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1,5-Benzosulfonamide anthracenedione analogues of mitoxantrone as antibacterial and anticancer agents

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The new 1,5-disubstituted 9,10 anthraquninone compounds have been synthesized and characterized by FT-IR, ^{1}H and ^{13}C NMR, and mass spectrometry. Mass fragmentation pattern confirms the structure of the synthesized analogues. The synthesized compounds (B1-B5) have been found active against Hela (cervix carcinoma), prostate cancer and breast cancer cell lines in comparison to mitoxantrone. B2 screened out to be best. IC_{50} value of B2 and mitoxantrone against Hela cell lines have been observed to be 17µg/mL and 2.5µg/mL respectively. DNA intercalation has been proposed as per cell cycle analysis of B2 on Hela cell lines which show major alteration in G0/G1 and S phase. The results are further supported by molecular docking study of (B1-B5) compounds and mitoxantrone with quadruplex terminals i-motif. The compounds have also been evaluated for antibacterial activities.

Keywords: Anthraquinone, cytotoxicity, prostate, cervix carcinoma, i-motif

Substituted 9,10-anthraquinone and sulfonamide feature has been the subject of research in wider areas like organic electronics, dyes and pharmacy. Anthracycline class of antibiotics has been used as an anticancer agents against diverse cancer types. Based on the structural features and configuration of molecules; number of mechanism are proposed. However mode of action at cellular level is still a question. Large number of anthracycline class of compounds are tested for their chemotherapeutic action[1-4]. Adriamycin, daunomycin and mitoxantrone are anthracycline class of drugs; used in cancer hospital worldwide for cancer treatment. Topoisomerase-II binding and DNA intercalation mechanism of action at cellular level has been proposed for said chemotherapeutic agents (Figure 1). These drugs also bind to telomeric junction and cause cell death[5-8].

Mitoxantrone is structurally different from adriamycin and daunomycin. Its structure is simple and symmetrical having 1,4 dihydroxy along with 5,8 dialkylamino substituent on 9,10 anthraquinone system[9]. Number of derivatives of mitoxantrone, adriamycin and daunomycin has been synthesized and evaluated for their biological activity[10-15]. As per literature study 9,10-anthraquinone is an important requirement for drug candidate to intercalate at DNA binding site. Functional group present on side arms and their position on tricyclic anthraquinone ring dictates the cytotoxic effect[16,17].

Molecules with R-SO_2-NH-R feature are proposed to be anticancer, antibacterial, antifungal, antiviral and antidiabetic agents[18-20]. Hence this study will develop the new path to design and synthesize new class of molecules having *i.e.* 9,10 anthraquinone skeleton with R-SO_2-NH-R feature having potential biological activities. In present research article we report the synthesis and biological evaluation of 1,5-benzosulfonamide di-substituted-9,10-anthraquinone derivatives having SO_2-NH, and amide feature with different amino acids in side arms at 1,5 position of anthraquinone chromophore (Figure 1). Cell cycle analysis of synthesized anthraquinone derivatives has been supported by docking simulations on i-motif DNA sequence.

Experimental Section

(B1-B5) have been synthesized in our laboratory under NTP condition. Characterization of all compounds was obtained using techniques as FT-IR on NICOLET 6700 supplied by Thermo scientific USA spectrometer using KBr pellets, ^{1}H and ^{13}C NMR on a Bruker AVANCE II 400 NMR spectrometer, and ESI-MS spectroscopic studies on XEVO G2-XS Q-TOF micro spectrometer as explained in supplementary file [ST1].

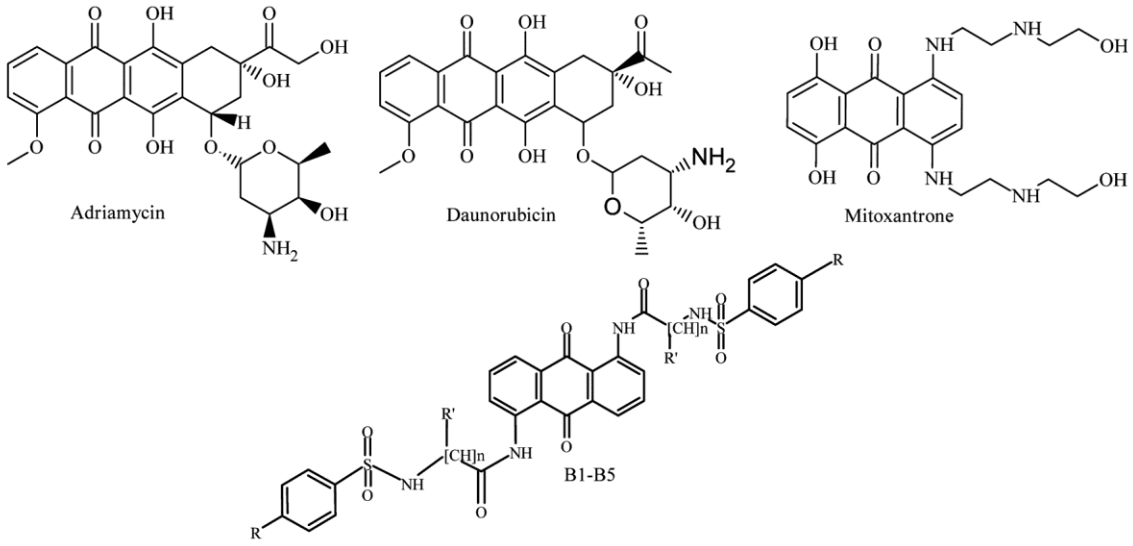


Figure 1 — Structure of the adriamycin, daunorubicin, mitoxantrone drugs and synthesized compounds (B1-B5)

General procedure of synthesis of anthraquinone derivatives (B1-B5)

Five new 1,5-diaminoanthraquinone derivatives were successfully synthesized by nucleophilic substitution reaction method by the treatment of (±)phenylalanine, (±)valine, (±)glycine, (±)beta-alanine (0.05m) (Table I) with benzene and toulene sulfonyl chloride (0.05m) in presence of NaOH solvent (1N) at 45-50°C for 4 hours stirring[21]. After the completion of reaction, the reaction-mixture was cooled to 5°C and diluted with concentrated HCl to maintain *p*H 6.5. The new product **A** was recrystallized out in hot water. The 0.1 mol of **A** in dry benzene (10 mL) was mixed with freshly distilled thionyl chloride (c) (5 mL). Reaction mixture was stirred at RT for 20 minutes and refluxed for 3 hours. The course of reaction was monitored by TLC. Excess of thionyl chloride was distilled off and residues left in reaction flask were of acid chloride of **A**. To the acid chloride; 1,5-diaminoanthraquinone (d) (0.1mol) (dissolved in 10 mL benzene at RT) was added drop wise with continuous stirring for 15 minutes followed by stirring of 4-6 hr to obtained final product **B** (Scheme I).

Cytotoxicity assay

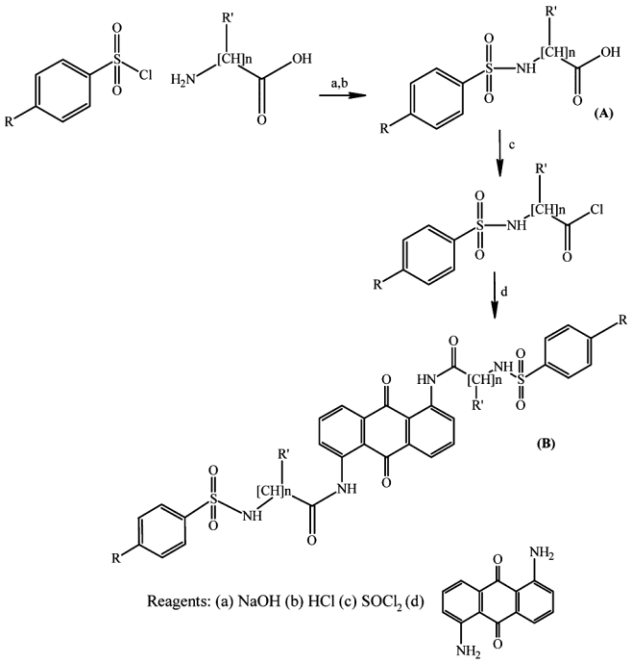
P155 Hela derivative (human cervix carcinoma), breast cancer (MCF-7) and prostate cancer (PC-3) cell lines were used to study the cytotoxic affect of compounds (B1-B5) in comparison to standard drug mitoxantonre. MTT and SRB methods were used to study the anti-proliferative effect of (B1-B5) compounds.

Table I — Substituent present in synthesized analogs (B1-B5)

Compd	R'	R
B1	$-CH_2C_6H_5$	H
B2	H	H
B3	$-CH_2C_6H_5$	$-CH_3$
B4	n=2	$-CH_3$
B5	$-CH(CH_3)_2$	$-CH_3$

Flow cytometry (Cell cycle assay)

Treated and untreated P155 Hela cells were cultured in the mixture of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1% antibiotic-antimycotic and 10% fetal bovine serum. The medium was centrifuged and trypsinized cells were washed thrice with cold phosphate buffer saline. Cells (density 5×10^5 cells/well) were then pelleted and incubated for24 hr at 37°C. The test analogues were dissolved in DMSO as a solvent to make 10 mg/mL stock solution. The cells monolayers were washed twice with freshly prepared phosphate buffered saline (PBS) and again centrifuged for 5 min. Further cell pellets were homogenized in 70% ethyl alcohol and fixed for 2h at 4°C. Fixed cells were washed with phosphate buffer saline (14 mL) and centrifuged for 5 min. Cell pellets were then mixed with propidium iodide (PI) solution containing 50µg/mL in 0.1% sodium citrate, 0.1% Triton-X 100 and 20 µg/mL RNaes A in dark for 30 minutes. Amis flow cytometer was used for processing samples. The cell cycle phase was calculated using IDEA software.



Scheme I — Synthesis of compounds (B1-B5)

Antibacterial and antifungal study

Reagents and chemicals

The nutrient broth growth medium used for antibacterial assay with phosphate buffered saline (PBS) (NaCl, Na_2HPO_4, NaH_2PO_4) as a diluent. 3 Gram-positive strains (*Salmonella aureus*, *Salmonella epidermidis* and *Salmonella citreus* and 3 gram negative (*Escherichia coli*, *Klebscilla pneumonia* and *Shigella flexneri)* micro-organisms were used for antibacterial study. Minimum inhibitory concentration (MIC) was calculated for synthesized analogues (B1-B5). *Mucor sps*. and *Candida albicans* fungal strains were used to check antifungal activity.

Sample preparation

Agar dilution method was used to study the antibacterial activity of (B1-B5). The plates used were incubated and test compounds were dissolved in methanol and poured into each petri dish. The agars were incubated for 24h at 37°C in BOD incubator. Then 2µL microbes grown in nutrient broth and 2µL of solutions of varying concentrations of each compound added to 96 micro-titre plates containing 200µL of nutrient broth each. Streptomycin was used as a reference drug.

Antioxidant activity by 2,2-diphenylpicrylhydrazyl (DPPH) assay

Spectrophotometric method was used to measure the reaction between 2,2-diphenylpicrylhydrazyl (DPPH) and synthesized analogs (B1-B5). To each concentration of synthesized compounds, DPPH solution was added. The reaction mixture was incubated for 30 min in a dark room. The mixture was shaken vigorously and allowed to stand at RT for 30 min. Absorbance was measured at 570 nm and the percentage of radical scavenging activity (RSA) was calculated using following equation:

$$RSA\ (\%) = [1\text{-}Abs._{sample}/Abs._{control}] \times 100$$

$Abs._{control}$ = Absorbance of the control without compounds.

$Abs._{sample}$= Absorbance of tested compounds

[Mitoxantrone was used as a standard drug. Lower absorbance of the reaction mixture indicated higher free radical activity].

Autodock protocol

Molecular docking was performed using Autodock 4.0 software (http://autodock.scripps.edu/). The structure of ligands is constructed using chem sketch (www.acdlabs.com) and saved as .mol file. The pdb file of i-motif is downloaded from RCSB protein data bank bearing PDB code (225d) *i.e*. the tetrameric DNA structure with protonated cytosine: cytosine base pairs. Polar hydrogens are added and grid box is generated using Autogrid tool. Docking simulations were performed using Lamarckian genetic algorithm (LGA). The ligands are ranked according to their binding energy and inhibition constant. The free energy of binding (Kcal/mol) of molecule at vicinity of an active site decided the potential interaction of ligand and macromolecule.

Results and Discussion

Chemistry

Structure of the compounds (B1-B5) are confirmed by various spectroscopic tools like FT-IR, ^{1}H NMR, ^{13}C NMR (Supplementary data). Presence of functional groups like C=O, O=S=O, C-N, N-H, C-H, C=C are identified. Bonding vibration are fixed and compared with literature. Similarly in order to check the number and type of protons and carbon atom in defined functional groups of the molecules; positions are marked and fixed in FT-IR, ^{1}H and ^{13}C NMR spectrogram and compared with literature.

Finally the mass of all the synthesized molecule (B1-B5) is calculated and proposed. We have laid down the fragmentation pattern based on the m/z values in mass spectra of one of the compound B1 (Figure 2). Molecular mass of B1 calculated to be M = 812 and ESI-MS resolved output confirms the same. $M^{+\cdot}$, $[M+H]^+$ and $[M+Na]^+$ are identified. $[M+H]^+$

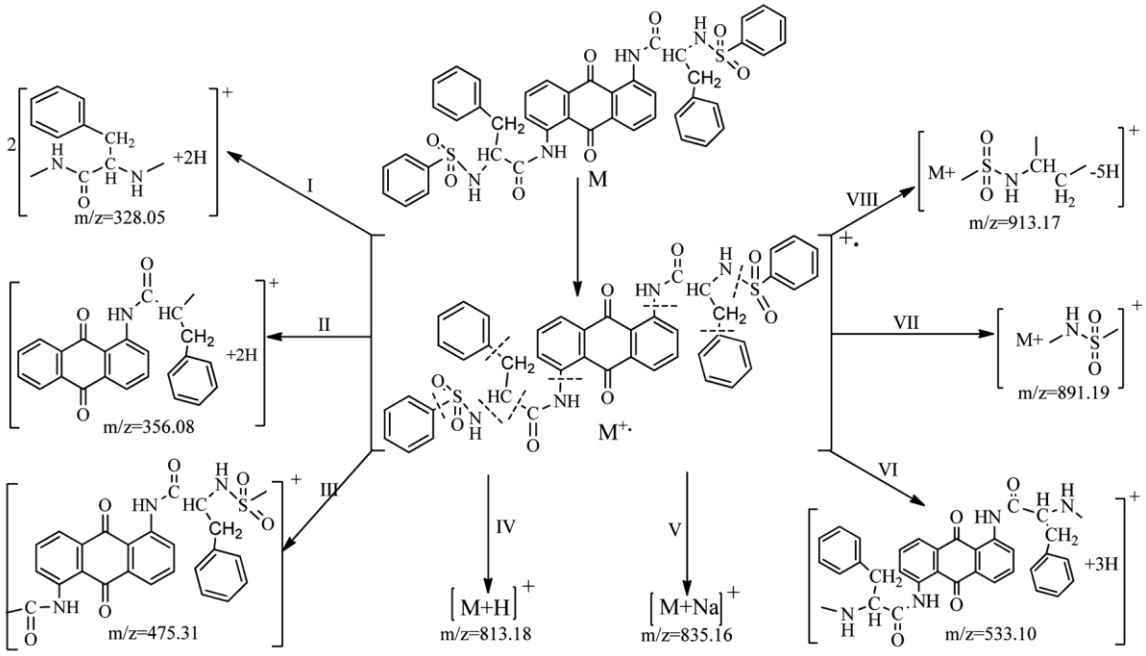


Figure 2 — Representation of mass fragmentation pattern of synthesized B1compound

(813.18) is the most intense peak indicates the stability of the compound B1. Stable fragments have been identified as per relative abundance in the mass spectra. All compounds (B2-B5) showed the similar pattern and hence confirm the structure of compounds.

In vitro cytotoxic screening

Synthesized di-substituted 9,10 anthraquinone analogues along with mitoxantrone are screened for their cytotoxic activities against Hep 2C (Hela derivative Human cell line) using MTT assay. Some of synthesized compounds (B1-B5) showed cytotoxicity at higher concentration against MCF-7 (Human Breast Cancer cell line) and PC-3 (Human Prostate Cancer cell line) using SRB method (Table II and Table III). All three cell lines were exposed to different concentration of synthesized di-substituted compounds (B1-B5) for a fixed hours in comparison to mitoxantrone (Figure 3).

Table II — IC_{50} value of (B1-B5) on Hep-2C determine by MTT assay

Hep-2C (Human cervix carcinoma)	IC_{50} (μg/mL)
B1	Not effective
B2	17
B3	13
B4	30
B5	Not effective
Mitoxantrone	2.5

Table III — GI_{50} values of (B1-B5) and MTX on MCF-7 and PC-3 by SRB Assay

Compd	MCF-7 (μg/mL)	PC-3 (μg/mL)
B1	NE	>80
B2	45.2	68.1
B3	>80	>80
B4	74.9	<10
B5	–	–
Mitoxantrone	<10	<10

Cell cycle (Flow cytometry) assay

As per cytotoxic study, B2 derivative of 1,5 di-substituted 9,10 anthraquinone compounds used for cell cycle analysis in order to understand the mechanism of action at cellular level[22]. The decrease in the population (~5%) of cell in the S phase has been observed (Table IV and Figure 4). G0/G1 phase activities are escalated by ~5%. However in comparison to control activities in G2/M phase it is dropped by ~1%. Complete disturbance has been observed in cell cycle. According to literature study, mitoxantrone significantly arrest S-phase of cell cycle and blocks G1 and G2 phases which suggests the obstruction in the synthesis phase[23]. DNA gets doubled in S phase where as RNA transcription and protein synthesis gets minimized. Fast activities in G0/G1 phase indicates the enhancement in growth phase. G0/G1 phase permits the cell to enter in S

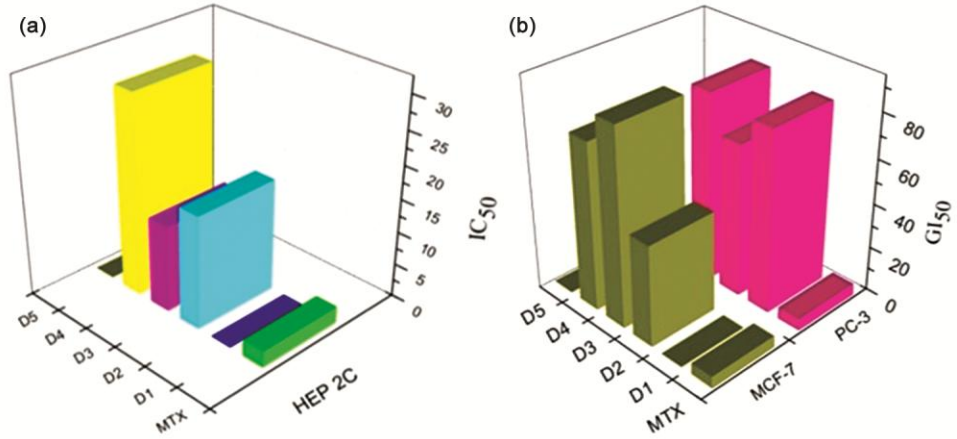


Figure 3 — **(a)** Percentage growth inhibition of Hep2C of (B1-B5); **(b)** Percentage growth inhibition of MCF-7 and PC3 of (B1-B5)

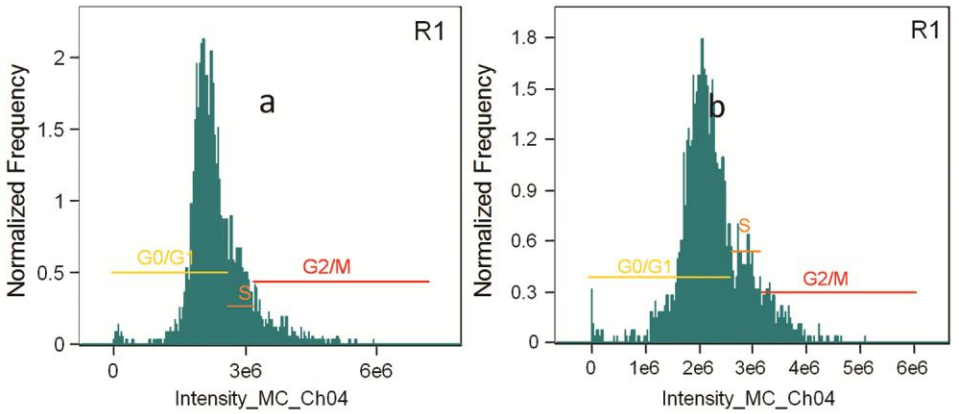


Figure 4 — Cell-cycle progression (a) control (b) B2

phase where as G2/M phase slows down initially and starts after DNA replication. It is a phase of protein synthesis and rapid cell growth to prepare for mitosis. Results clearly shows that B2 perturbs the cell distribution in all phases (G0/G1; S; G2/M) of cell cycle and S phase is most affected. Hence it is proposed that B2 block DNA replication mechanism (Figure 4). We have carried out similar studies on 1-substituted 9,10 anthraquinone analogues and surprisingly results are quite different[24]. It is pertinent to mention that by incorporating substituent chains/ group at different positions of anthraquinone ring has affected the biochemical mechanism of action of drug. In present case by blocking the 1,5 position simultaneously with the same functional group(B2) have affected the mechanism of action of substituted anthraquinone compounds.1-substitution did not target DNA while 1,5 substituted clearly indicates the DNA binding phenomena[24].

Table IV — Effect of control and B2 on cell cycle distribution

Sample	Distribution in Cell (%)		
	G_0/G_1-phase	S-phase	G_2/M-phase
B2 (5µg/mL)	78	12.8	7.56
Control	73.6	17.4	8.44

MIC (Minimum Inhibitory Concentration)

The minimum inhibitory concentration is the lowest concentration of test compound which inhibits the visible growth of microbe. The calculation of MICs involves semi-quantitative test protocol. The synthesized compounds (B1-B5) demonstrates their effectiveness in different concentrations against Gram positive and Gram negative bacteria. B2 is effective for all the strains except *S. flexneri* whereas some weak MIC values has also been observed. It could be due to structurally different side chains at N-1 and 5 position along with sulfonamide feature on the molecule (Table V and Table VI).

Table V — The zone of inhibition (24) hr on test compounds

Compd	Zone of inhibition (mm)					
	Gram +ve			Gram −ve		
	S. epidermidis	*S. aureus*	*S. citreus*	*K. pneumoniae*	*E. coli*	*S. flexneri*
B1	5.5	6.66	−	5	−	6.66
B2	11	15.33	16.33	6.66	11	−
B3	11	−	11	−	11	−
B4	5.5	11	−	−	6.66	6.66
B5	6.66	−	11	−	−	−
Streptomycin	16	20	40	16	20	32

Table VI — Antimicrobial activity of (B1-B5) with minimum inhibitory concentration

Compd	Gram +ve			Gram −ve		
	S. epidermidis	*S. aureus*	*S. citreus*	*K. pneumoniae*	*E. coli*	*S. flexneri*
B1	49	24.5	+	49	+	24.5
B2	12.25	6.12	3.06	24.5	12.25	+
B3	12.25	+	12.25	+	12.25	+
B4	49	12.25	−	+	24.5	+
B5	24.5	+	12.25	+	−	+
Streptomycin	0.765	3.062	1.55	6.1	3.062	0.765

Antifungal study

The study was done on two micro-organisms *i.e*. *Candida albicans* and *Mucor sps* but compounds are found to be ineffective on both the species.

Antioxidant activity

Limited number of anthracycline class of drugs used in chemotherapy due to their interference in redox mechanism. Anthraquinone class of compounds exerts peroxidating activity with production of the free radicals. The activity is initiated by the number of cellular oxy-reductase enzymes which has been proposed to be the main cause of cardio-toxicity. Anthraquinone class of drugs are attacked by oxido-reductase enzyme and produce O_2 radicals (oxygen radicals). Detailed study has been reported in literature on natural and synthetic anthraquinones class of compounds with different structural features to see their ability to undergo redox reactions[25]. Quinone functional groups on anthracycline ring favours or disfavours the oxidation-reduction/ free radical formation. It has been reported that Mitoxantrone, an anticancer drug showed poor affinity towards NADH dehydrogenation reaction, however, there are OH groups present at 1and 4 position with unmodified carbonyl group. The mechanism is not clearly drafted but it might be due to aromatic amino group with long chains at 5, 8 substituted positions on the anthraquinone ring.

Table VII — DPPH activity of synthesized compounds (B1-B5) at 200 µg/mL concentration

Compd	Percent scavenging
B1	Not effective
B2	Not effective
B3	Not effective
B4	Not effective
B5	Not effective

Highest level of flexibility of side arms and steric hindrance due to bulky groups with binding protein molecule could be another reason. So keeping in view, we have carried out DPPH analysis of (B1-B5) compounds to evaluate their potential to scavenge O_2 radical production (Table VII). Synthesized 1,5 di-substituted 9,10-anthraquinone compounds were not effective in stimulating free radical (O_2) oxygen production.

Molecular docking study

3'-end of eukaryotic chromosome consist of several kilos of polynucleotide bases with repeat of base sequence called telomere. These telomere ends of DNA are not preserved during cell division. Most of human cell can divide 50-60 times before it enters to apoptosis. However some type of cells for example germ cell, stem cell, *etc*. regulate telomerase to constant length by expressing a specialized RNA depended DNA polymerase called telomerase. As per literature this enzyme is nearly expressed in cancer

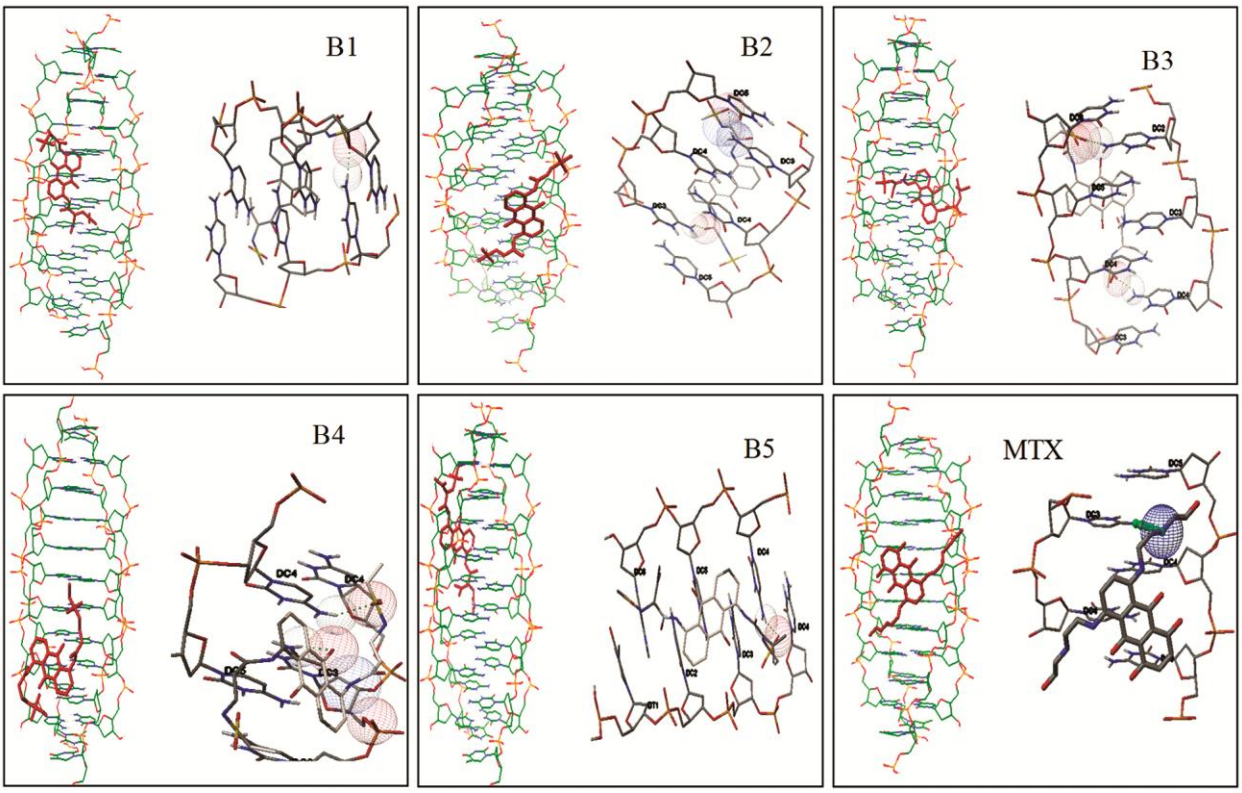


Figure 5 — Docked representation of (B1-B5) and MTX with i-motif

Table VIII — Inhibitory constant (K_i) Free energy of binding (ΔG_b) and bond length of (B1-B5) with d(TC_5)

Compd	Binding interactions	K_i (μM)	ΔG_b (Kcal/mol)	Number in cluster
B1	S=O…….CYS3	2.72	−7.59	5
B2	C=O…..DC3 S=O…DC3	19.04	−6.44	4
B3	S=O…..DC4 S=O…….DC2	166.11	−9.25	1
B4	S=O…..DC4 Aaq C=O…DC3	19.89	−6.41	3
B5	S=O……DC4	615.19	−8.47	2
MTX	NH……CYS4	63.67	−5.62	8

cells (Breast and gastric tumor cell)[26-29]. However enzyme was not detected in somatic cell. Anthraquinone compounds and its analogues are the first category of compounds to stabilized telomeric junction. Molecular docking study of (B1-B5) in comparison to MTX with d(TC_5) intermolecular i-motifs using autodock software has been analyzed. The docking study of five compounds showed large variation in inhibition constant (K_i)and the O=S=O group of the molecule bound to CYS residues of DNA *via* intermolecular hydrogen bonds (Table VIII). It has also been observed for (B1-B5) series where two side chains at 1,5 positions adopts extended confirmation at i-motif junction d(TCCCCC) with partial interaction of anthraquinone chromophore with tetrad base steps has also revealed by the binding affinity value. Some deformation at the tetrad junction has also been observed. This distorted conformation could be due to heavy functionalities at 1,5 side arms (Figure 5).

Conclusions

Objective of present study was to study effect of substituent groups and its position on tricyclic 9,10-anthraquinone ring. Our group is working on designing and synthesis of 1,2;1,5;1,4 and 1,8 substituent anthraquinone series. In present research paper we proposed the synthesis of 1,5 di-substituted 9,10-anthraquinone compounds. B2 and B3 1,5 di-substituted 9,10-anthraquinones showed anticancer activity in comparison to Mitoxantrone. The antibacterial activity of (B1-B5) compounds is significantly varied for six microbes due to their unique biocidal mechanism. The DPPH activity assay clears that compounds does not produce free radical. Cell cycle analysis of B2 clearly indicates the perturbation at S phase of cell cycle wherein DNA

gets doubled in S phase and RNA transcription and protein synthesis gets minimized. This observation clearly indicates the DNA binding mechanism of 1,5 di-substituted 9,10 anthraquinone molecules as per line of mitoxantrone. Molecular docking with i-motif indicates the binding of 1,5 di-substituted 9,10 anthraquinone molecules with DNA telomeric junction. Variation in cellular mechanism has been observed in comparison to 1-substituited analogues. Same can only be confirmed by in- vitro binding studies and will be reported subsequently.

Supplementary Information

Supplementary information is available in the website http://nopr.niscair.res.in/handle/123456789/58776.

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