and the flavone, quercetin [9] are competitive inhibitors at the ATP-binding site of kinases [10,11]. As the crystal structure of 5,6-dimethoxy-2-(4-methoxyphenyl)benzothiazole was solved [12], the preliminary analysis based on comparisons between polyhydroxylated 2-phenylbenzothiazoles and the adenine fragment of ATP suggested that suitably substituted benzothiazoles might mimic the ATP-competitive binding of genistein and quercetin at tyrosine kinases.

In this study, 10 derivatives of 2-phenyl-1,3-benzothiazole with halogen substitution on benzothiazole moiety and methyl/methoxy substitution at 4 position of the 2-phenyl ring were synthesized and evaluated for anticancer activity on the MCF-7 human breast cancer cell line. We have also tried to dock the synthesised

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Table 1
Physical data of the intermediates HB1a–HB10a (benzanilides).

Intermediate	R_1	R_2	% Yield	Mt. pt. (°C)	R_f[a]
HB1a	4-F	Me	77.5	176–184	0.549
HB2a	3,4-diF	Me	54.7	152–158	0.628
HB3a	4-Br	OMe	54.4	212–220	0.359
HB4a	4-F	OMe	54.8	186–194	0.292
HB5a	4-Br	Me	56	222–230	0.628
HB6a	3-Cl	OMe	82.8	126–136	0.546
HB7a	3-Br	OMe	42.2	116–120	0.47
HB8a	2-F	OMe	68	136–142	0.536
HB9a	3,4-diF	OMe	58.7	150–158	0.337
HB10a	2-F	Me	42	114–122	0.782

[a] n-Hexane:ethyl acetate, 4:1.

Table 2
Physical data of the intermediates HB1b–HB10b (thiobenzanilides).

Intermediate	R_1	R_2	% Yield	Mt. pt. (°C)	R_f
HB1b	4-F	Me	52.5	156–164	0.709[a]
HB2b	3,4-diF	Me	37.5	152–162	0.659[a]
HB3b	4-Br	OMe	62.3	176–182	0.488[a]
HB4b	4-F	OMe	77.9	206–212	0.376[a]
HB5b	4-Br	Me	54.4	198–206	0.686[a]
HB6b	3-Cl	OMe	24.8	130–136	0.574[b]
HB7b	3-Br	OMe	45.1	150–154	0.458[a]
HB8b	2-F	OMe	33.6	125–132	0.533[a]
HB9b	3,4-diF	OMe	66	160–168	0.35[a]
HB10b	2-F	Me	15.5	120–124	0.565[a]

[a] n-Hexane:ethyl acetate, 4:1.
[b] n-Hexane:ethyl acetate, 4.5:0.5.

compounds with the crystal structure of EGFR to explore the possible anticancer mechanism of our compounds. Prior compounds have been reported [13,14] to compare the anticancer activity of such compounds.

2. Materials and methods

2.1. Synthesis

2.1.1. Reagents

All the chemicals and solvents used were of AR-grade and LR-grade and obtained from Sigma-Aldrich, Sisco Research Laboratories, Qualigens, Rankem, S.D. Fine, Hi-Media and Merck and were used without further purification.

2.1.2. TLC analysis

Thin-layer chromatography (TLC) was performed on pre-coated aluminium plates (Silicagel 60 F254, Merck). Plates were visualized by UV light and iodine vapour.

2.1.3. Equipment

Melting points were measured on a electrothermal melting point apparatus Toshniwal apparatus (Toshniwal Company, Bangalore, India) and are uncorrected. Infrared (IR) spectra were recorded as KBr pellets with an FTIR-8300 spectrophotometer (Shimadzu). Proton magnetic resonance (^{1}H NMR) spectra were recorded in DMSO-d_6 (Merck) on a AMX-400 NMR spectrometer (IISc, Bangalore, India). Mass spectra were recorded on a GCMS (QP 5050A, Shimadzu Corporation, Japan). Absorption maxima were taken on a UV-Visible spectrophotometer-2450 (Shimadzu).

2.1.3.1. Synthesis of benzanilides (HB1a–10a). 0.02 mol of an appropriately substituted aniline was dissolved in 20 mL of pyridine. To this, 0.02 mol of appropriately substituted benzoyl chloride was added with dropwise addition. The reaction mixture was maintained at reflux for 2 h and then poured into water. The precipitate was collected, washed with water, dried and recrystallized from methanol. Yields and physical characteristics are listed in Table 1.

2.1.3.2. Synthesis of thiobenzanilides (HB1b–10b). A mixture of the substituted benzanilides (0.01 mol) and Lawesson's reagent (0.6 mol equiv.) was refluxed in chlorobenzene (5–10 mL) for 3 h to produce a clear solution. The solution was cooled and the product precipitated. This solid was collected and recrystallized from methanol. Yields and physical characteristics are listed in Table 2.

2.1.3.3. Synthesis of 2-phenyl-1,3-benzothiazoles (HB1c–10c). Substituted thiobenzanilides (0.01 mol) were wetted with a little ethanol (5.0 mL)and 30% aqueous sodium hydroxide solution (8.0 mol equiv.) was added. The mixture was diluted with water to provide a final solution/suspension of 10% aqueous sodium hydroxide. Aliquots of this mixture (1.0 mL) were added at 1 min intervals to a stirred solution of potassium ferricyanide (4.0 mol equiv.) in water at 80–90 °C. The reaction mixture was heated for an additional 30 min and then allowed to cool. The solid was collected, washed with water and recrystallized from methanol. Yields and physical characteristics are listed in Table 3.

2.1.3.4. HB1c [6-fluoro-2-(4-methylphenyl)-1,3-benzothiazole]. Yield: 52.1%. IR (KBr): 817.85 (C–H), 1606.76 (C=N), 702.11 (C–S), 1192.05 (C–F) cm^{-1}. ^{1}H NMR (DMSO): δ 2.4 (s, 3H, CH_3 of Ar), δ 7.25–7.9 (m, 7H, Ar). GC–MS: 243 (M^+).

2.1.3.5. HB2c [5,6-difluoro-2-(4-methylphenyl)-1,3-benzothiazole]. Yield: 53.9%. IR (KBr): 817.85 (C–H), 1608.69 (C=N), 709.83 (C–S), 1197.83 (C–F) cm^{-1}. ^{1}H NMR (DMSO): δ 2.4 (s, 3H, CH_3 of Ar), δ 7.25–7.9 (m, 6H, Ar). GC–MS: 261 (M^+).

2.1.3.6. HB3c [6-bromo-2-(4-methoxyphenyl)-1,3-benzothiazole]. Yield: 65.1%. IR (KBr): 819.77 (C–H), 1681.33 (C=N), 692.47 (C–S), 561.3 (C–Br), 1026.16, 1246.06 (C–O–C) cm^{-1}. ^{1}H NMR (DMSO): δ 3.85 (s, 3H, OCH_3), δ 7–8 (m, 7H, Ar). GC–MS: 321 (M^++1).

2.1.3.7. HB4c [6-fluoro-2-(4-methoxyphenyl)-1,3-benzothiazole]. Yield: 40.6%. IR (KBr): 821.70 (C–H), 1602.99 (C=N), 702.11 (C–S), 1193.98 (C–F), 1031.95, 1249.91 (C–O–C) cm^{-1}. ^{1}H NMR (DMSO): δ 3.85 (s, 3H, OCH_3), δ 7–8 (m, 7H, Ar). GC–MS: 259 (M^+).

2.1.3.8. HB5c [6-bromo-2-(4-methylphenyl)-1,3-benzothiazole]. Yield: 47.8%. IR (KBr): 831.35 (C–H), 1608.69 (C=N), 692.47 (C–S), 561.3 (C–Br), 1481.38 (C=C) cm^{-1}.

2.1.3.9. HB6c [5-chloro-2-(4-methoxyphenyl)-1,3-benzothiazole]. Yield: 60.4%. IR (KBr): 827.49 (C–H), 1606.76 (C=N), 690.54 (C–S), 1112.96 (C–Cl), 1485.24 (C=C) cm^{-1}.

2.1.3.10. HB7c [5-bromo-2-(4-methoxyphenyl)-1,3-benzothiazole]. Yield: 52.1%. IR (KBr): 831.35 (C–H), 1608.69 (C=N), 694.4 (C–S), 551.66 (C–Br), 1485.24 (C=C) cm^{-1}.

2.1.3.11. HB8c [5-fluoro-2-(4-methoxyphenyl)-1,3-benzothiazole]. Yield: 41.9%. IR (KBr): 825.56 (C–H), 1602.9 (C=N), 692.47 (C–S), 1222.91 (C–F), 1485.24 (C=C) cm^{-1}.

2.1.3.12. HB9c [5,6-difluoro-2-(4-methoxyphenyl)-1,3-benzothiazole]. Yield: 64.1%. IR (KBr): 833.28 (C–H), 1602.9 (C=N), 702.11 (C–S), 1209.41 (C–F), 1455.3 (C=C) cm^{-1}.

2.1.3.13. HB9c [4-fluoro-2-(4-methylphenyl)-1,3-benzothiazole]. Yield: 25.7%. IR (KBr): 819.77 (C–H), 1610.61 (C=N), 659.68 (C–S), 1188.19 (C–F), 1473.66 (C=C) cm^{-1}.

Table 3
Physical data of the intermediates HB1c–HB10c (benzothiazoles).

Compound	R_1	R_2	% Yield	Mt. pt. (°C)	R_f	Elemental analysis calculated (found)
HB1c	6-F	Me	52.1	118–126	0.689[a]	C-69.11% (69.10%) H-4.14% (4.13%) N-5.76% (5.75%)
HB2c	5,6-diF	Me	53.9	150–160	0.86[b]	C-64.35% (64.32%) H-3.47% (3.45%) N-5.36% (5.35%)
HB3c	6-Br	OMe	65.1	160–168	0.615[b]	C-52.51% (52.50%) H-3.15% (3.14%) N-4.37% (4.36%)
HB4c	6-F	OMe	40.6	120–128	0.899[c]	C-64.85% (64.83%) H-3.89% (3.88%) N-5.4% (5.41%)
HB5c	6-Br	Me	47.8	168–174	0.966[b]	C-55.28% (55.27%) H-3.31% (3.30%) N-4.60% (4.61%)
HB6c[d]	5-Br 7-Br	OMe OMe	60.4	116–122	0.614[b] 0.693	C-52.51% (52.50%) H-3.15% (3.14%) N-4.37% (4.36%)
HB7c[d]	5-Cl 7-Cl	OMe OMe	52.1	128–136	0.558[b] 0.628	C-60.98% (60.97%) H-3.66% (3.65%) N-5.08%(5.07%)
HB8c	4-F	OMe	41.9	112–118	0.678[b]	C-64.85% (64.83%) H-3.89% (3.88%) N-5.4% (5.41%)
HB9c	5,6-diF	OMe	64.1	132–140	0.655[b]	C-60.63% (60.62%) H-3.27% (3.26%) N-5.05% (5.06%)
HB10c	4-F	Me	25.7	140–148	0.891[b]	C-69.11% (69.10%) H-4.14% (4.15%) N-5.76% (5.75%)

[a] n-Hexane: 5.
[b] n-Hexane:ethyl acetate, 4.5:0.5.
[c] n-Hexane:ethyl acetate, 4:1.
[d] Gives regioisomers.

2.2. Biological evaluation and docking studies

2.2.1. Anticancer activity

Growth of breast cancer cells was quantitated by the ability of living cells to reduce the yellow MTT to purple formazan products [15]. The amount of formazan product formed is directly proportional to the number of living cells.

Synthesized compounds were prepared as 4.0 mM top stock solutions, dissolved in DMSO. MCF-7 human breast cancer cells (human breast adenocarcinoma cell line originally obtained in 1973 from Michigan Cancer Foundation) were cultivated at 37 °C in an atmosphere of 5% CO_2 in Dubecco's modified Eagle's minimal medium (DMEM) supplemented with 3.0 mM L-glutamine with 10% fetal bovine serum were routinely subcultured twice weekly to maintain in continuous logarithmic growth. Cells were trypsinized for the passage into the well plate and plated at 10,000 cells/well in 100 μL of medium in 96-well plates. Cells were allowed to adhere to the surface of well plates. After 24 h, medium was removed and 100 μL of drug solutions (prepared at 12.5, 25, 50 and 100 μM concentrations) were added into the wells. 100 μL of fresh medium without cells was added as control. 4 wells were used for each concentration of drug solution, while 4 wells were reserved for cell culture control, which contained the corresponding amount of DMSO. The total drug exposure was 72 h. After 72 h, contents of the well were removed and 20 μL of MTT solution (5 mg in 1 mL of phosphate buffer saline) was added to each well. Incubation at 37 °C for 4 h allowed reduction of MTT by mitochondrial dehydrogenase to an insoluble formazan product. Well contents were removed and the formazan product was solubilised by addition of 100 μL DMSO. The purple colour was produced. Absorbance of each well was read on Tenac 200 plate reader at 570 nm. From the absorbance, the % inhibition was calculated as% growth inhibition $= \frac{A_c - A_t}{A_c} \times 100$where A_c is the mean absorbance of control and A_t is the mean absorbance of test (Table 4).

2.2.2. Docking studies

The involvement of the EGFR family of tyrosine kinases in cancer proliferation suggests that an inhibitor which blocks the tyrosine kinase activity of the entire EGFR family, could have significant therapeutic potential [16]. So we selected EGFR as a biological target for carrying out the docking study of our synthesized compounds

Table 4
% Growth inhibition of MCF-7 cells.

Compound	% Growth inhibition			
	12.5 μM	25 μM	50 μM	100 μM
HB1c	5.2	8.26	29.33	62.8
HB2c	0.67	6.38	23.25	55.18
HB3c	16.78	23.8	42.17	60.64
HB4c	8.49	14.57	50.16	62.06
HB5c	5.41	4.77	15.29	57.14
HB6c	4.96	12.68	21.56	37.08
HB7c	2.75	9.28	11.58	34.49
HB8c	8.3	13.25	21.23	34.92
HB9c	0.48	1.67	11.08	20.46
HB10c	3.28	10.63	25.35	42.79

Table 5
Docking scores of the final synthesised compounds.

Compound	Dock score
HB1c	−44.8585
HB2c	−49.4539
HB3c	−48.1762
HB4c	−48.2842
HB5c	−47.4309
HB6c	−50.2033
HB7c	−48.6359
HB8c	−47.4589
HB9c	−52.0807
HB10c	−51.0141

and learn the mechanism of activity. The crystal structure of EGFR kinase domain in complex with an irreversible inhibitor (PDB ID: 2J5F) was obtained from the protein data bank. The crystal structure was refined using vLife Science's MDS 3.0 software [17]. The refinement of the crude PDB structure of receptor was done by completing the incomplete residues. The co-crystallized ligand lying within the receptor was modified by assigning missing bond order and hybridization states. The side chain hydrogens were then added to the crystal structure and their positions were optimized up to the rms gradient 1 by aggregating the other part of the receptor. The optimized receptor was then saved as mol file and used for docking simulation.

The 2D structure of the compounds HB1c–HBl0c were built and then converted into the 3D with the help of vLife MDS 3.0 software [18]. The 3D structures were then energetically minimized up to the rms gradient of 0.01 using Merck Molecular Force Field (MMFF) [19]. Conformers of all the synthesized ligands were then generated by Monte Carlo method. In doing so, all rotatable bonds of the ligands were selected and number of seeds used for searching the conformational space was set as 5. All conformers were then energetically minimized up to the rms gradient of 0.01 and then saved in separate folder [20]. The active site selection was done by choosing the cavity having maximum hydrophobic surface area. The docking simulation was done using GRIP batch docking.

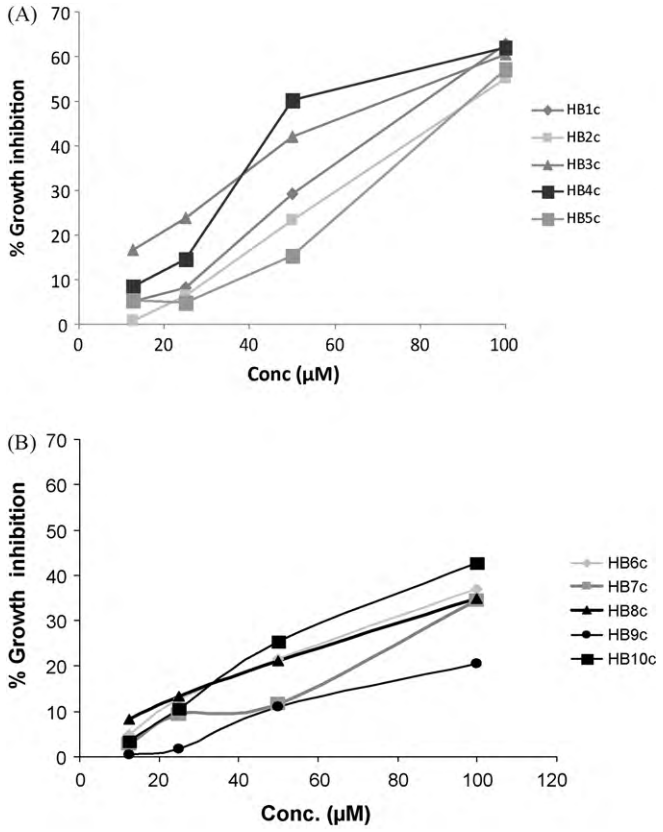
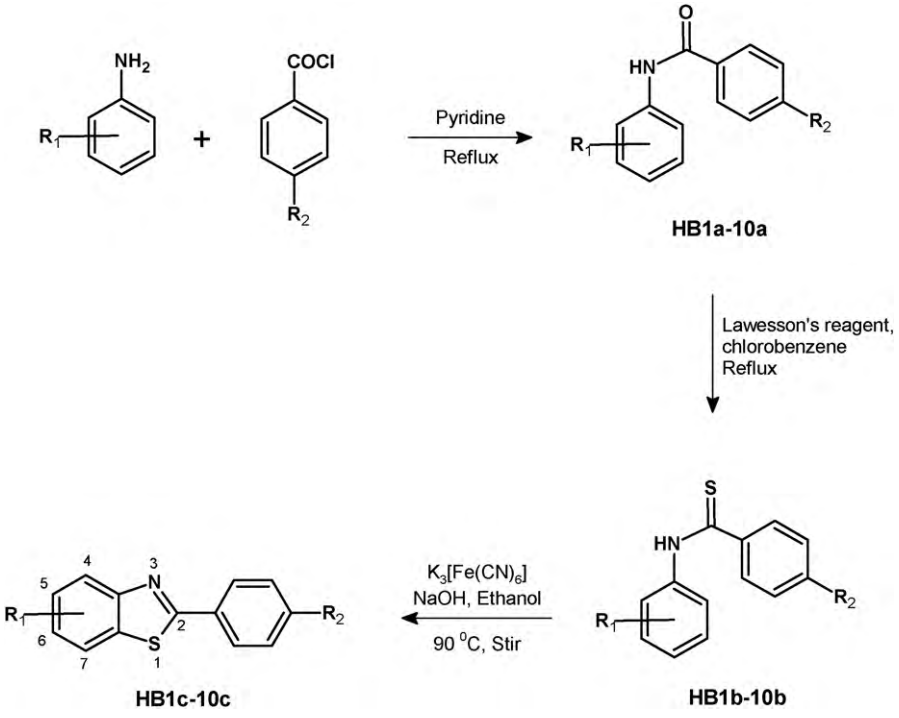


Fig. 1. Graph of % growth inhibition of MCF-7 cells by HB1c–HB5c (A) and HB6c–HB10c (B).

In this, all generated conformers of one ligand were put as one batch in GRIP docking wizard. Likewise, the batches for all other ligands were put. All the conformers were virtually docked at the defined cavity of the receptor. The parameters fixed for docking



Scheme 1.

simulation were like this – number of placements: 30, rotation angle: 30°, exhaustive method, scoring function: dock score. By rotation angle, the ligand gets rotated for different poses. By placements, the method will check all the 30 possible placements into the active site pocket and will result out few best placements out of 30. For each ligand, all the conformers with their best placements and their dock score will be saved in output folder. The method also highlights the best placement of best conformer of one particular ligand which is having best (minimum) dock score. In the results of docking, we have listed only best conformers and the dock score for each ligand in Table 5. The ligand forming most stable drug-receptor complex is the one which is having minimum dock score. After docking simulation, the best docked conformer of each ligand and receptor were merged and their complexes were then energetically optimized by defining the radius of 10 Å measured from the docked ligand. Stepwise energy optimization was done by first hydrogens , second side chains and finally the backbone of receptor [21]. The optimized complexes were then checked for various interaction of ligand with receptor like hydrogen bonding, hydrophobic bonding and van der Waal's interaction. No compound was found to exhibit hydrogen bonding with the receptor. So all the docked compounds were analyzed for various types of other interactions like hydrophobic bonding and van der Waal's interaction. All the compounds were found to form hydrophobic bonding by 'C' atom of methyl and methoxy group. Some of the common residues involved in this type of interaction are Leu178, Lys745, Thr790 and Val726. All the compounds were found to exhibit large number of van der Waal's bonding with wide range of residues. So it was very difficult to find out common residues involved in this type of interaction. Only Val726 was found to be the common residue involved in docking of all the ligands (Fig. 2B and C).

3. Results and discussion

Four derivatives of 2-phenyl-1,3-benzothiazole in which mono or di-halogenated substitution on benzothiazole moiety and methyl group at para position of 2-phenyl ring were synthesized. Same type of another six derivatives was also synthesized but they possessed methoxy group at para position of 2-phenyl ring as shown in Scheme 1.

In the case of cyclization of HB6b [*N*-(3-chlorophenyl)-4-methoxybenzenecarbothioamide], a mixture of the 5-chloro and its regioisomer 7-chloro methoxybenozthiazoles (HB6c) was formed . Similarly from HB7b [*N*-(3-bromophenyl)-4-methoxybenzenecarbothioamide], a mixture of the 5-bromo and 7-bromo substituted isomers (HB7c) was formed. It was very difficult to separate these mixtures of regioisomers. Therefore, the anticancer potential of HB6c and HB7c was found out by considering them as a mixture of regioisomers. In all other cases, only a single methyl and methoxy benzothiazole isomer was formed [22].

Structures, yields and physical characteristics of all methyl and methoxybenzanilides, thiobenzanilides and halogenated 2-(4-methylphenyl)-benzothiazoles and 2-(4-methoxyphenyl)-benzothiazoles are given in Tables 1–3 respectively.

In MTT assay, the anticancer activity of all the synthesized compounds was found out at 12.5, 25, 50 and 100 μM concentration. The % growth inhibition of MCF-7 cells (presented in Fig. 1 and listed in Table 4) was calculated from the measured absorbance of formazan product produced by living cells. At 12.5 μM concentration, compound HB3c showed highest cell growth inhibition, while HB1c, 4c, 5c, 6c and 8c showed moderate inhibition and HB2c and HB9c showed least inhibition. At 25 μM concentration, HB3c exhibited highest activity, while HB4c, 6c, 8c and 10c exhibited moderate activity and HB5c and 9c exhibited least activity. At 50 μM concentration, HB4c showed highest activity, while HB1c, 2c and 10c showed moderate activity and HB7c and 9c showed least activity. At 100 μM concentration, HB1c exhibited highest inhibitory activity, while HB6c, 7c and 8c exhibited moderate activity and HB9c showed least inhibitory activity. These results indicated that benzothiazole derivatives we synthesised are able to inhibit epidermal growth factor receptor (EGFR) in human breast cell line, MCF-7.

From the dock score, compounds HB9c, HB10c and HB6c were found to have highest negative dock score ranging from −52.0 to −50.0 (Table 5). It means that these formed most stable drug-receptor complex amongst other compounds. All the docked compounds were analyzed for various types of interactions like

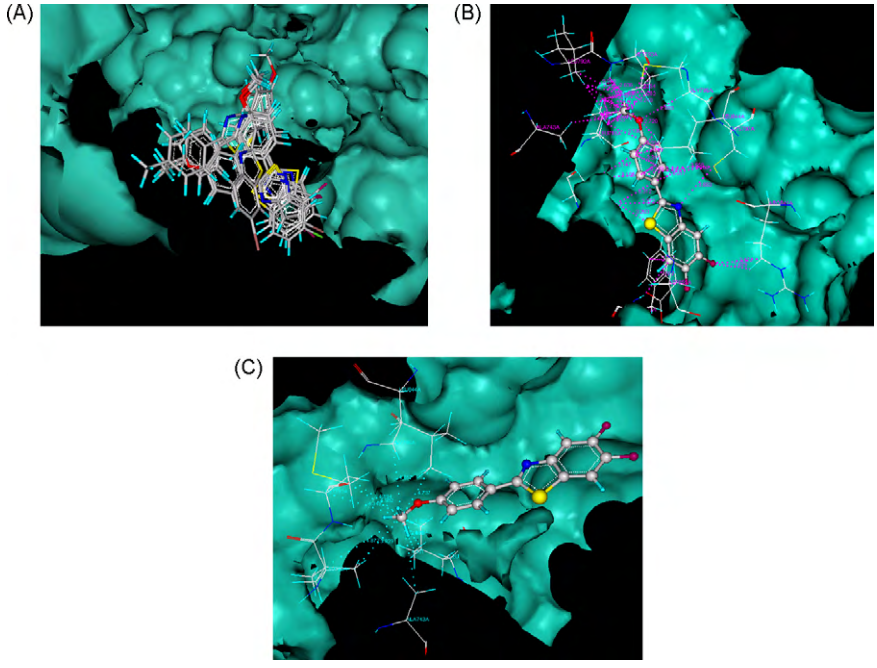


Fig. 2. (A) Docking position of all synthesised compounds in the receptor cavity. (B) van der Waal's interaction of HB9c (ball and stick) with the receptor residues (wire) shown by pink colour dotted lines. (C) Hydrophobic interaction of HB9c (ball and stick) with the receptor residues (wire) by 'C' atom of methoxy group shown by light green dotted lines.

hydrophobic bonding and van der Waal's interaction because no compound was found to exhibit hydrogen bonding with the receptor. Fig. 2A shows the docking position of all the compounds in the receptor cavity.

4. Conclusion

As the concentration of compound being tested increased, the *in vitro* anticancer activity also increased. Compound HB9c [5,6-difluoro-2-(4-methoxyphenyl)-1,3-benzothiazole] was found to be least active amongst all the tested compounds at all the concentration levels. It may be concluded that, compounds having mono bromo or mono fluoro substitution on benzothiazole moiety exhibit good anticancer activity against MCF-7 breast cancer cells. On the other hand, difluoro substitution on benzothiazole ring, reduces the activity to greater extent. Exact correlation between methyl/methoxy substitution on 2-phenyl ring and anticancer activity is not established. The docking score of the synthesised compounds could not be correlated with the i*n vitro* anticancer activity and conclusion could not be drawn on their exact mechanism of action. So further molecular modification on 2-phenyl-1,3-benzothiazole is required in order to arrive at more accurate structure activity relationship with their anticancer activity on breast cancer cell lines or different EGFR crystal structure could be selected from the PDB to study their mechanism of action.

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