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IN SILICO DESIGN, SYNTHESIS AND STUDY OF NOVEL N-SUBSTITUTED PHTHALIMIDE DERIVATIVES FOR ITS ANTI-CANCER ACTIVITY AGAINST TARGETED ENZYMES IN BREAST CANCER

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ABSTRACT

The estimated number of breast cancer fatalities among women worldwide in 2020 was 6,85,000, or 15% of all cancer deaths among women. In India, there were an estimated 1,78,361 new cases and 90,408 deaths from the disease, according to Global statistics in 2020. Breast cancer is one of the second most common cancer in India and causes the greatest number of cancer-related deaths among women. The development of multi-drug resistance and undesirable side effects leads to investigating the new anticancer agents. In order to overcome the new cancers, the present work focussed on to develop some novel anti-breast cancer agents. The study involved "*In-silico* design, synthesis and study of novel N-substituted phthalimide derivatives for its anti-cancer activity against targeted enzymes in breast cancer. The purity of synthesized compounds was determined by melting point determination and thin layer chromatography. The structures of the synthesized compounds were characterized using FT-IR, $_{1}$H-NMR, $_{13}$C-NMR and Mass spectral analysis. Molinspiration software were used to study the Drug likeness properties of the synthesized compounds. All the synthesized compounds obeyed the Lipinski rule of Five. SWISS ADME online web tool was used to predict the *in silico* ADME studies and OSIRIS property explorer were used to study the toxicity profile. Molecular docking studies for all the designed compounds were done using Autodock software version 4.2 against the various target enzymes such as Dihydrofolate reductase, Poly (ADP-Ribose) polymerase and Epidermal Growth factor receptor. The compound DPAL showed best binding energy of 10.24&-9.31 kcal/mol when compared to the binding energy of standard Methotrexate -4.44 and Talazoparib-6.59 kcal/mol against the target Dihyrofolate reductase (DHFR) and Poly (ADP-ribose) polymerase(PARP-I).The compound DPSML showed best binding energy of -8.37 kcal/mol compared with the standard Lapatinib -6.99 kcal/mol against the Epidermal growth factor receptor (EGFR).The present research study showed promising response in the molecular docking studies against the targeted enzyme in breast cancer. Hence, this research work will be further continued by designing few more novel analogues and study of its potential against the targeted enzyme for its anticancer activity against the breast cancer cell lines.

Keywords: Phthalimide, Breast cancer, Molecular docking, Dihydrofolate reductase, Epidermal growth factor receptor, Autodock4.2

INTRODUCTION

The most common kind of cancer known globally is Breast cancer. It accounts for 23% of all cancer cases, 14% of cancer deaths, and is now the most common cancer among women in developing nations **[1]**. It is also the most frequently diagnosed cancer and the leading cause of cancer death among females. TNBC has a 1% immune staining expression of the estrogen and progesterone receptors and is human epidermal growth factor receptor 2 negative according to clinical testing **[2]**. Chemotherapy is currently the cornerstone for breast cancer treatment; nevertheless, its toxicity in normal cells and developed tumor resistance to the drug employed are thought to be the main hurdles. With certain secondary causes including virus-mediated genetic disruptions and many more, environmental factors and dietary habits are the main sources of BC induction. Therefore, the creation of new potent and secure anti-breast cancer treatments is still urgently needed **[3]**.

There is a need to create a new class of anticancer drugs that specifically target Breast cancer cells despite substantial research and quick advancements in cancer treatment. One of the intriguing candidates that has garnered a lot of attention from medicinal chemists in recent studies is phthalimide. Although there are many commercially accessible anticancer medications, there are still problems with drug resistance, toxicity and therapy. Phthalimide (isoindoline1,3-dione) has frequently been used in the development of potential anti-tumor **[4]**, immunomodulatory **[5]**, anti-angiogenic **[6]**, anti-microbial **[7]** and anti-inflammatory **[8]**. Phthalimides have a hydrophobic chemical structure, which boosts their ability to pass biological membranes *in vivo* **[9]**.

Based on the biological importance of phthalimide derivative with limited toxicity and less multi drug resistance, the present study deals with*in silico* design, synthesis, molecular docking and study of novel N-substituted phthalimide derivatives against targeted enzyme for breast cancer such as Dihyrofolate reductase (6DAV), Epidermal growth factor receptor (6TFY), Poly (ADP-ribose) polymerase (2RIQ). To make a novel derivative, phthalic anhydride was combined with a number of primary amines, and then it was further reacted with lamivudine to produce the appropriate N-substituted phthalimide derivative.

MATERIALS AND METHODS:

The compounds were designed by Chemsketch software. The designed compounds were subjected to *insilico* toxicity studies (OSIRIS) and evaluation of Lipinski rule of five properties. AutoDock software version 4.2 was used for Molecular docking study.

EXPERIMENTAL:

Analytical grade chemicals and reagents were employed throughout the experiment. Utilizing the one end sealed open capillary tube approach, the melting points of the produced compounds were ascertained. Using chloroform: ethyl acetate (9:1) and chloroform: ethyl acetate: methanol (5:3:2) spots, TLC was used to monitor the reaction's progress on a precoated silica gel plate. With the help of FT-IR, ^{1}H-NMR, ^{13}C-NMR, and mass spectrometry, the synthesized chemical was identified.

CHEMISTRY:

Step1: Equimolar concentration of primary amine and phthalic anhydride (0.001 mole) in 5ml of glacial acetic acid were taken in a round bottom flask and refluxed for 20-30 minutes. The N-substituted phthalimide were separates out on cooling. The formed product was recrystallized using ethanol or glacial acetic acid **(Figure 1)**.

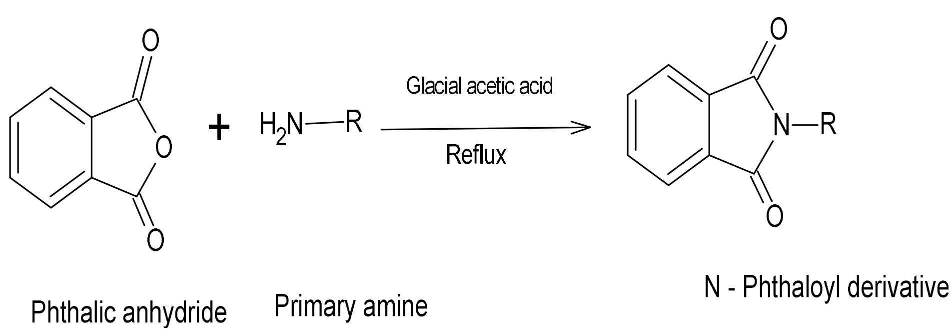


Figure 1: Synthesis of N-phthaloyl derivatives

Step 2: In a reaction flask, 1 mole of the N-phthaloyl derivative produced in Step 1 was added along with 1.2 moles of potassium hydroxide in methanol and 1 mole of

Lamivudine. The reaction mixture was then stirred using a magnetic stirrer at room temperature until it was fully completed. Using the ratio of chloroform: ethyl acetate: methanol [5:3:2], the reaction was seen by TLC. Glacial acetic acid was used to recrystallize the produced crude product **(Figure 2)**.

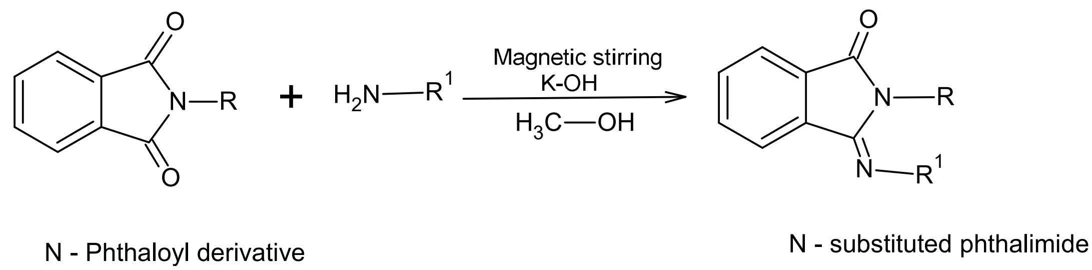


Figure 2: Synthesis of N-substituted phthalimide

PHYSICAL AND SPECTROSCOPIC DATA OF SYNTHESIZED PRODUCTS:

Physicochemical properties: The synthesized compounds were screened for their physicochemical properties of the synthesized compound. The purity of the synthesized compound was determined by melting point and Thin Layer Chromatography. Elemental analysis of all synthesized compound were determined by Chemsketch software.

Spectroscopic data for the Synthesized Product: The structure of the synthesized compounds was characterized byvarious spectroscopy.

Compound DPSAL:

^{1}H NMR; δ ppm: 5.3 (-OH), 8.0 (-CH), 3.91 (CH_2), 7.9 (-CH), FT-IR; (KBr, cm^{-1}): 1267 (C-H) aromatic, 1085 (S=O), 3142 (-OH), 1483 (C=C) aromatic, 1714 (C=O), 1318 (C-N), Mass (m/z): 515

***IN SILICO* STUDIES:**

Evaluation of Lipinski's rule of five and drug likeliness properties:

The synthesized compounds were evaluated for their Lipinski's rule of five and drug likeliness properties using molinspiration software.

ADMET properties:

The various pharmacokinetic properties were evaluated using Osiris property explorer.

Molecular docking study:

Ligand Preparation: Structure of synthesized compounds were drawn using MARVINSKETCH software and ligands are saved in pdb file format.

Protein Preparation: The crystal structure of protein Dihydro folate reductase (PDB Id: 6DAV) **[10]**, PARP-I ((PDB Id: 2RIQ) **[11]** and Epidermal Growth Factor Receptor (PDB Id: 6TFY) **[12]** was downloaded from RCSB PDB (www.rcsb.org) and the protein is saved in pdb file format. Remove the heteroatoms from the pdb file. Protein were prepared by adding polar hydrogen and charges to be assigned using AutoDock Tool V.1.5.6. The crystal structure of all the targets were given in **Figure 3, 4 and 5**. The active site of the target protein were identified using Pdbsum online tool.

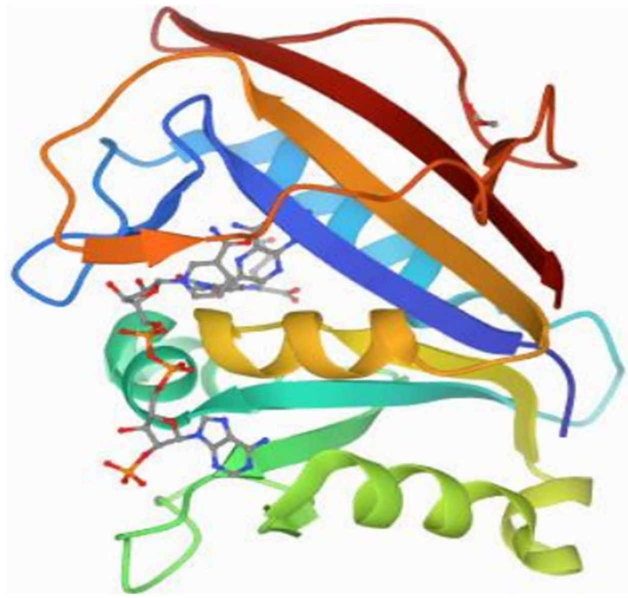


Figure 3: Crystal structure of the target Dihydrofolate reductase (PDB ID: 6DAV)

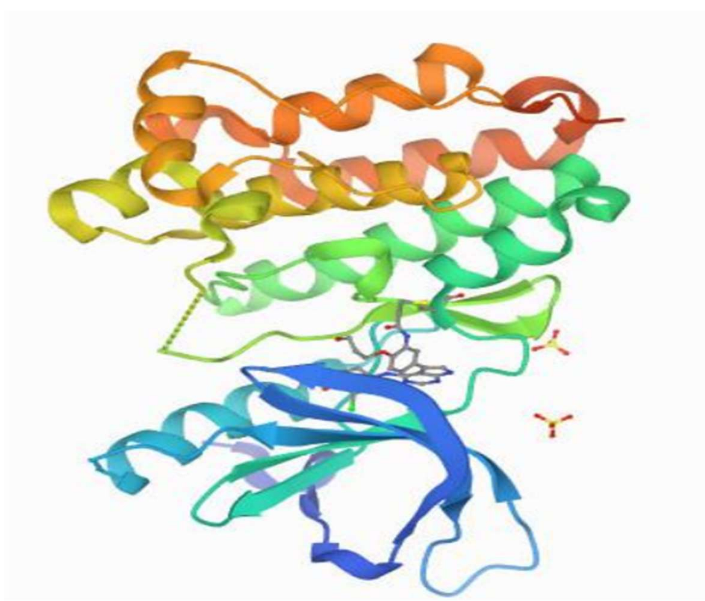


Figure 4: Crystal structure of the target Epidermal Growth factor receptor (PDB ID: 6TFY)

Figure 5: Crystal structure of the target Poly ADP-Ribose Polymerase (PDB ID: 2RIQ)

Grid Generation: A three-dimensional (3D) method called grid box generation arranges the molecules in a cubic grid and calculates the interaction energies between each grid point's molecule and a predetermined probe. The 60*60*60-inch 3D grid box having (x, y, z) coordinates were assigned. The AutoGrid application was used to choose the macromolecule and ligand in PDBQT format, after which the GPF (grid parameter file) was created and the AutoGrid-provided GLG (grid log file) was run.

Docking: With around 10 rotatable bonds in the ligand, Lamarckian Genetic Algorithm, one of the most effective conformation search methods, is offered by AutoDock. The chosen protein and ligand from the AutoGrid choices were docked, and the resulting DPF (docking parameter file) and DLG (docking log file) were generated. The final docked coordinates are recorded by AutoDock in the docking log file.

Evaluation of *In Vitro* Cytotoxicity Activity: Method: MTT ASSAY

DMEM media with 1 X Antibiotic Antimycotic Solution and 10% fetal bovine serum (Himedia, India) were used to plate the breast cancer (MDA-MB 231) cell line at a density of 1 104 cells per well in separate 96-well plates in a CO2 incubator at 37 C with 5% CO2. The cells were treated with various test concentrations of the medication in serum-free media and incubated for 24 hours after a 200 L wash with 1X PBS. The medium was aspirated from the cells at the end of the treatment period. A CO2 incubator was used to incubate the mixture for 4 hours at 37 °C with 0.5 mg/mL MTT made in 1X PBS.After incubation, 200 L of PBS were used to wash the cells of the MTT-containing solution. Before dissolving, 100 L of DMSO were carefully combined with the generated crystals. The change of color intensity was measured at 570 nm. As it ages, the formazan dye turns purple-blue. The absorbance at 570 nm was measured using a microplate reader.

RESULTS:

Synthetic Methodology:

Phthalic anhydride was combined with a number of primary amines to generate a series of seven unique designed phthalimide derivatives, which were then condensed with lamivudine to create N-substituted phthalimide derivatives **(Table 1)**. TLC was used to check that the reaction had finished using the solvent system of chloroform, ethyl acetate, and methanol [5:3:2]. Melting point measurements were used to assess the purity of the synthesized compounds and were performed without correction. The range of the synthesized compounds' percentage yield was determined to be between 41.3% and 63.3%. The physiochemical characteristics of the synthesized compounds were screened and are shown in **Table 2**. ^{1}H- NMR, IR, and MASS spectral analyses were used to analyze the chemical structures of the produced compounds. **Table 3** displays the produced compounds' derived elemental analysis data.

***In Silico* Studies:**

The features of the synthesized compounds that resembled drugs were examined using the Molinspiration program. Molecular weight, hydrogen bond donors, hydrogen bond acceptors, and log p value are among the different factors evaluated. **Table 4** displays the findings and demonstrates that all synthesized compounds adhered to Lipinski's rule of five attributes and had an excellent oral bioavailability. The findings of the assessment of the drug similarity score for the various targets were presented in **Table 5**.

Molecular Docking study:

The structure of the designed compounds were subjected to molecular docking study using AutoDock Version 4.2 software to predict the interaction between the designed compounds and various residues in the target enzymes.

i) **Dihydrohydro Folate Reductase**: The binding energy of compounds ranges from -8.34 to -10.24 kcal/mol. The results were shown in **Table 6**. Compound DPAL showed best binding affinity of -10.24 kcal/mol compared to the standard Methotrexate (-4.44 kcal/mol). Compound DPAL formed one hydrogen bond with the residue Arg 71 and the interatomic distance was found to be 1.934 A°.whereas the standard drug Methotrexate also produced one hydrogen bond with the residue Arg 71 with the distance of 2.080A°. The docked pose of the compound DPAL and standard Methotrexate were visualized using chimera as shown in **Figure 6 and 7**.

ii) **Epidermal Growth Factor Receptor:** The binding energy of compounds ranges between -7.03 to -8.37 kcal/mol

(Table 7). Compound DPSML showed better binding affinity of -8.37 kcal/mol compared with the standard Lapatinib (-6.99 kcal/mol). Compound DPSML interacted with the target by forming three hydrogen bonds with the residue Lys 823, Asn 771, Asn 826 with an interatomic distance of 1.832, 1.995 and 2.104 A° respectively. The best docked pose of the compound DPSML and standard Lapatinib were visualized using chimera as shown in **Figure 8 and 9**.

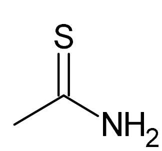
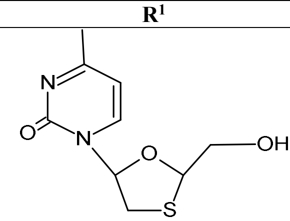
iii) **Poly (ADP-ribose) polymerase:** The binding energy of compounds ranges from -7.87 to -9.68 kcal/mol (Table 8). The compound DPPAL produced the binding energy of -9.68 kcal/mol and also two hydrogen bonds were formed with the target residue Arg 282 and Arg 330 with the interatomic distance of 1.581 and 1.875 A° respectively. Whereas the Talazoparib (reference drug) showed a binding energy of -6.59 kcal/mol and also formed two hydrogen bonds with the target ARG 282, Phe 304. was depicted in the table 1.7. The best docked pose of the compound DPPAL and standard Talazoparib were visualized using chimera as shown in **Figure 10 and 11**.

Toxicity Assessment: Using the Osiris Property Explorer software, the toxicity profile of each synthesized molecule was examined. Except for three compounds (DPAL, DPPL, and DPSAL), the results showed that the majority of the examined compounds were deemed to be low risk when compared to the standard Methotrexate (**Table 9**).

Pharmacological Evaluation:

***In-vitro* anti-cancer activity:** Compounds DPTL and DPSAL were chosen for anti-cancer evaluation against Breast cancer cell line MDA-MB-231 utilizing MTT assay based on the potential docking score and good yield (Table 10). Compound DPTL had an excellent binding score, demonstrated 57.83% cytotoxicity at 500 µM, and poses a lower danger than normal methotrexate, which poses a higher risk. Compound DPSAL likewise produced a strong binding score and shown high risk 51.71% cytotoxicity at 500 µM **(Table 10)**.

Table: 1 Scheme details:

S. No.	Compound Code	R	R[1]
1	DPTL		

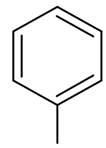
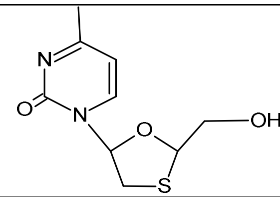
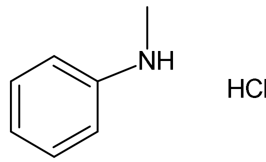
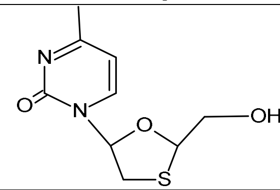
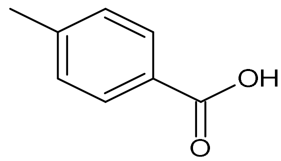
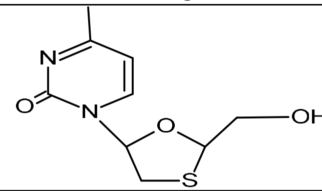
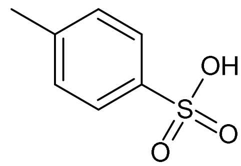
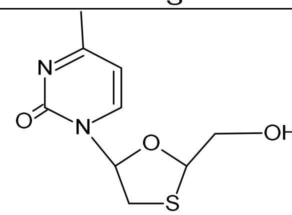
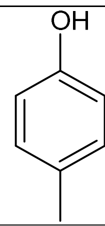
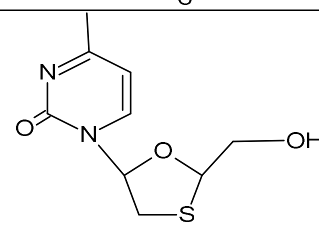
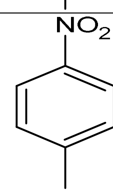
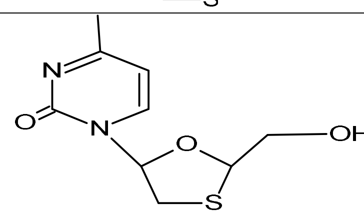
2	**DPAL**		
3	**DPPL**		
4	**DPPAL**		
5	**DPSAL**		
6	**DPAPL**		
7	**DPNAL**		

Table 2: Physicochemical Properties of the synthesized compounds

S. No.	Compound Code	Molecular Formula	Molecular Weight (in grams)	%Yield	Melting Point	R_f Value
1	DPTL	$C_{17}H_{15}N_5O_4S_2$	417.46	63.3	190^0C	0.5
2	DPAL	$C_{22}H_{18}N_4O_4S$	434.47	53.3	280^0C	0.8
3	DPPL	$C_{22}H_{20}N_5O_4SCl$	485.94	46.4	290^0C	0.9
4	DPPAL	$C_{23}H_{18}N_4O_6S$	478.47	41.30	190^0C	0.7
5	DPSAL	$C_{22}H_{18}N_4O_7S_2$	514.53	78.78	200^0C	0.4
6	DPAPL	$C_{22}H_{18}N_4O_5S$	450.46	54.05	195^0C	0.8
7	DPNAL	$C_{22}H_{17}N_5O_6S$	479.46	57.14	210^0C	0.8

Table 3: Elemental Analysis

Compound Code	C%	H%	N%	S%	O%	Cl%
DPTL	48.91	3.62	16.78	15.36	15.33	-
DPAL	60.82	4.18	12.90	7.38	14.73	-
DPPL	54.38	4.15	14.41	6.86	13.17	7.30
DPPAL	57.73	3.79	11.71	6.70	20.06	-
DPSAL	51.35	3.53	10.89	12.46	21.77	-
DPAPL	58.66	4.03	12.44	7.1	17.76	-
DPNAL	55.11	3.57	14.61	6.6	20.02	-

Table 4: Molecular Properties of designed compounds:

Compound code	Mi log P	TPSA	n atoms	n ON	n OHNH	n violations	n rotb	volume
DPTL	0.93	124.75	28	9	3	0	4	330.53
DPAL	2.39	98.73	31	8	1	0	4	362.79
DPPL	2.57	110.75	32	9	2	0	5	375.19
DPPAL	2.30	136.03	34	10	2	0	5	389.79
DPSAL	-0.62	153.10	35	11	2	2	5	402.24
DPAPL	1.91	118.96	32	9	2	0	4	370.81
DPNAL	2.35	144.55	34	11	1	1	5	386.12

Table 5: Drug Likeness properties of the compounds by Molinspiration software:

S. No.	Compound code	GPCRligand	Ion channel modulator	Kinase inhibitor	Nuclear receptor ligand	Protease inhibitor	Enzyme inhibitor
1	DPTL	0.07	-0.65	-0.24	-0.98	-0.36	0.36
	DPAL	0.07	-0.45	-0.22	-0.66	-0.26	0.41
3	DPPL	0.02	-0.64	-0.23	-0.73	-0.42	0.47
4	DPPAL	0.05	-0.44	-0.25	-0.54	-0.24	0.39
5	DPSAL	0.14	-0.35	-0.28	-0.78	-0.10	0.46
6	DPAPL	-0.05	-0.91	-0.43	-0.86	-0.28	0.07
7	DPNAL	0.09	-0.55	-0.21	-0.83	-0.28	0.55

Table 6: Molecular Interactions of designed compounds against the target Dihydro folate reductase (Pdb ID:-6DAV)

S. No.	Compound Code	No. of Hydrogen Bonds formed	H-Bond Interaction	H-Bond Distance (A°)	Binding Energy (kcal/mol)
1	Standard (Methotrexate)	1	Arg71	2.080	-4.44
2	DPTL	2	Ser60; Ser120	2.029; 2.003	-8.34
3	DPAL	1	Arg71	1.934	-10.24
4	DPPL	1	Ser60	2.704	-9.04
5	DPPAL	2	Arg33; Asn65	2.064; 2.132	-9.36
6	DPSAL	1	Arg71	1.685	-10.21
7	DPAPL	1	Arg33	2.155	-8.60
8	DPNAL	2	Asn65; Arg33	2.189; 1.811	-9.39

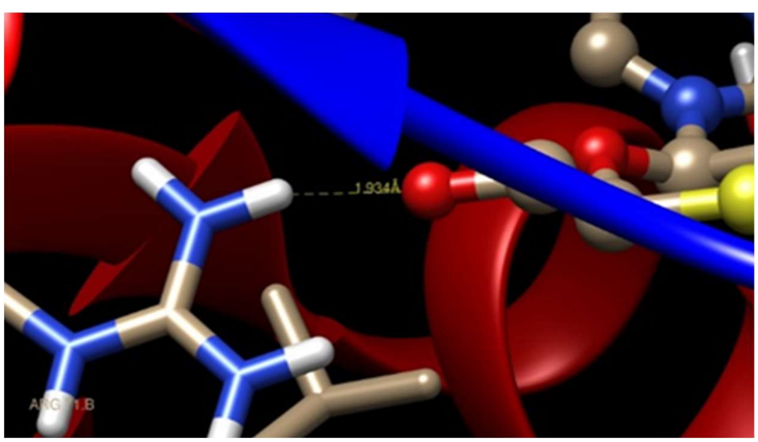


Figure 6: Binding mode of compound DPAL in the active site of the target Dihydrohydro Folate Reductase

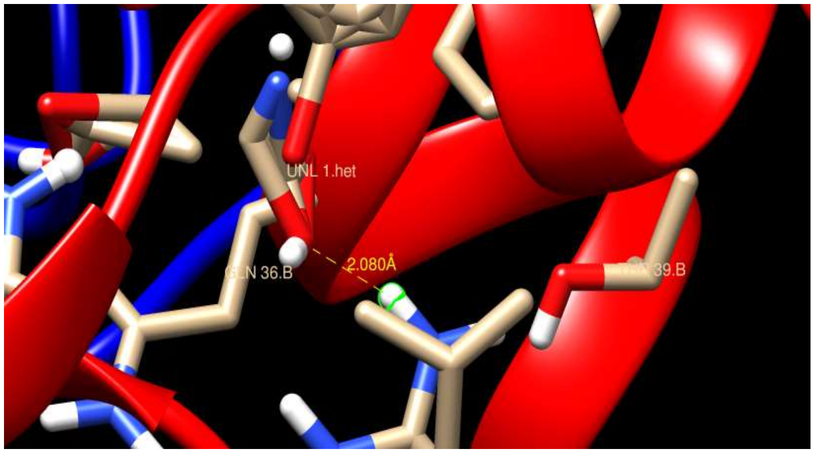


Figure 7: Binding mode of standard drug Methotrexate in the active site of the target Dihydrohydro Folate Reductase

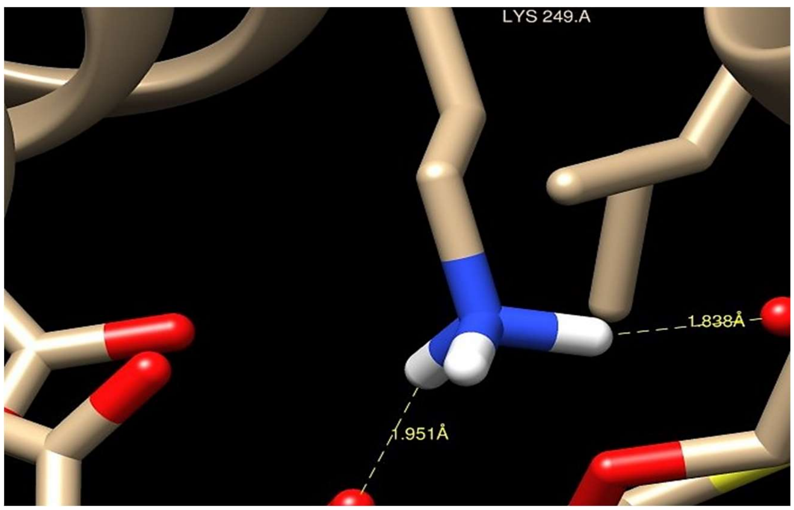


Figure 8: Binding mode of compound DPAL in the active site of the target Poly ADP-ribose polymerase

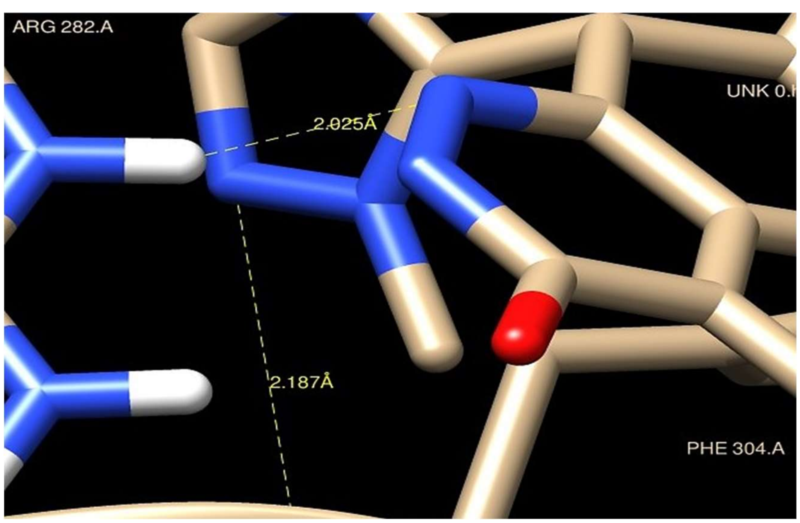


Figure 9: Binding mode of standard Talazoparib in the active site of the target Poly ADP-ribose polymerase-I

Table 7: Molecular Interactions of designed compounds against Epidermal Growth Factor Receptor (PDB ID: -6TFY)

S. No.	Compound Code	No. of Hydrogen Bonds formed	H-Bond Interaction	H-Bond Distance (A°)	Binding Energy (kcal/mol)
1	Standard (Lapatinib)	1	Ser969	1.991	-6.99
2	DPTL	1	Gly796	2.886	-7.47
3	DPAL	2	Lys823	2.063	-7.50
4	DPPL	2	Asn771;Asn826	2.136;1.824	-7.03
5	DPPAL	2	Asn826; Lys823	2.046; 1.861	-7.71
6	DPSAL	3	Lys823;Asn771; Asn826	1.832; 1.995 2.104	-8.13
7	DPAPL	1	Gln820	1.782	-7.71
8	DPNAL	2	Asn826; Lys823	1.992; 2.137	-8.02

Table 8: Molecular Interactions of designed compounds againstthe target Poly ADP Ribose Polymerase (PDB id: 2RIQ)

S. No.	Compound Code	No. of Hydrogen Bonds formed	H-Bond Interaction	H-Bond Distance (A°)	Binding Energy (kcal/mol)
1	Standard (Talazoparib)	2	Arg282; Phe304	2.025; 2.187	-6.59
2	DPTL	2	Val303; Leu292	2.148; 2.181	-7.87
3	DPAL	2	Lys249	1.951; 1.838	-9.31
4	DPPL	2	Gln270; Val303	2; 2.135	-8.86
5	DPPAL	3	Arg282; Arg330;	1.581; 1.875	-9.68
6	DPSAL	1	Arg282	-1.637	-8.13
7	DPAPL	2	Lys249; Arg282	2.142; 2.038	-8.37
8	DPNAL	3	Lys249; Val303	2.092; 1.942	-8.35

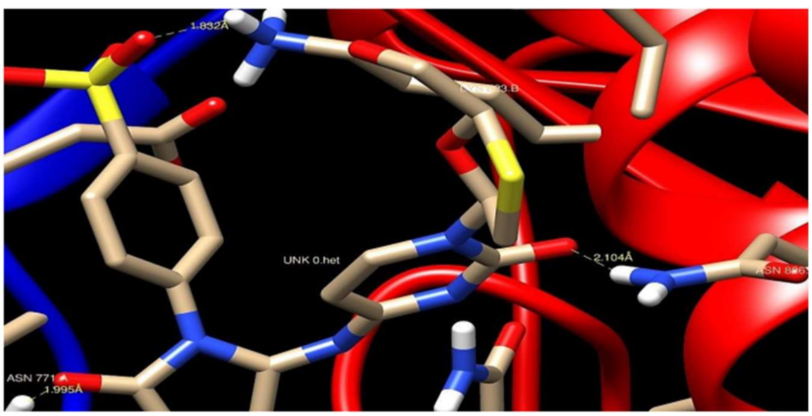


Figure 10: Binding mode of compound DPSAL in the active site of the target Epidermal Growth Factor Receptor

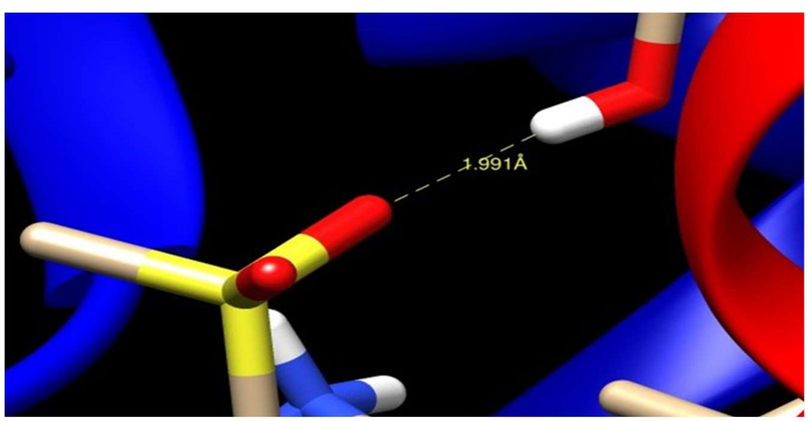


Figure 11: Binding mode of standard Lapatinib in the active site of the target Epidermal Growth Factor Receptor

Table 9: Toxicity Assessment by OSIRIS Property Explorer

Compound Code	Mutagenic	Tumorigenic	Irritant	Reproductive
DPTL	Green	Green	Green	Green
DPAL	Red	Orange	Orange	Red
DPPL	Green	Red	Green	Green
DPPAL	Green	Green	Green	Green
DPSAL	Red	Green	Red	Green
DPAPL	Green	Green	Green	Green
DPNAL	Green	Green	Green	Green
METHOTREXATE	Green	Red	Green	Green

Green: Low Risk, Orange: Medium risk, Red: High risk

Table 10*: Invitro* anticancer activity of the compound DPTL and DPSAL against MBA-MB 231 cell line

Concentration(µm)	% Cytotoxicity	
	DPTL	DPSAL
500	57.83	51.71
250	54.68	39.96
125	43.32	44.41
62.5	29.68	37.12
31.25	2.10	19.26

DISCUSSION:

Most of the breast cancer drugs develops multi-drug resistance and toxicity, there is a need for the development of new anti-cancer agents targeting BC cells. Phthalimide scaffold is an important heterocyclic compound possessing wide range of pharmacological applications. The present study involves designed to develop series of N-substituted phthalimide derivatives were subjected to docking studies. The designed compounds were synthesized using conventional method by condensing phthalic anhydride with various substituted primary amines and further reacted with Lamivudine to produced corresponding N-substituted phthalimide derivative. Based on the potential docking scores and good yield, the compounds were selected for their *in vitro* cytotoxic activity against breast cancer cell lines. Compound DPTL showed 57.83% cytotoxic activity at the concentration of 500µM and produced less toxicity compared to standard (methotrexate). So, the compound DPTL is potential candidate for the treatment of breast cancer.

CONCLUSION:

By combining phthalic anhydride with various primary amines, novel N-substituted phthalimide derivatives were created, and these compounds were then produced by reacting them with lamivudine. Using the solvent system chloroform: ethyl acetate: methanol (5:3:2), melting point analysis and thin layer chromatography were used to evaluate the purity of produced compounds. Using Molinspiration software, the physiochemical characteristics of the synthesized compounds were examined, with the parameters set in accordance with the Lipinski rule of five attributes. The docking studies were performed for all the designed compounds using Autodock software version 4.2 against the target enzymes Dihdydrofolate reductase, Epidermal Growth Factor Receptor and Poly ADP Ribose Polymerase for anti-cancer activity. Most of the designed compounds showed good binding affinity against the selected targets when compared to standard drug (Methotrexate, Lapatinib, Talazoparib). It showed promising response in the docking studies against the targeted enzymefor breast cancer and low risk of toxicity when compared to standard (Methotrexate). By using the MTT assay, compound DPSAL and DPTL were tested for their in vitro cytotoxic activity against the MDA-MB-231 cell line (a breast cancer cell line) and showed, at a concentration of 500 M, 51.71 and 57.83% cytotoxicity, respectively. It was concluded that the phthalimide derivatives are significant and useful scaffolds for the synthesis of lead drug for its potential anticancer action against breast cancer cell lines. The results demonstrated condensation of phthalimide with lamivudine undergoes nucleophilic substitution reaction.

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