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Research Article

IN SILICO DESIGN, SYNTHESIS AND CHARACTERIZATION OF SOME NOVEL BENZOTHIAZOLE DERIVATIVES AS ANTICANCER AGENTS

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ABSTRACT

Objectives: Cancer is a disease characterized by uncontrollable, irreversible, independent, autonomous, uncoordinated, relatively unlimited, and abnormal overgrowth of tissues. Breast cancer is the second most common type of cancer after lung cancer. The aim of the study is to carry out the docking studies, synthesis, and antitumor activities of benzothiazole derivatives containing oxadiazole groups or amino groups.

Methods: The docking studies of benzothiazole derivatives were done with known anticancer targets like estrogen receptor using Argus lab and AutoDock programs and compared with the standard drug tamoxifen. Based on the results obtained from the molecular modeling studies, the derivatives were selected for the synthesis. The synthesized compounds were characterized by melting point, thin layer chromatography, InfraRed, ^{1}H NMR, ^{13}CNMR, mass spectral data, and screened for their *in-vitro* anticancer activities.

Results: The docking scores obtained for benzothiazole derivatives (BT1, BT2, BT3, BT4) and std. tamoxifen from the preliminary docking program using Argus Lab were −9.68, −9.4, −9.59, −11.1988, −9.71 and using AutoDock program were −6.29, −5.25, −7.19, −7.48, −3.86, respectively. All the four derivatives were synthesized, characterized, and subjected to *in vitro* anticancer screening by MTT assay in breast cancer (MCF-7) cell lines. Compounds DBT1, DBT2, and DBT3 were the most active compounds against MCF-7 cell lines with inhibitory concentration 50% of 70.0, 64.0 and 65.0, respectively.

Conclusion: All the four derivatives show good docking scores when compared to standard drug tamoxifen and can be concluded that all the synthesized benzothiazole ligands show good anticancer property.

Keywords: Benzothiazole, Oxadiazole, Estrogen receptor, Anticancer targets.

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INTRODUCTION

Benzothiazole is a heterocyclic compound which possesses various biological activities. It is of great scientific interest nowadays. Benzothiazoles are fused membered rings which consist of thiazole ring fused with benzene ring. They are widely found in bio-organic and medicinal chemistry with application in drug discovery [1]. Benzothiazole moieties are part of compounds showing numerous biological activities such as antifungal [2], antiepileptic [3] anticancer [4], anti-inflammatory (COX-inhibitors) [5], antidiabetic [6], anticonvulsant [7], antimicrobial [8] diuretic [9], antitubercular [10], schictosomicidal [11], and anthelmintic [12] activities. Benzothiazole is used in research as a starting material for the synthesis of various bioactive structures. Its aromaticity makes it a relatively stable compound [13]. Drug design, sometimes referred to as rational drug design or more simply rational design, is the inventive process of finding new medications based on the knowledge of a biological target. The drug is most commonly an organic small molecule that activates or inhibits the function of a biomolecule such as a protein, which in turn results in a therapeutic benefit to the patient [14] Docking is a method which predicts the preferred orientation of one molecule to the second when bound to each other to form a stable complex. Breast cancer is a type of cancer originating from breast tissue, most commonly from the inner lining of milk ducts or the lobules that supply the ducts with milk.

METHODS

In silico molecular modeling

In silico drug, designing is a technique used for the design of new drug molecules, and the identification of drug targets using various bioinformatic tools with the help of computers. Analysis of Lipinski's rule of five and determination of toxicity parameter was carried out.

Target identification and retrieval

Crystallographic structures of the target of interest were obtained from Protein Data Bank (PDB) and saved in standard three dimensional (3D) coordinate format. The selected target and its PDB ID are mentioned in Table 1.

Estrogen receptor (ER) [15]

Breast cancer, the most common malignancy in women, was already known to be associated with the steroid hormone estrogen. The discovery of the ER provided us not only with a powerful predictive and prognostic marker but also an efficient target for the treatment of hormone-dependent breast cancer with antiestrogens [16].

3D structures of inhibitors

The chemical structures of inhibitors were designed, and the structure was analyzed using Chemsketch. Molecular docking and visual inspection are carried out. The list of compounds undergone for docking studies was mentioned in Table 2.

Experimental part

Preparation of 4, 6-disubstituted-1,3-benzothiazol-2-amine derivatives

Substituted aniline and potassium thiocyanate were dissolved in ethanol containing 2 ml concentrated hydrochloric acid (HCL). To this, bromine in glacial acetic acid was added, and the reaction mixture was refluxed for 1 hr. Then, the mixture was poured into crushed ice. The precipitate obtained was strained well. It was then filtered, washed

with water and dried. The crude precipitate was recrystallized from rectified spirit [17]. The reaction is shown in Fig. 1.

Preparation of 2-(5-substituted-1, 3, 4-oxadiazole-2-yl)-1, 3-benzothiazole

Step 1: Preparation of ethyl-2-benzothiazole carboxylate

Ethyl-2-benzothiazole carboxylate was synthesized by the following method. A mixture of *o*-aminothiophenol (0.1 mol) and diethyl oxalate (0.2 mol) was heated at mild reflux for 4 hrs, during which the temperature decreases from 147°C to 93°C. After cooling, the mixture was poured into a solution consisting of 50 ml of concentrated HCL, 150 ml of water and 70 ml of ethanol. The oil got dissolved and a solid formed, upon stirring. The mixture was cooled. The product was filtered and washed with aq. Ethanol, then dried and finally recrystallized from pet ether.

Step 2: Preparation of 1, 3-benzothiazole-2-carboxyhydrazide

Into a clean, dry 100 mL round bottomed flask, the ethyl-2-benzothiazole carboxylate (1) (0.01 mol) was dissolved in ethanol (60 mL). The hydrazine hydrate (0.02 mol) (99%) was added drop by drop with constant stirring and the content were refluxed for 8 hrs, cooled to room temperature. The solid separated was filtered and washed with water, dried and finally crystallized from ethanol.

Step 3: Preparation of 2-(5-substituted-1, 3, 4-oxadiazole-2-yl)-1, 3-Benzothiazole.

A mixture of 1, 3-benzothiazole-2-carboxyhydrazide (2) (0.01 mol), appropriate aromatic acid (0.02 mol) and phosphoryl chloride (10 mL) was refluxed on water bath for 6-8 hrs. After cooling to room temperature, it was poured into the crushed ice with stirring. The solid thus obtained was filtered, washed with water and crystallized from ethanol [18]. The reaction is shown in Fig. 2.

Table 1: Selected target and its PDB ID

Target	PDB ID
Estrogen receptor	3ERT

Spectral analysis

All the synthesized compounds were characterized by InfraRed (IR), NMR, and mass spectroscopy.

Pharmacological evaluation

Anticancer activity

Chemicals

3-(4,5–dimethyl thiazol–2–yl)–5–diphenyltetrazolium bromide (MTT), fetal bovine serum (FBS), phosphate buffered saline (PBS), Dulbecco's Modified Eagle's Medium (DMEM), and Trypsin were obtained from Sigma-Aldrich Co, St Louis, USA. Ethylenediaminetetraacetic acid (EDTA), glucose and antibiotics from Hi-Media Laboratories Ltd., Mumbai. Dimethyl sulfoxide (DMSO) and Propanol from E. Merck Ltd., Mumbai, India.

Cell lines and culture medium

MCF-7 cell line was procured from National Centre for Cell Sciences, Pune, India. Stock cells were cultured in DMEM, supplemented with 10% inactivated FBS, penicillin (100 IU/ml), streptomycin (100 g/ml), and amphotericin B (5 g/ml) in an humidified atmosphere of 5% CO_2 at 37°C until confluent. The cells were dissociated with trypsin phosphate versene glucose solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm^2 culture flasks and all experiments were carried out in 96 microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

Preparation of test solutions

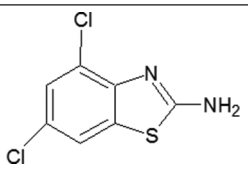
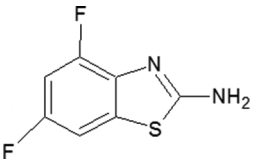
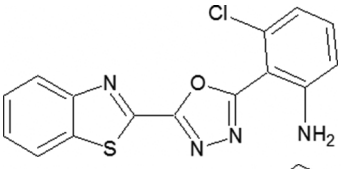
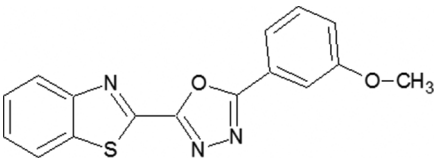
For cytotoxicity studies, each test drug was weighed and mixed to obtain the desired concentration and dissolved in distilled DMSO and volume was made up with DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration. Serial two-fold dilutions were prepared from this for carrying out cytotoxic studies.

Determination of cell viability by MTT assay [19,20]

Procedure

The monolayer cell culture was trypsinized, and the cell count was adjusted to 1.0×10^5 cells/ml using DMEM containing 10% FBS. To

Table 2: Compounds undergoing docking study

Compound code	Structure	Chemical name
DBT1		4,6-dichloro-1,3-benzothiazol-2-amine
DBT2		4,6-difluoro-1,3-benzothiazol-2-amine
DBT3		2-[5-(1,3-benzothiazol-2-yl)-1,3, 4-oxadiazol-2-yl]-3-chloroaniline
DBT4		2-[5-(3-methoxyphenyl)-1,3, 4-oxadiazol-2-yl]-1,3-benzothiazole

each well of the 96 well microtiter plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 hrs, when a partial monolayer was formed, the supernatant was flicked off. The monolayer was washed once with the medium. 100 µl of different test concentrations of test drugs were added on to the partial monolayer in microtitre plates. The plates were then incubated at 37°C for 3 days in 5% CO_2 atmosphere, and microscopic examination was carried out and observations were noted every 24 hrs interval. After 72 hrs, the drug solutions in the wells were discarded and 50 µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 hrs at 37°C in 5% CO_2 atmosphere. The supernatant was removed and 100 µl of propanol was added, and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (cytotoxic concentration$_{50}$) values is generated from the dose-response curves for each cell line.

$$\text{Percentage growth inhibition} = 100 - \left(\frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}} \right) \times 100$$

RESULT AND DISCUSSION

Molecular docking

Benzothiazole derivatives were subjected to molecular docking against ER. The resulting docking score helped to select the more potent derivatives for the synthesis. *In silico* studies were done using different softwares such as ArgusLab, AutoDock, ACD/Chemsketch, and molinspiration. ArgusLab serves as a primary docking tool. The prepared ligands were validated by docking operation using ArgusLab and AutoDock. The docking score of the ligands was compared with the reference standards. The docking scores obtained from the preliminary docking program using ArgusLab were listed in Table 3.

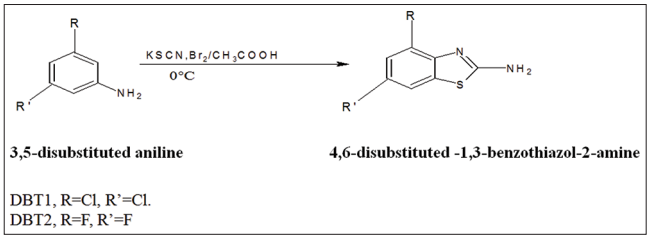


Fig. 1: Reaction of 4,6-disubstituted -1,3-benzothiazol-2-amine derivatives

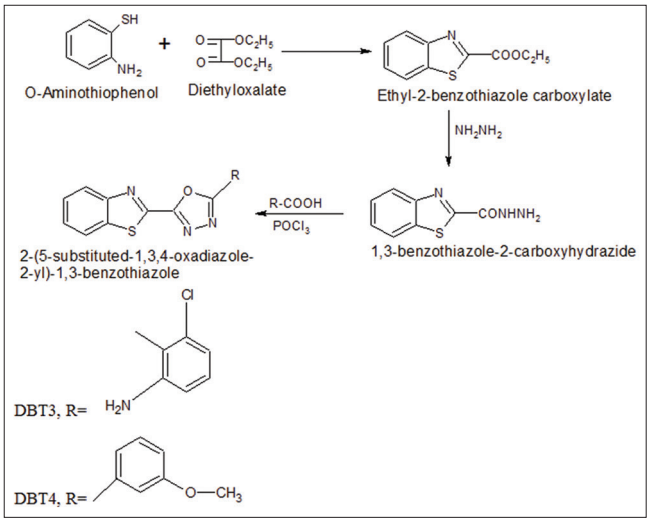


Fig. 2: Reaction of 2-(5-substituted-1, 3, 4-oxadiazole-2-yl)-1, 3-benzothiazole

The docking scores were obtained from the analogs against ER receptors. All the compounds show good docking scores when compared to standard drug. Docking score of the compounds targeted ER was compared with the score of the drug Tamoxifen which is used as a potent drug for the treatment of breast cancer. Benzothiazole derivatives were docked with the crystallographic structures of the targets by AutoDock version 4.0 screening program as shown in Table 4. The analogs were examined for their binding energies and hydrogen bonding. The conformations with the highest binding energies and greater number of hydrogen bonds of all the ligands were taken in consideration for ranking the analogs. It is listed in Table 4.

All the analogs show higher docking scores when compared to standard drug. AutoDock shows lowest docking scores than ArgusLab but have more accuracy. All the ligands show good docking scores when compared to standard drug. The interactions were stronger (energetically lesser) for all the ligands which are used for docking simulation.

AutoDock screening program also helped to know about the hydrogen bonding interactions of all the derived compounds. It showed in Table 5.

Residues ARG 374, ARG 928, ARG 926, ASN 375, ARG 376, ARG 346, THR 206, TYR 385, HIS 386, THR 212, TRP 345, MET 421, and HIS 524 were predicted as active site in the target proteins, ER. Number of hydrogen bonding will considerably increase the affinity of ligand-target interaction. AutoDock results show that most of the benzothiazole derivatives show higher hydrogen bonding between the ligand-target interactions. Some of the derivatives showing oxadiazole moieties show more than two hydrogen bonds (commonly ARG 374, ASN 375

Table 3:Argus Lab docking scores for the designed Benzothiazole derivatives

Compound code	Docking score (kcal/mol)
	ER
DBT1	−9.68
DBT2	−9.4
DBT3	−9.59
DBT4	−11.1988
Tamoxifen (Std)	−9.71

Table 4: Ligand-target hydrogen bonding interactions

Compound code	Hydrogen bonding interactions
	ER
DBT1	HIS 524
DBT2	ARG 346
DBT3	TRP 345
	ARG346
DBT4	MET 421

Table 5: AutoDock docking scores

Compound code	Docking score (kcal/mol
	ER
DBT1	−6.29
DBT2	−5.25
DBT3	−7.19
DBT4	−7.48
Tamoxifen (Std)	−3.86

or ARG 376). This hydrogen bonding interactions help to increase the binding energy of ligand-protein interactions.

Some of the high docking scores ligand-target complexes with different hydrogen bonding interactions generated by AutoDock 4.0 program were shown in Fig. 4. in a separate file.

In silico analysis

Drug-likeness assessment

The derived analogs were evaluated for their drug-likeness. It was done by calculating the parameters like Lipinski rule of 5 and some of their extension parameters like number of rotatable bonds and cLog P. The drug-likeness assessments of the compounds were shown in Table 6.

This result showed that the value of all the derivatives relies within the optimal range. All the compounds had the molecular weight <500 daltons and possessed number of hydrogen bond donors and hydrogen bond acceptors of all the analogs below 5 and 10, respectively. All the values of partition coefficient and number of rotatable bonds were coming under the limit of 5. All these data indicate that violations are unlikely and the compounds may form an orally active drug.

Toxicity parameters by means of ADMET predictor

ADMET predictor was a computer software system for predictive modeling of absorption, distribution, metabolism, elimination, and toxicity of chemical substances in the human body. The results for the toxicity parameters using ADMET predictor were shown in Table 7.

BBB: 0-very high penetration, 1-high penetration, 2-medium penetration, 3-low penetration, and 4-undefined. Absorption level: 0-good absorption, 1-moderate absorption, 2-poor absorption, and 3-very low absorption. Solubility level: 0-very high solubility, 1-high solubility, 2-medium solubility, 3-low solubility, and 4-undefined. Hepatotoxicity: 0-non-toxic, unlikely to cause dose-dependent liver injuries; 1-toxic, likely to cause dose-dependent liver injuries. CYP2D6 Inhibition: 0-not a likely inhibitor; 1-potential inhibitor. Plasma protein binding (PPB) level: 0-binding is <90% (No markers flagged and AlogP98 < 4.0); 1-binding is = 90% (flagged at 90% or AlogP98 = 4.0); 2-binding is = 95% (flagged at 95% or AlogP98 = 5.0).

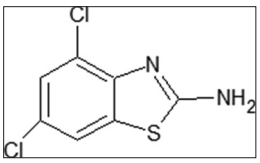
Toxicity studies were carried out using ADMET SAR to determine their pharmacokinetic (ADMET) properties. A high degree of PPB implies high lipophilicity which makes the drug long-acting. All the compounds show high and medium penetration to penetrate blood–brain barrier. BT1and BT4 showed very good level of intestinal absorption after oral administration. BT2 has medium aqueous solubility, and all other compounds show low solubility. All the compounds show dose-dependent hepatotoxicity. CYP2D6 is the enzyme which metabolizes the 20% of the drug; all the compounds showed inhibition against CYP2D6. All the derivatives showed <90% binding to plasma proteins.

Synthesis

Benzothiazole analogs were screened by *in silico* molecular docking and analysis showed better activity against the selected anticancer targets. The selected derivatives were synthesized in two-step process by refluxing benzothiazolyl carboxyhydrazide with different aryl acids in phosphoryl chloride to give substituted benzothiazole derivatives. Prepared four compounds are DBT1, DBT2, DBT3, and DBT4.

Characterization of prepared compounds

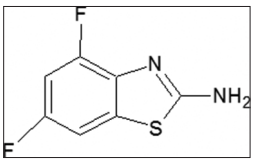
DBT1



Molecular formula: $C_7H_4C_{l2}N_2S$ molecular Weight: 219.09
Melting point: 194-196°C percentage yield: 58.2%
R_f value: 0.64 (Pet.ether: Ethylacetate (1:1))

IR (KBr) 3367, 3455 (N-H str. Of 1º amine), 3071 (arom. –CH str), 1457 (C=C str in aromatic hydrocarbons), 1292 (C-N str. Of amino grp), 773 (C-Clstr).;^{1}H NMR (DMSO, 400 MHz) δ5.563 (s, 1H), δ7.49 (s, 1H), δ7.42 (s, 1H)^{13}C NMR (DMSO, 100MHz) δ160.33, 157.28, 149.09, 148.79, 130.90, 129.94, 129.87, 129.57, 126.56, 121.80, 120.43; ESI MS (m/z relative abundance) 220 [$(M+H)^+$, 28], 211 [$(C_7H_3Cl_2NS)^+$], 171 [$(C_7H_7Cl_2)^+$,100].

DBT2



Molecular formula: $C_7H_4F_2N_2S$ molecular Weight: 186.18
Melting point: 216-220°C percentage yield: 42.23%
R_f value: 0.72 (ethylacetate: acetone(9:1))

IR (KBr) 3284, 3154 (N-H str. Of 1º amine), 3081 (arom. –CH str), 1493 (C=C str in aromatic hydrocarbons), 1256 (C-N str. Of amino grp), 1141 (C-F str);^{1}H NMR (DMSO, 400 MHz) δ6.697 (s, 1 H), δ6.694 (s, 1H), δ6.676 (s, 1H);^{13}C NMR (DMSO, 100MHz) δ163.44, 161.01, 160.29, 159.02, 149.06, 149.00, 148.88, 130.68, 130.59, 129.91, 123.46; ESI MS (m/z relative abundance) 187 [$(M+H)^+$], 178 [$(C_7H_3F_2N_2S)^+$], 170 [$(C_7H_2F_2S)^+$, 100].

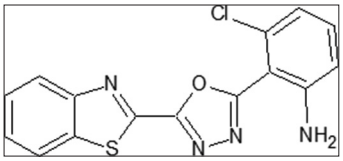
Table 6: Drug-likeness assessments of the benzothiazole derivatives

Compound code	Molecular weight	Number of HBA	Number of HBD	C Log P	Number of ROT.B	n violation
DBT1	219.096	2	2	3.238	0	0
DBT2	186.186	2	2	2.209	0	0
DBT3	328.784	2	5	4.348	2	0
DBT4	309.35	0	5	4.316	3	0

Table 7: Results of ADMET ox parameters

Compound code	BBB level	Absorption level	Solubility level	Hepatotoxicity	CYP2D6 inhibition	PPB level
DBT1	1	0	3	1	1	1
DBT2	1	1	2	1	1	1
DBT3	1	1	3	1	1	1
DBT4	0	0	3	1	1	1

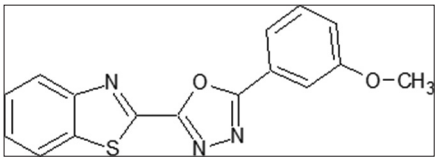
DBT3



Molecular formula: $C_{15}H_9ClN_4OS$ molecular Weight: 328
Melting point: 192-196°C percentage yield: 16.20%
R_f value: 0.6 (chloroform: Methanol (9:1))

IR (KBr) 3476, 3367 (N-H str. Of 1º amine), 3029 (arom. –CH str), 1577 (C=C str in aromatic hydrocarbons), 1266 (C-N str. Of amino grp), 1126 (C-O-C str), 804 (C-Clstr).;^{1}H NMR (DMSO, 400 MHz) δ6.58 (s, 1H), δ6.606 (d, J=1.2, 1H), δ6.697 (t, J=1.2, 2H), δ7.093 (t, J=8, 2H);^{13}C NMR (DMSO, 100MHz) δ160, 167, 157, 158, 148.83,148.80, 133.10, 130.57, 129.86, 127.08, 126.67, 126.17, 120.43, 112.94, 112.51, 102.23, 55.99, 45.58, 40.11, 39.91, 39.70, 39.49, 39.28, 39.07; ESI MS (m/z relative abundance) 330 [(M+2)$^+$,], 259 [($C1_4H_8ClN_2O$)$^+$], 243 [($C_{14}H_8ClN_2$)$^+$, 100.

DBT4



Molecular formula: $C_{16}H_{11}N_3O_2S$ molecular Weight: 309
Melting point: 190-194°C percentage yield: 18.20%
R_f value: 0.7 (ethylacetate: Acetone (9:1))

IR (KBr) 3072 (arom. –CH str),1457 (C=C str in aromatic hydrocarbons), 1105 (C-O-C str), 2832 (C-O-CH_3^{st}r);^{1}H NMR (DMSO, 400 MHz) δ3.87 (s, 1H), δ7.169 (s, 1H), δ7.625 (s, 1H), δ7.176 (d, J=1.2,1H), δ7.4 (t, J=8, 2H);^{13}C NMR (DMSO, 100MHz) δ160, 169, 157, 148.83, 148.80, 133, 130.57, 129.86, 127.08, 126.67,125, 120.43, 112.94, 112.51, 102.23, 55.49, 55.46, 45.58, 40.11, 39.91, 39.70, 39.49, 39.28, 39.07, 38.86; ESI MS (m/z relative abundance) 309 [(M)$^+$,16], 277 [($C_{16}H_{11}N_3O_2$)$^+$, 27], 225 [($C1_5H_{10}N_2$)$^+$, 100].

IR spectrum clearly suggested the formation of expected compounds.

H^1 and C^{13} NMR were carried out using DMSO as solvent. C^{13} NMR signals H and^{13}C NMR spectral data led to confirm the structure of targeted derivatives.

***In vitro* screening**

In vitro cytotoxicity against MCF-7 cell lines by MTT assay

In vitro anticancer screening was carried out by MTT assay in breast cancer (MCF-7) cell lines. Four of the synthesized compounds were submitted to cytotoxicity study. Medium of cancer cell lines without samples were served as control. It is listed in Table 8.

Graphical representation of cytotoxic effect of prepared derivatives
The graph was plotted between concentration and percentage inhibition using Graph Pad Prism software.

All the compounds were screened for *in vitro* anticancer activity at different concentrations of 1000, 500, 250, 125, and 62.5 µg/mL. All the compounds possess good to moderate anticancer activity. Compounds DBT1, DBT2, and DBT3 were the most active compounds against MCF-7 cell lines with inhibitory concentration 50% of 70.0, 64.0, and 65.0, respectively.

Table 8: Percentage cytotoxicity of prepared compounds against breast cancer (MCF-7) cell lines

Code of compound	Test concentration (µg/ml)	Percentage cytotoxicity	CTC_{50} (µg/Ml)
DBT1	1000	84.13±0.2	70.0
	500	81.34±0.4	
	250	79.86±0.2	
	125	78.29±0.2	
	62.5	48.28±0.3	
DBT2	1000	74.29±0.6	64.0
	500	72.46±0.5	
	250	67.32±0.5	
	125	61.52±0.2	
	62.5	50.47±0.2	
DBT3	1000	88.13±0.2	65.0
	500	82.46±0.4	
	250	68.19±0.3	
	125	52.49±0.2	
	62.5	49.76±0.3	
DBT4	1000	82.39±0.1	118.00
	500	75.32±0.2	
	250	70.46±0.1	
	125	51.32±0.1	
	62.5	38.97±0.2	

CTC: Cytotoxic concentration

CONCLUSION

All the derivatives show good docking scores when compared to standard drug and can be concluded that all designed ligands can be potent anticancer agents. The analysis of drug-likeness and toxicity studies strongly suggested the possibility of oral activity of the drug. As the concentration of compound being tested increased, the *in-vitro* anticancer activity also increased. Substitution on the 5th position of oxadiazole ring with aromatic group increases the activity. Introduction of an ethylene bridge between oxadiazole and aromatic substitution resulted in an analog with best binding potency. From the studied compounds, it was noticed that the presence of electron donating groups and electronegative halogens improve the activity of the compounds (DBT1, DBT2, DBT3, and DBT4). This revealed that the designed and the synthesized benzothiazole analogs had good anticancer activity.

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