RESEARCH ARTICLE

Design, Synthesis and Bio-evaluation of New Phenothiazine Derivatives of Sulfonamide Dyes as Anticancer Agents

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A series of new phenothiazine derivatives of sulfonamide dyes was designed, synthesized & evaluated for their potent anti-cancer activities. The compounds were designed on the basis of molecular docking studies. Firstly they were docked with three different anticancer target enzymes viz. topoisomerase-II, aromatase and CDK2 and on the basis of dock score the designed compounds were synthesized. All synthesized compounds were bio-evaluated for their in-vitro anti-cancer action which displayed adequate results in agreement with the outcome of docking study

Introduction

Cancer, the 2nd main reason of death after cardiac disease, asserting more than 7 million deaths in last decade, the most current global statistics for cancer [**1**]. As compared to men women are prone to more types of cancer [**2,3**]. In the year 2030 12 million deaths is expected as the death from cancer worldwide are likely to keep on growing. Whereas the principal means of cancer treatment are surgery, radiation, and chemotherapy, [**4**] the beleaguered chemotherapy acts on specific noticeable molecular anomalies for few tumors, and which lessens destruction to normal live cells, is appealing progressively hopefully popular [**5**].

Drug discovery-development, is a prolonged and expensive procedure and so, computational methods are required for generating the lead and their optimization in the process of discovery-development by significantly plummeting the phase time. The in-silico approach are being paid more attention which can lead the enhancement for designing novel anticancer drug after the development of the first peptide-based HIV protease inhibitors and inhibitors of the H5N1 avian influenza [**6**]. Molecular docking can be understood as an optimization test that illustrates the "best-fit" ligand orientation. Ligand binds to a specific protein and is used to deduce the assembly of the intermolecular complex formed with minimum binding energy for predicting affinity and activity. Generating drug-receptor interactions through molecular docking studies can be taken as a modern drug designing, process [**7**]. In chemotherapy for cancer the major focus of research includes the discovery, characterization and development of novel and safe cancer chemo representative agents [**8**]. In cancer therapy cytotoxicity and geno-toxicity of anticancer drugs to the healthy cells are foremost problems and may induce secondary malignancy and leads to many side-effects, like pain, nausea, vomiting, loss of hair and that in turn, limits the efficacy of the attempted therapy. Recently, an apprehensive research has been carried out for the finding and developing novel selective anti-cancer agents, which are devoid of number of the terrible side effects of regular anti-cancer agents [**9**]. A broadly used anti-psychotic drugs that chiefly act on central dopamine receptors and inhibits tumour cell proliferation are drugs of Phenothiazine derivatives which are relatively secure. They show varying degree of antiemetic, sedative, anti-psychotic, hypothermic and analgesic effects. The drug chlorpromazine, a drug from the same class being studied and found that it can enhance the cytotoxic effects of tamoxifen on breast cancer. Resistance to chemotherapeutic drug can be reversed cisplatin, a Phenothiazine drugs by enhancing the sensitivity [**10**]. Phenothiazine derivatives constitutes an important class of thiazines heterocyclic ring system and possess diverse activities as neuroleptics tranquilizers, antimalarial, anti-Parkinson, anti-convulsant, anti-histaminic, antiviral, anti-helminthic, anticancer, antibacterial and CNS-depressant [**11**]. Phenothiazines are inexpensive and widely available, and therefore have been examined as anticancer drugs. In search for better active moieties a slight variation in nucleus makes a striking difference in the bioactivity [**12**].

Recent reports on anti-cancer agents, on structurally novel sulfonamide derivatives found to show substantial antitumor activity in vitro and/or in vivo [**13**]. The sulfonamide antimicrobial drugs were the first effective chemotherapeutic agents but the rapid development of widespread resistance diminished the usefulness of sulfonamides. An evaluation of azo dyes was done and prontosil was found to protect against and cure streptococcal infections in mice. The structure-activity study on the sulfonamide azo dyes was performed and the

reductive cleavage of azo linkage to release the active antibacterial product, sulfonamide, was concluded. Further, sulphonamide containing compounds have enormous potential as pharmaceutical and agricultural agents, due to their diverse biological profile. They have extensively been documented for their wide variety of pharmacological activities such as antimicrobial, insulin releasing, anti-diabetic, diuretic, anti-carbonic anhydrase, anti-thyroid, anti HIV and anti-tumour activity among others. Already it has been exploited clinically for treating a variety of diseases like epilepsy, glaucoma, congestive heart failure, gastric, mountain sickness, and duodenal ulcers or as diuretic agents, but a little explored as antitumor drugs [**14**]. Current advances in bio-medical sciences and combinatorial chemistry have lead to the design and synthesis of hundreds of new anti-neoplastic agents and by understanding the drug action we can ration ally design newer drugs with selectivity and reduced side effects. However, the exact biology of cancer still remains angiomatous at large, offering a lot of scope for the research targeting the malignant cells [**15**]. The combination of two or more pharmacophores in a single molecule is one of the major approaches for designing of new biologically active moiety. Structural fragments with heterocyclic moieties are most obvious among all drugs [**16**].

Estrogen receptor-positive cases of breast cancer accounting around 70% of the total cases, contains receptors that attach to the estrogen hormone [**17**]. Estrogen levels are lowered by aromatase inhibition. These drugs lower estrogen levels in females whose ovaries aren't making estrogen (say a women with menopause) [**18**].

Recent evidence suggests that Cycline Dependent Kinase 2 (CDK2) seems to have a essential role in the G2 phase of the cell cycle progression that has led to an active pursuit of small molecule inhibitors of this enzyme may be helpful in treatment against cancer and other hyper-proliferative disorders [**19**].

The physiological function of Topoisomerase II is explained in mechanistic detail elsewhere [**20**].

In concurrence with our efforts to develop broad spectrum phenothiazine derivatives of sulfonamide dyes as anticancer agents that are based on the analysis of the interaction of the anticancer drug target enzymes (topoisomerase II, aromatase and CDK2) with the designed ligands, using **ArgusLab 4.0.** In order to (i) mock-up the binding interaction between a series of phenothiazine derivatives of sulfonamide dyes and the three enzymes, and (ii) evaluate the effects of structural variation of phenothiazine derivatives of sulfonamide dyes. Various phenothiazine derivatives of sulfonamide dyes in which the **R**-groups were changed (Table 1), were docked in silico, onto the three anticancer drug target enzymes. And thus before actual synthesis the molecules were designed and selected [**21**].

The diazotization technique through protonation of nitrous acid under acidic conditions is renowned [**22**]. But looking to their environmental incompatibility, [**23,24**] silica sulfuric acid (SiO_2-O-SO_3H), a environmentally benign solid acid, was used [**25**].

Considering the above findings about importance of phenothiazine and sulfonamide derivatives as anti-cancer agent, we designed novel phenothiazine derivatives containing sulfonamide moiety as three different anti-cancer target enzyme inhibitors viz. topoisomerase-II, aromatase and CDK2. Due to the presence of a polar pocket in the active site of aromatase enzyme, the hydrophilic sulfonamide group was connected to the phenothiazine scaffold as a proton acceptor or donor to combine their positive effects. In this study, we tried describing the designing via docking study, synthesis and evaluation of4-(4-((10H-phenothiazin-10-yl) sulfonyl) phenyl) diazenyl derivatives **5a-g** as anti-cancer agents.

Results and discussion

Modeling of the three enzymes (topoisomerase II, aromatase and CDK2) and ligands

On precise analysis of the active site of all the three enzyme is recapitulated according to docking dataset. The active site of Aromatase is contemplated to be Ala438, Arg115, Arg145, Ilu132, Ilu152, Ilu372, Ilu477, Gly439, Met311, Thr310, Try224, Val370 and other amino acids around in the site. The residues contributing to the catalytic cleft of CDK2 are Ala31, Ala144, Asp86, Gln85, His84, Ilu10, Leu134, Lys89, Phe80, Val18, etc. The active site of Topoisomerase II is considered to be Arg81, Asn51, Glu55, Ile48, Ile98, Met83, Pro84, Thr94, Val76, Val174, etc.

The quick evaluation of binding energies for topoisomerase II, aromatase and CDK2, structure-based drug design: molecular docking studies was carried out. First, we examined all the designed phenothiazine derivatives of sulfonamide dyes (**5a-5g**) with three anticancer drug target enzymes **(**topoisomerase II, aromatase and CDK2). The the ligand binding affinities was estimated as energy score (kcal/mol). The compounds displaying the maximum binding affinity, least dock score, is the one forming the most stable ligand-enzyme complex. The binding models were assessed from the number of hydrogen bonds. As shown in Table 1 all the designed ligands showed better dock score with aromatase enzyme as compare to topoisomerase-II and CDK2. But the dock score of almost all ligands with topoisomerase II and CDK2 were found to be better as compared to doxorubicin, a known topoisomerase II inhibitor and flavopiridol, a known CDK2 inhibitor respectively.

The amino acid residues at the active site of aromatase forms two H-bond with the -SO_2-and –OH group of **5a**, particularly with the catalytic amino acid Gly439 and Arg115 respectively. But the same compound does not form any hydrogen bond with topoisomerase II as there is no catalytic amino acid present at appropriate distance to the -SO_2- and –OH group. Compound **5e** and **5f**, with resorcinol and phenol substituents respectively,

has shown reasonably high dock score as compared to other compounds (comparison in **Table 1**).

The best docked pose of **5f** with topoisomerase II, aromatase and CDK2 is shown in Figure1A, 2A and 3A respectively. Similarly, to the binding pose of **5f** in the active site of enzyme crystal structure, the -SO_2- and –OH group seems to form hydrogen bond with amino acid residues (Figure 1B, 2B and 3B). The docked structure has several interactions with the residues inside the active site of enzyme. Further, the Authors submit that the probable reason for high dock score of compounds **5e** and **5f** over other compounds (5a-5d and 5g) is the presence of only –OH group in resorcinol and phenol without any other functional group which leads to the formation of H-bond more easily as compared to other compounds in which there are either two functional groups or it is bicyclic. Thus, there will be less steric hindrance in case of compounds **5e** and **5f** that makes the binding of compounds 5e and 5f with the active sites of enzyme more feasible as compared to the other compounds (5a-5d and 5g). In the general docking report, the diazo linked aromatic rings packs in the up-cleft of the binding pocket and the phenothiazine ring takes up the position against the pocket. The diazo linked aromatic ring adopting a non-coplanar conformation with the phenothiazine ring to be positioned in the centre of the binding pocket. The -SO_2- group of **5f** bonds through a H atom with the catalytic amino acid Gly439 in the active site of Aromatase, and the distance between the O_2 of -SO_2- and the NH group in the side chain of Gly439 is 2.22 Å, indicating that **5f** interacts certainly with Gly439. The –OH group of **5f** forms hydrogen bonds with the Leu298 in the active site of CDK2 with bond length 2.90 Å.

Likewise, to the binding position of the -SO_2- and –OH group of **5f** with topoisomerase II, aromatase and CDK2, the other compounds also retain interactions with the catalytic residues in the active site of all the three enzymes, suggesting that the designed compounds **(5a-5g)** are capable of inhibiting more than one enzyme. Thus, predicting the broad spectrum anticancer property of the designed compounds. In addition, we attempted to dock Doxorubicin, Letrazol and Flavopiridol into the active site of topoisomerase II, aromatase and CDK2 respectively to make a comparative study of the binding affinity of these standard inhibitors with the designed compounds.

Table 1. Dock scores and no. of H-bonds of the designed compounds **5a-5g**, standard inhibitors (Doxorubicin, Letrazol and Flavopiridol) docked on the active site of DNA topoisomerase II, Aromatase and CDK2.

Compound	Dock Score (Kcal/mol) /No. of H-Bonds					
	Topoisomerase II		Aromatase		CDK2	
5a	-7.487	-	-13.738	2	-12.138	1
5b	-9.560	2	-14.736	2	-11.526	-
5c	-10.262	1	-12.300	1	-12.042	-
5d	-9.893	1	-11.599	1	-12.055	1
5e	-9.815	1	-13.927	2	-11.909	2
5f	-10.533	3	-14.198	2	-12.548	1
5g	-9.500	3	-12.702	3	-10.994	-
Doxorubicin	-8.198	6	---------	----	---------	------
Letrazol	-------	-------	-10.941	1	---------	------
Flavopiridol	-------	-------	---------	----	-10.069	2

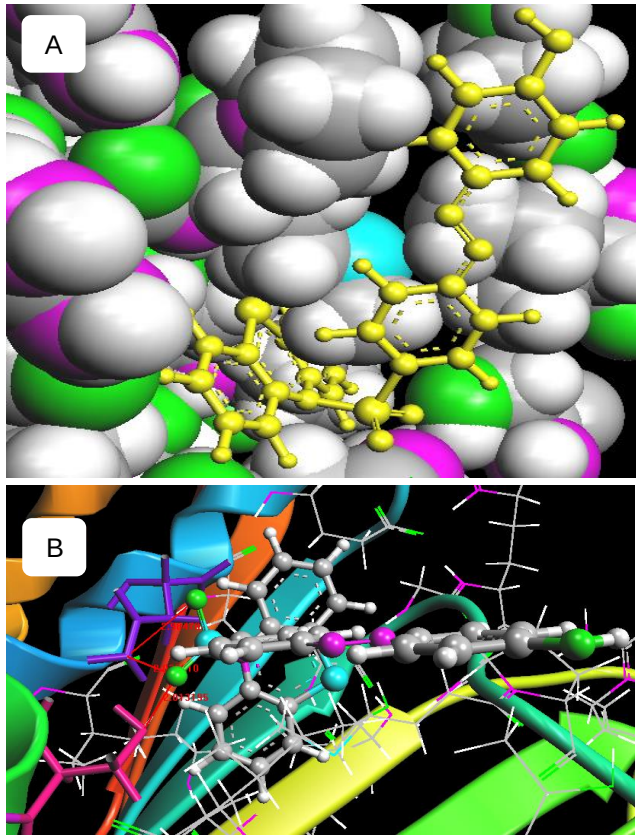


Fig. 1. Docking of compounds **5f** with Topoisomerase II. **(A)** Binding pose of compound **5f i**n the pocket (active site) of Topoisomerase II. **(B)** Formation of hydrogen bond (red) with amino acids Asn51(purple) and Ser124(pink) in the active site of enzyme.

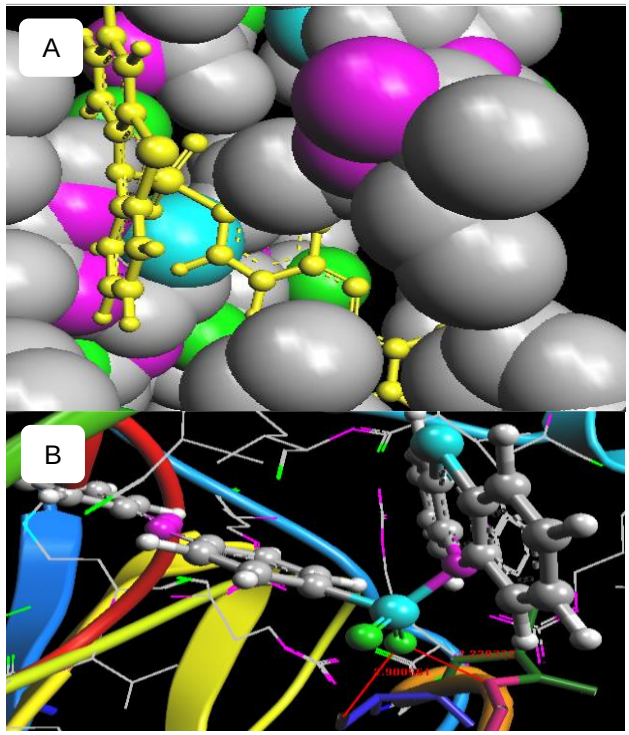


Fig. 2. Docking of compounds **5f** with Aromatase.**(A)** Binding pose of compound **5f i**n pocket (active site) of Aromatase enzyme. **(B)** Formation of hydrogen bond (red) with amino acids Gly439(pink) and Cys437(indigo) in the active site of enzyme.

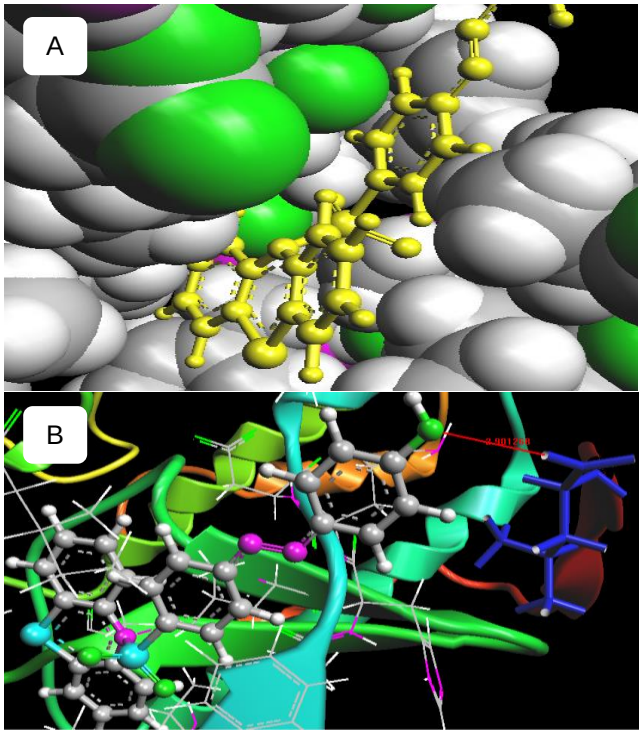
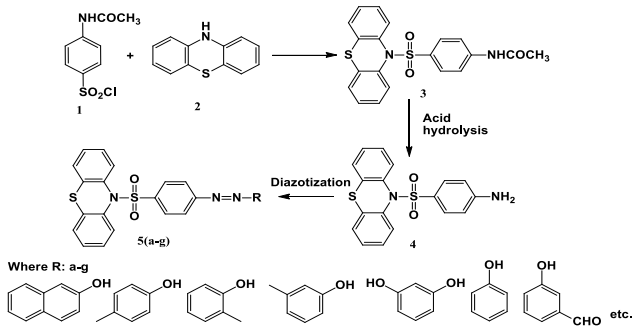


Fig. 3. Docking of compound **5f** with CDK2. **(A)** Binding pose of compound **5f i**n the pocket (active site) of CDK2 enzyme. **(B)** Formation of hydrogen bond (red) with amino acid Leu298(indigo) in the active site of enzyme.



Scheme 1. General protocol for synthesis of 4-((4-(10H-phenothiazin-10-ylsulfonyl)phenyl) diazenyl) phenol derivatives **(5a-g)**.

Synthesis

Being leaded by the docking outcomes, we designed, synthesized and characterized the above 7 phenothiazine derivatives of sulfonamides dyes (**Scheme 1**). This paper describes the simplistic and original method for the synthesis of sulfonamide dyes through grinding under solvent-free conditions (without using conventional acids or bases) looking at the poor thermal stability of the diazonium salts they were synthesized around 0-10 °C and were handled below 0°C prior to the actual synthesis [**30**]. In our study, the aryl diazonium salts with dry silica supported sulfuric acid was adequately stable at room temperature. In our new method, N-(4-((10H-phenothiazin-10-yl)sulfonyl)phenyl)acetamide **(4)**, were rapidly converted, at room temperature to the corresponding azo dyes in the presence of $NaNO_2$ and silica supported sulfuric acid.

Anticancer evaluation

As shown in **Fig. 4**, in MDA-MB-231 cell line, all the compounds **(a-g)** determined a clear dose-dependent inhibitory effect on cell growth. The compounds **b** and **e** (in which diazo is linked to p-cresol and resorcinol) elicited excellent anticancer activity in a dose dependent manner as compared to other compounds in the series. However, all other compounds exhibited significant decrease in cell viability at 20 µM concentration. Remarkably, a 24 h treatment with compound **a-g** (20 µM) was able to prevent MDA-MB-231 cell growth.

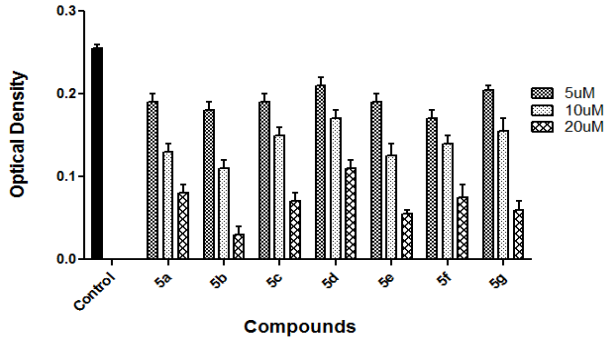


Fig. 4. Optical Densities of different doses of compounds (**5a-5g**) on MDA-MB-231 cell proliferation using MTT assay.

Data analysis

Cell survival has been calculated using the formula: Cell Viability (%) = [(absorbance of treated cells - absorbance of culture medium)/(absorbance of untreated cells - absorbance of culture medium)] x100.[31] The percentage viability of cells was measured by MTT assay. **Table 2** shows the minimum viability percentage of the maximum Drug concentrations in µM.

Table 2. Anti proliferative effects of phenothiazine derivatives of sulfonamides dyes.

Compounds	%Cell Viability shown by compounds of three different concentrations		
	5µM	10µM	20µM
5a	74.50	50.98	31.37
5b	70.58	43.13	11.76
5c	74.50	58.82	27.45
5d	82.35	66.66	43.13
5e	74.50	49.01	21.56
5f	66.66	54.90	29.41
5g	80.39	60.78	23.52
Control		100	

Experimental

Docking studies

The binding affinity of the synthesized sulfonamide derivatives were compared, by docking them into the empty binding site of the experimentally known crystal structure of the anti-cancer drug target enzymes (topoisomerase II, aromatase and CDK2) [**26**]. All docking studies were performed using '**ArgusLab 4.0.1**'.

Preparation of protein structure

The crystal structure of the aromatase (PDB code-pdb 4kq8), CDK2 (PDB code-pdb 1ckp) and DNA topoisomerase II (PDB code-pdb 4g0u) was recovered from the Protein Data Bank (http://www.pdb.org). All bound water and cofactors were removed from the protein.

Preparation of the ligand structures

Firstly a ligand was prepared from the moiety that was already present in the active site of the enzyme and then the binding sites group was created from that ligand. After creating the active site, that ligand was removed from the protein. A set of 7 phenothiazine derivatives of sulfonamides dyes structures were designed to bind in the active site of aromatase, CDK2 and DNA topoisomerase II. Their 3D structures were constructed using ChemDraw 3D pro12.0 software, and then they were energetically minimized with MM2, Jop Type with show every iterations and minimum RMS gradient of 0.01, and saved as pdb file. The compounds considered for the study are listed in Table 1.

Protein-ligand docking using ArgusLab 4.0.1

All the three enzymes viz. aromatase, CDK2 and DNA topoisomerase II protein were docked against the constructed structures of sulfonamides using Argus Lab 4.0.1 (Mark A. Thompson, Planaria Software LLC, Seattle, WA, USA, http://www.arguslab.com). The interaction helps finding favorable binding geometries of the ligand with the protein. Ligands were flexibly docked into aromatase, CDK2 and DNA topoisomerase II protein using Argus Dock, and scored by AScore, according to established protocol [**27**]. The pose having the highest dock score was selected for further analysis.

Synthesis

Analytical grade solvents, reagents and catalyst are of obtained from a commercial source and used directly without any further purification. The purity of compounds were checked consistently by TLC using silica gel-G coated Al-plates (Merck, 60F254) & were visualized under UV light. Melting points were determined using open tube capillary. Bruker FTIR-αE was used to record IR spectra; Bruker-Avance II 400, Varian-Gemini spectrophotometer using DMSO-*d6* solvent was used to record ^{1}HNMR (400MHz) spectra and ^{13}C NMR (100MHz) spectra of the synthesized compounds with TMS as the internal standard. EI-MS spectra were determined Thermo Fisher, San Jose, CA, USA mass spectrometer. The C, H, N, and S of compounds was performed on Perkin Elmer 2400 CHN analyzer. The results were found to be in good agreement with the calculated values.

General procedures for the synthesis of N-(4-((10H-phenothiazin-10-yl)sulfonyl) phenyl) acetamide (3)

To the solution of substituted phenothiazine **2** (1 equiv) in DMF, triethyl amnie (1 equiv) were added slowly. To this mixture acetamido benzene-sulfonyl chloride **1** (1 equiv) were added with stirring. The mixture was stirred at 150 °C for 15-16 hours. The reaction progress was monitored by TLC. After completion of the reaction, it was quenched with ice water and the precipitated product was filtered off. The sulfonamide was recrystallized from ethanol. The acetamide group of 4-acetamido-benzenesulfonamide **3** was acid hydrolyzed without affecting the sulfonamide group which was further neutralized with Na_2CO_3 to isolate the product [**28**].

Preparation of sulfonamide dyes on silica sulfuric acid support

A general procedure is described below for the preparation of 1-((4-((10H-phenothiazin-10-yl) sulfonyl) phenyl) diazenyl)naphthalen-2-ol **5a** and all other sulfonamide dyes were prepared in the similar manner.

Representative procedure for the synthesis of 1-((4-((10H-phenothiazin-10-yl)sulfonyl)phenyl)diazenyl) naphthalen-2-ol (5a): The mixtures 4-((10H-phenothiazin-10-yl)sulfonyl)aniline **4**(2.8mm), SSA(1g) and sodium nitrite (2.8mm) were mixed, ground in an agate mortar for 10 mins. at room temperature. Then naphthol (2.8mm) was added to it and ground for 10 min. TLC was used to monitor with mixture of ethyl acetate and petroleum ether (1:1; v/v) as solvent. The synthesized dye was extracted using petroleum ether.

In-vitro anti-cancer activity

Compounds (A-E) were screened using the MTT assay for in vitro anti-cancer activity against MDA-MB-231 cell line. The conversion of soluble yellowish MTT into the insoluble purple formazan by active mitochondrial lactate dehydrogenase of living cells.[29]

Cell viability assay (MTT method)

10000 cells were seeded/well of 96 wells culture plate and grown for 24 hrs (h) in specific culture media. Further, cells were cured with DMSO or drug concentrations for specific time periods. After specific time periods media was aspirated. Then 100 µl solution of 5mg/ml MTT metabolite solution in 1xPBS was added to each well with 4hr incubation at 37°C in CO_2 incubator. The plates were further removed from incubator and MTT solution was aspirated out from the cultured plates. The formazan crystals were dissolved, 100 µl DMSO was added and incubated for 10-15 minutes to get a purple color. Wells were read at 570 nm using Synergy H1 multi-mode microplate reader.

Conclusion

A cogent approach to the design and synthesis of new phenothiazine derivatives of sulfonamides dyes is being highly developed. The "**ArgusLab 4.0.1**" has been used to mock-up the binding interaction for a list of sulfonamides dyes with Topoisomerase II, Aromatase and CDK2, the target enzymes for many anticancer agents. This offers guidance in the design of phenothiazine derivatives of

sulfonamides dyes for using in cancer therapy and abet in the selection of agents that are compatible to MDA-MB-231 cell line. This general approach can be applied to the development of new therapeutic pharmaceuticals for specific enzyme and other macromolecular target molecules. A series of 1-(4-((10H-phenothiazin-10-yl) sulfonyl)phenyl)diazenyl derivatives **5a-g**was synthesized in appreciable yield. The synthesized compounds were evaluated for their in vitro anticancer activity. Cytotoxic activity results indicated that most of the synthesized compounds were active against the MDA-MB-231 breast cancer cell lines.The results of anticancer activity were found to be in agreement with the outcome of docking study.

Acknowledgement

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Conflicts of interest
There are no conflicts to declare.

Keywords

Docking, topoisomerase-II, aromatase enzyme, CDK2, phenothiazine derivatives of sulfonamide, anticancer evaluation

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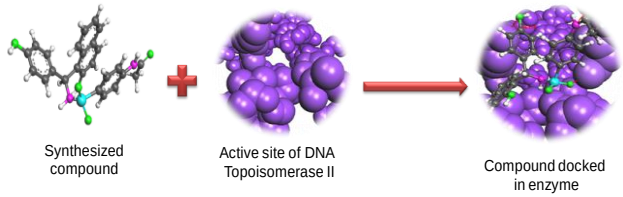
Graphical abstract



Author biography

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