Indian Journal of Advances in Chemical Science

Article

Spectroscopic Investigation of Steroidal Benzothiazoles as DNA-Binding Agents and their Role in Cytotoxicity

Ayaz Mahmood Dar[1]*, Shafia Mir[2], Manzoor Ahmad Gatoo[3], Shamsuzzaman[1]

[1]Department of Chemistry, Aligarh Muslim University, Aligarh 202 002, UP, India, [2]Department of Chemistry, OPJS University, Churu 331001, Rajasthan, India, [3]Department of Biochemistry, JNMC Aligarh Muslim University, Aligarh 202 002, UP, India

ABSTRACT

The new steroidal benzathiazole derivatives (**4-6**) were synthesized by the reaction of steroidal ketones (**1-3**) with 2-aminothiophenol in chloroform. After characterization by spectral and analytical data, the DNA interaction studies of one of the compounds were carried out by ultraviolet–visible, fluorescence spectroscopy, viscosity measurements, circular dichroism, molecular docking, and gel electrophoresis. The compound binds to DNA preferentially through electrostatic and hydrophobic interactions with Kb; 4.5 $\times 10^4$ M^{-1} revealing its potential propensity toward computed tomography DNA. Gel electrophoresis demonstrated that compound **5** showed a strong interaction during the concentration-dependent cleavage activity with pBR322 DNA. The molecular docking study suggested the intercalation of compounds in the minor groove of DNA. During *in vitro* cytotoxicity (3-(4,5-dimethylthizao1-2-y1)-2,5-diphenyltetrazolium bromide assay), compound (**4-6**) revealed potential toxicity against the different human cancer cells. Western blotting analysis clearly indicates that compound **5** causes apoptosis in HL-60 cancer cells. The results revealed that compound **5** has better prospectus to act as a cancer chemotherapeutic candidate, which warrants further *in vivo* anticancer investigations.

Key words: Benzothiazole, DNA binding, 3-(4,5-Dimethylthizao1-2-y1)-2,5-diphenyltetrazolium bromide assay, Western blotting.

1. INTRODUCTION

Steroids have always attracted considerable attention because of being a fundamental class of biologically signaling molecules. In addition to their profound physiological and clinical importance [1], they have the potential to be developed as drugs for the treatment of a large number of diseases including cardiovascular, autoimmune, and brain tumors, breast cancer, prostate cancer, and osteoarthritis [2-4]. Most of the steroid-based pharmaceuticals are semisynthetic compounds prepared by connecting a special functionality to the core structure of a steroid [5]. The advantage of employing hydrophobic steroid units is their ability to interact with cell membranes and thus pave the way for biological activity of such hybrid molecules [5].

Benzothiazole moiety containing molecules possess an extensive range of biological activities including anticonvulsant [6], antiviral [7], antitubercular [8], antimalarial [9], anthelmintic [10], photosensitizing [11], diuretic [12], analgesic [13], anticancer [14], antimicrobial [15], antioxidant, anti-inflammatory [13], and antidiabetics [16] and other activities [17]. The role of benzothiazole moiety in oncology was assessed during the development and screening of tyrosine kinase inhibitors during the late 20th century. Keeping in view the applications of these benzothiazole derivatives and in continuation of our previous work [18] herein, we report the synthesis of new steroidal benzothiazoles as metal-free DNA-binding agents. Furthermore, these compounds have also been screened for *in vitro* anticancer activity. It is indeed interesting to note that these compounds show significantly potential anticancer behavior against human cancer cells selected for the study.

2. EXPERIMENTAL

2.1. Material and Methods

Chemicals were purchased from Merck and Sigma-Aldrich as "synthesis grade" and used without further purification. Melting points were determined on a Kofler apparatus and are uncorrected. The infrared (IR) spectra were recorded on KBr pellets with Pye Unicam SP3-100 Spectrophotometer and values are given in cm^{-1}. ^1H and ^{13}C-NMR spectra were run in $CDCl_3$ on a JEOL Eclipse (400 MHz) instrument with TMS as internal standard and values are given in ppm (δ). Mass spectra were recorded on a JEOL SX 102/DA-6000 Mass Spectrometer. Thin-layer chromatography plates were coated with silica gel G and exposed to iodine vapors to check the homogeneity as well as the progress of reaction. Sodium sulfate (anhydrous) was used as a drying agent. pUC 19 DNA was purchased from GeNei (India) and used for the agarose gel experiment without further purification. Double-stranded calf thymus DNA, purchased from Sigma, was dissolved in a 0.1M Tris-buffer.

2.2. General Method for the Synthesis of Steroidal Spiro-benzathiazoles (4-6)

Steroidal ketones [19] (3.52 mmol) were dissolved in 20 ml of chloroform and three drops of $BF_3 \cdot Et_2O$ were added. Then, freshly prepared 2-aminothiophenol (10.56 mmol) was added dropwise. The mixture was refluxed for 12 h and then cooled to room temperature. After completion of reaction, the solvent was reduced to the three fourth of the original volume under reduced pressure. The reaction mixture was taken in ether, washed with water, and dried over Na_2SO_4. Evaporation of solvents and recrystallization from methanol afforded

***Corresponding author:**
E-mail: ayazchem09@gmail.com

ISSN NO: 2320-0898 (p); 2320-0928 (e)
DOI: 10.22607/IJACS.2021.903001

Received: 17th January 2021;
Accepted: 02nd February2021

respective products (**4-6**).

2.2.1. 3β-Acetoxy-5α-cholestano-6-spirobenzothiazole (4)

Yield: 75%; solid; mp: 152°C; anal. calcd. for $C_{35}H_{53}NO_2S$: C 76.17, H 9.68, N 2.54; found C 76.05, H 9.45, N 2.31. IR (KBr, cm^{-1}): 3260 (NH), 1720 ($OCOCH_3$), 3035, 1593 (aromatic), 1380 (C-N), 1085 (C-O), 634 (C-S). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.3 (s, 1H, NH, exchangeable with D_2O), 6.30–6.24 (*m*, 4H, aromatic), 4.7 (*m*, 1H, C3 α-H, *W½* = 15 Hz), 2.03 (*s*, 3H, $OCOCH_3$), 1.8 (*d*, 2H, C_4-H_2), 1.9 (*d*, 2H, C_7-H_2), 1.18 (*s*, 3H, C_{10}-CH_3), 0.70 (*s*, 3H, C_{13}-CH_3), 0.97 and 0.83 (other methyl protons). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 174, 134, 129, 128, 126, 124, 122, 70, 48, 46, 42, 39, 35, 26, 24, 22, 20, 17. ESI MS: 551 [M^+].

2.2.2. 3β-Chloro-5α-cholestano-6-spirobenzothiazole (5)

Yield: 73%; solid; m p: 142°C; anal. calcd. for $C_{33}H_{50}ClNS$: C 75.03, H 9.54, N 2.65; found C 74.22, H 9.06, N 2.24. IR (KBr, cm^{-1}): 3290 (NH), 3033, 1600 (aromatic), 1382 (C-N), 740 (C-Cl), 638 (C-S). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.0 (s, 1H, NH, exchangeable with D_2O), 6.22–6.13 (*m*, 4H, aromatic), 3.9 (*m*, 1H, C_3 α-H, *W½* = 17 Hz), 1.6 (*d*, 2H, C_4-H_2), 1.8 (*d*, 2H, C_7-H_2), 1.2 (*s*, 3H, C_{10}-CH_3), 0.70 (*s*, 3H, C_{13}-CH_3), 0.97 and 0.83 (other methyl protons). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 132, 130, 127, 124, 122, 121, 59, 44, 40, 38, 36, 31, 27, 25, 21, 19, 17. ESI MS: 527/529 [M^+].

2.2.3. 5α-cholestano-6-spirobenzothiazole (6)

Yield: 70%; solid; m p: 146°C; anal. calcd. for $C_3H_{51}NS$: C 80.26, H 9.06, N 2.84; found C 79.26, H 8.68, N 2.53. IR (KBr, cm^{-1}): 3267 (NH), 3025, 1615 (aromatic), 1380 (C-N), 740 (C-Cl), 632 (C-S). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.7 (s, 1H, NH exchangeable with D_2O), 6.20-6.15 (*m*, 4H, aromatic), 1.55 (*d*, 2H, C_4-H_2), 1.9 (*d*, 2H, C_7-H_2), 1.23 (*s*, 3H, C_{10}-CH_3), 0.75 (*s*, 3H, C_{13}-CH_3), 0.96 and 0.80 (other methyl protons). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 137, 135, 131, 129, 127, 124, 46, 44, 40, 38, 36, 31, 27, 25, 21, 19, 17. ESI MS: 493 [M^+].

2.3. DNA-binding experiments

2.3.1. Electronic absorption and fluorescence spectroscopy

DNA-binding experiments, including absorption spectral traces and emission spectroscopy, conformed to the standard methods and practices reported in the literature [20]. While measuring the absorption spectra, an equal amount of DNA was added to both the compound solution and the reference solution to eliminate the absorbance of the DNA itself, Tris-buffer was subtracted through baseline correction.

2.3.2. DNA cleavage experiments

Cleavage experiments were performed with Axygen agarose electrophoresis [21] connected to a Genei 50–500 V power supply, visualized and photographed by the Vilber-INFINITY gel documentation system. Cleavage experiments of supercoiled pBR322 DNA (300 ng) by compound **5 (**1.0–5.0 µM) in a 5 mM Tris-HCl/50 mM NaCl buffer at pH 7.2 were carried out and the reaction followed by agarose gel electrophoresis. The samples were incubated for 1 h at 37°C. A loading buffer, containing 25% bromophenol blue, 0.25% xylene cyanol, and 30% glycerol, was added and electrophoresis was carried out at 60 V for 1 h in a Tris-HCl buffer using a 1% agarose gel containing 1.0 mg/mL of ethidium bromide.

2.3.3. Viscosity measurements

Viscosity measurements were done using an Ubbelohde viscometer maintained at a constant temperature at 25°C. DNA samples approximately 200 base pairs in average length were prepared by sonication to minimize complexities arising from DNA flexibility. Flow time was measured with a digital stopwatch, and sample was measured three times, and an average flow time was calculated. Relative viscosities for DNA in the presence and absence of compound were calculated from the relation $\eta = (t–t°)/t°$, where, t is the observed flow time of the DNA-containing solution and t° is the flow time of buffer alone. Data were presented as $(\eta/\eta°)^{1/3}$ versus binding ratio, where, η is the viscosity of DNA in the presence of compounds and $\eta°$ is the viscosity of DNA alone.

2.3.4. Circular dichroism measurements

Circular dichroism measurements were recorded on a JASCO (J-810) spectropolarimeter by keeping the concentration of DNA constant (5×10^{-5} M) while varying the compound concentration (ri=[Compound]/[DNA]=0.0, 0.02, 0.04, 0.06, and 0.08). The optical chamber of the circular dichroism (CD) spectrometer was deoxygenated with dry nitrogen before use and kept in a nitrogen atmosphere during experiments. All observed CD spectra were corrected for the buffer signal.

2.3.5. Molecular docking

The rigid molecular docking studies were performed using **HEX 6.1** software [22]. The initial structure of the compounds **7** was generated by Mercury modeling software. The molecules of compounds **7-9** were optimized for use in the following docking study. The crystal structure of the B–DNA dodecamer $d(CGCAAATTTCGC)_2$ (PDB ID: 1BNA) was downloaded from the Protein Data Bank. All calculations were carried out on an Intel CORE i5, 2.6 GHz based machine running MS Windows 7 as the operating system. Visualization of the docked pose has been done using PyMol molecular graphics programs.

2.4. In vitro cytotoxicity

Human cancer cell lines **SW480** (colon adenocarcinoma), **A549** (lung carcinoma), **HepG2** (hepatic carcinoma), **HeLa** (cervical cancer), **MCF-7** (breast cancer cells), and **HL-60** (leukemia) were taken for the study. The cells lines were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 10 U penicillin, and 100 µg/ml streptomycin at 37°C with 5% CO_2 in a humidified atmosphere **NL-20** (normal lung cells) and **HPC** (normal pulp cells) which were grown at 37°C with 5% CO_2, 95% air under the humidified conditions. Fresh medium was given every 2nd day and on the day before, the experiments were done. Cells were passaged at pre-confluent densities, using a solution containing 0.05% trypsin and 0.5 mM EDTA. The anticancer activity *in vitro* was measured using the 3-(4,5-dimethylthizao1-2-y1)-2,5-diphenyltetrazolium bromide (MTT) assay. The assay was carried out according to known protocol [23]. Exponentially growing cells were harvested and plated in 96-well plates at a concentration of 1×10^4 cells/well. After 24 h incubation at 37°C under a humidified 5% CO_2 to allow cell attachment, the cells in the wells were respectively treated with target compounds at various concentrations for 48 h. The concentration of dimethyl sulfoxide (DMSO) was always kept below 1.25%, which was found to be non-toxic to the cells. A solution of MTT was prepared at 5 mg/ml in phosphate-buffered saline. Twenty microliters of this solution were added to each well. After incubation for 4 h at 37°C in a humidified incubator with 5% CO_2, the medium/MTT mixtures were removed, and the formazan crystals formed by the mitochondrial dehydrogenase activity of vital cells were dissolved in 100 µl of DMSO per well. Effects of the drug cell viability were calculated using cell treated with DMSO as control. IC_{50} is the concentration in "µM" required for 50% inhibition of cell growth as compared to that of control. IC_{50} values shown in Table 1 were determined from the linear portion of the curve by calculating the concentration of agent that reduced absorbance in treated cells, compared to control cells, by 50%. Evaluation is based on mean values from three independent experiments, each comprising at least six microcultures per concentration level.

Table 1: Anticancer activity data of compound **4-6** against human cancer cell lines.

Compound	IC_{50} (µmol)[a]							
	SW480	A549	HepG2	HeLa	HL60	MCF7	NL20	HPC
4	17.38	>50	32.11	19.61	16.21	17.75	64.23	82.11
5	19.52	17.41	22.53	19.27	14.35	21.42	77.12	66.43
6	24.13	20.37	38.12	>50	21.26	25.12	>100	72.15
Doxorubicin	12.45	9.12	13.21	16.76	14.37	14.21	>100	>100
Cisplatin	3.52	12.1	9.63	9.43	7.83	9.3	63.35	61.17

Cell growth inhibition was analyzed by the MTT assay. [a] IC_{50} is the concentration of compound that inhibits 50% of cell growth.

2.5. Western Blot Analysis

HL-60 cells were grown on 100 mm tissue culture discs at a density of 1 × 10^6 cells per plate and incubated overnight. Then, the cells were exposed to compound **5** (0, 10, 20, and 30 µM) for 48 h before harvest. The cell lysate was prepared using modified RIPA lysis buffer (50 mM tris, 150 mM NaCl, 0.5 mM deoxycholate, 1% NP-40, 0.1% SDS, 1mMNa3VO4, 5 m MEDTA, 1 m MPMSF, 2 m MDTT, 10 m M*β*-glycerophosphate, 50 mM NAF, 0.5% triton X-100, and protease inhibitor cocktail). Protein estimation was done by Bradford's method. Proteins were separated in 10% SDS-PAGE and transferred to PVDF membrane. Membranes were blocked overnight with 10% skimmed milk in 1 × TBS-T (Tris-buffered saline containing 0.05% of Tween-20) at 4°C and immunoblotted with antibodies anti-Bcl-XL, anti-Bax, and anti-PARP. Detection of signals was done using ECL Western blotting reagent and chemiluminescence was exposed to Kodak X-Omat films. Antibody, anti-*β*-actin was used as loading control. All the antibodies were procured from cell signaling technology, CA, USA [18].

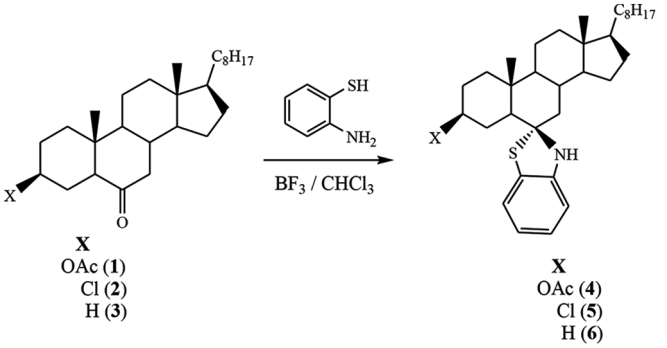
3. RESULTS AND DISCUSSION

3.1. Chemistry

Development of highly functional molecules from simple building blocks has always been the curiosity of synthetic chemists. Hence, we here in report the convenient route for the synthesis of new steroidal benzothiazoles (**4-6**) by reacting steroidal ketones (**1-3**) with 2-aminothiophenol in chloroform for the period of about 12 h under reflux conditions (Scheme 1) and on the completion of reaction, products were obtained in better yields (70–75%).

The mechanism (Scheme 2) for the formation of compounds (**4-6**) involves the first nucleophilic attack from sulfur atom of 2-aminothiophenol moiety on carbonyl group of steroidal ketone and subsequent removal of water molecule that leads to the formation of products (**4-6**). The structures of these compounds were characterized by spectral (IR, ^{1}H-NMR, ^{13}C-NMR, and MS) and analytical methods.

The characterization studies showed good agreement with proposed structures of steroidal benzothiazoles (**4-6**) (Scheme 1). In their IR spectra, the presence of absorption bands in the range 3260–3290 shows the presence of NH and as the medium intensity absorption bands at 3025–3035 and 1593–1615 cm^{-1} confirm the presence of aromatic *CH* and *C=C* groups, respectively, in the compounds (**4-6**). The absorption bands at 632–638 and 1380–1382 cm^{-1} were ascribed to C-S and C-N groups, respectively. In ^{1}H-NMR study of compounds (**4-6**), the downfield singlet at *δ* 8.3-7.7 was ascribed to N*H* while as the presence of multiplet at *δ* 6.13–6.30 was assigned to four aromatic protons of benzathiazole ring. In ^{13}C-NMR study, the signals at *δ* 137-122, *δ* 44–46, and *δ* 42–48 confirm the presence of C=C (aromatic), *C*-S, and *C*-N groups, respectively, in the products (**4-6**). Finally, the presence of distinct molecular ion peak [M^+] at m/z: 551, 527/529, and 493 in the MS also proved the formation of compounds (**4-6**). The strategy can also be applied to diverse ketones, in that way, benzothiazoles may also allow further modifications on the substituted heterocyclic systems.



Scheme 1: Formation of steroidal benzothiazoles from steroidal ketones.

3.2. DNA-Binding Studies

3.2.1. Absorption spectroscopy

Compound **5** exhibited bands at 254 nm, respectively, in the ultraviolet (UV) region which are assigned to the π-π* transitions, due to long living triplet excited state of the two double bonds in conjugation (Figure 1). The UV region exhibited an increase in absorption intensity "hyperchromic" effect with a red shift of 2–4 nm in π-π* region on increasing concentration of computed tomography (CT) DNA (0–0.4 × 10^{-4} M). The strong hyperchromic effect with a significant red shift is suggestive of higher binding propensity to CT DNA, possibly by electrostatic mode, and stabilization of the compound DNA adduct.

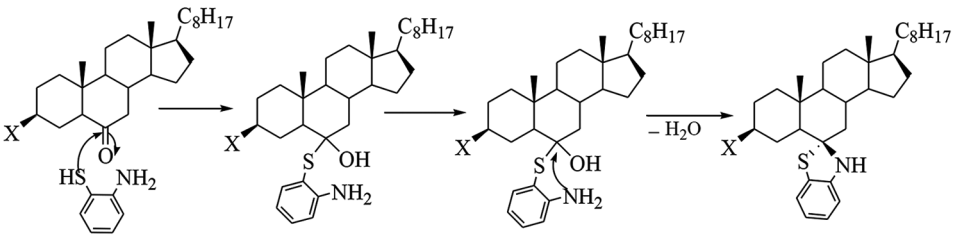
The intrinsic binding constants K_b of the compounds were determined to evaluate quantitatively the binding strengths of compound **5** with CT DNA, with Eq. (1) by monitoring the changes in absorbance of the π-π* bands with increasing concentration of CT DNA:

$$[DNA]/|\varepsilon_a-\varepsilon_f| = [DNA]/|\varepsilon_b-\varepsilon_f| + 1/K_b|\varepsilon_b-\varepsilon_f| \quad (1)$$

Where, [DNA] represents the concentration of DNA, ε_a, ε_f, and ε_b are the apparent extinction coefficient Aobs/[M], the extinction coefficient for free compound and the extinction coefficient for compound in the fully bound form, respectively. In the plots of [DNA]/εa-εf versus [DNA], Kb is given by the ratio of the slope to the intercept. The intrinsic binding constant of compounds **5** was found to be 4.5 ×10^4 M^{-1} revealing potential propensity toward CT DNA.

3.2.2. Emission spectroscopy

The fluorescence study was used to investigate the interaction of compound **5** in the presence and absence of CT DNA. The compound



Scheme 2: Mechanism for the formation of steroidal benzothiazoles from steroidal ketones.

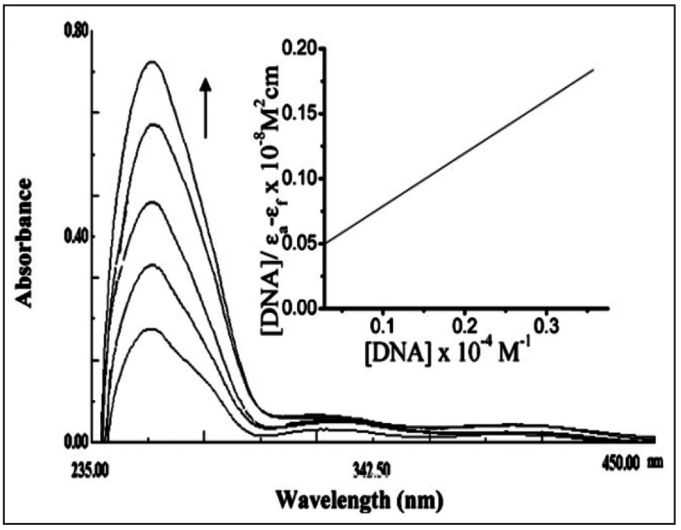


Figure 1: Variation of ultraviolet–visible absorption for compound **5** with increase in the concentration of computed tomography DNA in buffer 5 mM Tris-HCl/50 mM NaCl, pH = 7.2 at 25°C. Inset: Plots of (DNA)/ε_a-ε_f versus (DNA).

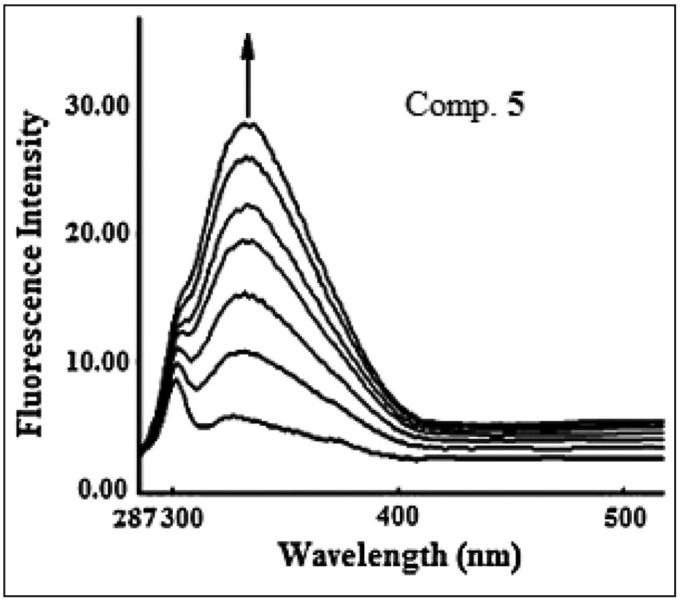


Figure 2: Emission spectra of compound 5, in the absence and presence of computed tomography DNA in buffer 5 mM Tris-HCl/50 mM NaCl, pH = 7.2 at 25°C.

5 when excited at 265 nm, exhibited emission bands at 336 nm. Compound **5** emit weak luminescence in 5 mM Tris-HCl, 50 mM NaCl buffer at ambient temperature in the absence of CT DNA. The increase in the emission intensity of compound **5** with increasing CT DNA concentration is shown in Figure 2. Since it is found that compounds with increased hydrophobicity show greater increase in emission intensities on binding to polyelectrolytes, namely, DNA, thus, we conclude that it is the hydrophobicity of both the molecules which is mainly responsible for some interaction between the CT DNA and compounds. The CT DNA-binding ability of the compound **5** was obtained from Scatchard equation [24], with $K = 4.3 \times 10^4\ M^{-1}$ for compound. These results are consistent with the findings obtained from UV–Vis spectral studies.

3.2.3. DNA cleavage

The DNA cleavage was controlled by the relaxation of supercoiled circular form of pBR322 DNA into the nicked and linear form. When a circular plasmid DNA is subjected to agarose gel electrophoresis, the fastest migration will be observed for supercoiled form (Form I). If one strand is cleaved, the supercoils will relax to produce a slower moving open circular form (Form II). If both strands are cleaved, a linear form (Form III) will be generated that migrates in between Form I and Form II. We carried out cleavage studies of DNA using compound **5** only. Its DNA cleavage ability was investigated using pBR322 DNA. In the absence of any external additives, the compound **5** cleaved double-stranded supercoiled plasmid DNA (SC form: Form I) (300 ng) in 5 mM Tris-HCl/50mM NaCl buffer into nicked circular form (NC form: Form II) after 1 h of incubation at physiological pH 7.2 and temperature 25°C. Keeping the DNA concentration constant (300 ng), the concentration of compound **5** was varied (2.0–10.0 μM) and the cleavage reaction was further monitored by gel electrophoresis. The results revealed concentration-dependent electrophoretic cleavage clearly showing the conversion of SC form (Form I) to NC form (Form II) with increase in concentration of compound **5**.

At 4 μM concentration, compound **5** exhibited efficient nuclease activity. At still higher concentrations, there was complete conversion of SC form into NC form. The presence of Form I and II of pBR322 DNA indicated that compound **5** is involved in double-stranded DNA cleavage (Figure 3).

3.2.4. Viscosity measurements

The viscosity of a DNA solution is sensitive to the addition of organic compounds bound by intercalation. In general, a classical intercalation of a compound into DNA causes a significant increase in the viscosity of a DNA solution because of an increase in the separation of the base pairs at the intercalation site, and hence, an increase in the overall DNA molecular length. A non-classical intercalation of compound could bend the DNA helix, reduces its effective length and its viscosity. The relative changes in viscosity were measured using CT DNA with increasing concentration of the compound **5**. The effects of compound on the relative viscosity of rod-like DNA are shown in Figure 4. On increasing the concentrations of compound **5**, the relative viscosity of the DNA increases. These results show that compound **5** intercalates between the DNA base pairs.

3.2.5. CD spectra

In the CD spectrum of B-DNA, the positive band is due to the base stacking (270 nm), while the negative one (240 nm) corresponds

to the right-handed helicity [25]. In this study, the CD spectra of B-DNA were recorded in the presence of different molar ratios of the steroid compounds (**4-6**). The changes in the CD signals of BDNA, as observed in the interaction with the compounds, can be assigned to the corresponding changes in DNA morphology [26]. Classical intercalative molecules tend to enhance the intensities of bands due to a strong base stacking interactions and stable DNA conformations, while simple groove binding and electrostatic interactions demonstrate less perturbation or no perturbation on the base stacking and helicity bands. Furthermore, it should be stated that intercalated compounds which disrupt interactions between DNA bases and weaken base stacking cause a decrease in the intensities of CD bands [27]. The CD spectra of DNA in the presence of the compounds are illustrated in Figure 5.

3.2.6. Molecular docking

In our experiment, molecular docking studies of compound (**4-6**) with DNA duplex of sequence d(CGCGAATTCGCG)$_2$ dodecamer (PDB ID: 1BNA) were performed to predict the chosen binding site along with preferred orientation of the molecules inside the DNA groove. It is evident from Figure 6 that compound **4** interacts in the minor groove of the nucleotide base pairs of DNA and in this configuration; the acetoxy group at 3*β*-axial position remains inclined toward the phosphodiester bond of DNA and forms one hydrogen bond with the nitrogen of 20th thiamine (T 20) of DNA. The compound **5** did not show any hydrogen bond but literature reveals that existence of intercalating forces such as van der Waals forces or hydrophobic is much more important than hydrogen bonding of the compound to the base pairs of DNA [22]. The compound **6** shows normal electrostatic interaction with nucleotide base pairs of DNA through major groove by -NH of the benzothiazole moiety and the hydrogen bond formation occurs with nitrogen of 6th adenine of DNA. The resulting binding energies of docked steroid benzothiazole-DNA complexes (**4-6**) were found to be –341.83, –319.78, and –308.9 KJ mol^{-1}, respectively.

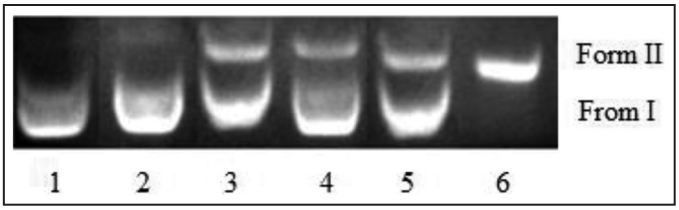


Figure 3: Agarose gel electrophoresis patterns of pBR322 plasmid DNA (300 ng) cleaved by compound **5** (1.0–5.0 µM), after 1 h incubation time (concentration dependent) lane 1: control; lane 2: 2.0 µM **5**+DNA; lane 3: 4.0 µM **5** +DNA; lane 4: 6.0 µM **5** +DNA. Lane 5: 8.0 µM **5** +DNA; lane 6: 10.0 µM **5** +DNA in buffer (5 mM Tris-HCl/50mM NaCl, pH 7.2 at 25°C).

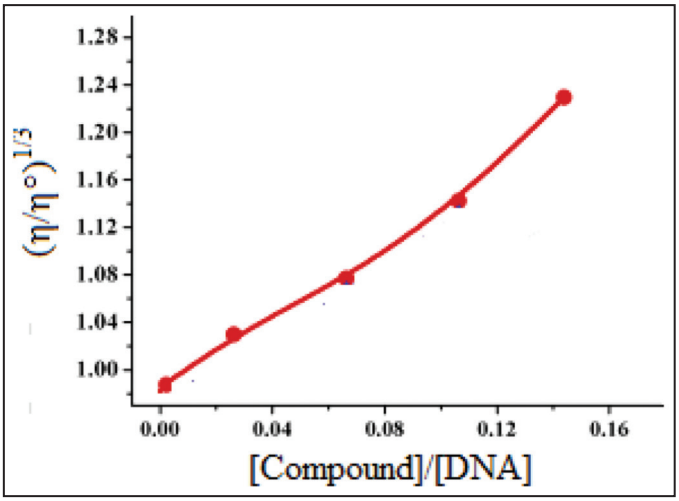


Figure 4: Relative viscosity changes of computed tomography DNA solution (200 mM) in the presence of different concentration of compound **5**.

3.3. In vitro anticancer activity

The *in vitro* cytotoxicity was measured using the MTT assay [23] during which the conversion of the soluble yellowish MTT to the insoluble purple formazan by active mitochondrial lactate dehydrogenase of living cells has been used to develop an assay system for measurement of cell proliferation. The data reported in Table 1 indicate that compounds **4-6** showed different levels of anticancer inhibition. The compound **4** showed minimum IC_{50}=16.21 (HL-60), 17.38 (SW480), 17.75 (MCF-7), and 19.61 (HeLa), while compound **5** showed minimum IC_{50}=14.35 (HL-60), 17.41 (A549), 21.42 (MCF-7), and 19.27 (HeLa). The inhibitions shown by compound **6** are ≥ 20 µmol l^{-1} (as shown in Table 1). Thus, compound **5** can be considered potential anticancer agent (IC_{50}=14.35 [HL-60]) among

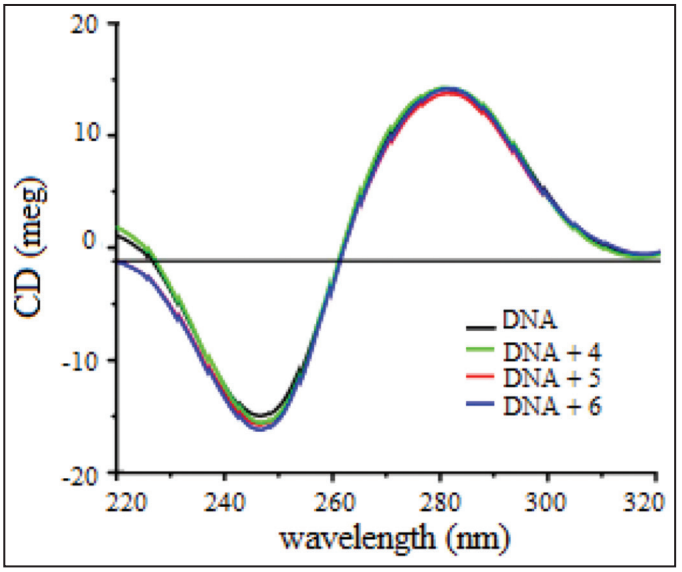


Figure 5: Circular dichroism spectra of CT DNA (black), in the presence of 4 (green), 5 (red), and 6 (blue).

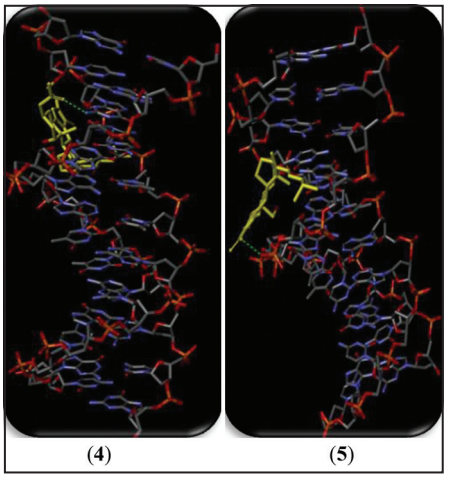


Figure 6: Molecular docked model of compound (4 and 5) with DNA dodecamer duplex of sequenced (CGCGAATTCGCG)2 (PDB ID: 1BNA) and the green dashed lines showing hydrogen bond interaction between them.

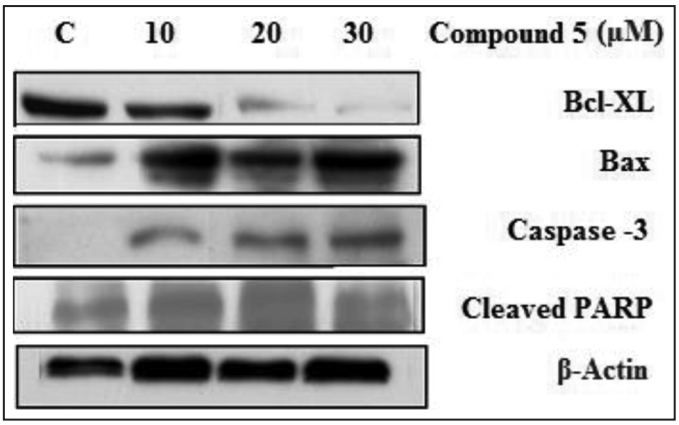


Figure 7: Western blot analysis of whole cell extracts. Lower panel (*β*-actin) represents equal loading of each lane.

compounds. The cytotoxicity screening data also suggest that the appending of benzothiazole scaffold to the steroid nucleus is one of the factors responsible for the cytotoxicity of the compounds (**4-6**).

From Table 1, it is clear that compounds **4** and **5** are showing better cytotoxicity against SW480, HL-60, and MCF-7 cell lines by showing IC_{50} close to that of doxorubicin, thus can be considered as potential cytotoxic agents. Compound **5** even showed IC_{50} same to that of doxorubicin against HL-60 cell line. Further, modifications and derivatization may lead to the development of more active cytotoxic agents.

To confirm the cytotoxicity of new compounds, the compounds **4-6** were tested with some non-cancer cell lines NL-20 (lung) and HPC (pulp) during which none of the synthesized compounds were found toxic, all the compounds showed IC_{50} >60. This also suggests that these steroidal benzothiazoles can be used specifically for the treatment of cancer cells without showing toxicity to the non-cancer cells.

3.4. Western Blot Analysis

To confirm the apoptotic effect of compound **5** in HL-60 cells, a study of protein levels of some apoptotic markers such as Caspase-3, Bax, Bcl-XL, and PARP cleavage was performed by Western blot analysis (Figure 7). The expression of Bcl-XL was reduced when cells were exposed to compound **5** of increasing concentrations (0, 10, 20, and 30 μM). In contrast to Bcl-XL, the expression of Bax and cleaved Caspase-3 was increased. The level of cleaved PARP product (104 KDa) was higher in the treated cells than untreated cells and increased with increasing concentrations of the compound **5**. As expected, the expression of *β*-actin, which served as a loading control, remained unaltered. These relative expressions of relevant apoptotic markers including the increasing Bax/Bcl-XL ratio, cleaved Caspase-3, and PARP cleavage clearly indicate that compound **5** causes apoptosis in HL-60 cancer cells.

4. CONCLUSION

We synthesized steroidal benzothiazoles from steroidal ketones and characterized. From spectroscopic titration, compounds bind to DNA (CT DNA) effectively through electrostatic and hydrophobic interactions. The absorption and fluorescence studies reveal the stabilization of the energy levels of the compounds in the presence of DNA. The DNA cleavage, viscosity studies, CD spectra, and molecular modeling studies revealed the potential binding interaction capacity of these compounds with DNA molecule. From *in vitro* cytotoxicity screening, it is clear that steroidal benzothiazoles were found to be potential cytotoxic agents against the panel of cancer cell lines particularly compound **5** against HL-60 cell line. These relative expressions of relevant apoptotic markers including the increasing Bax/Bcl-XL ratio, cleaved Caspase-3, and PARP cleavage clearly indicate that compound **5** causes apoptosis in HL-60 cancer cells. Hence, the present study has shown that these synthesized steroidal benzothiazoles can be used as a template for future development through modification and derivatization to design more potent and selective cytotoxic and DNA-binding agents.

5. ACKNOWLEDGMENTS

The author AMD is thankful to Prof. Shamsuzzaman for his able guidance and is also thankful to the Department of Chemistry, Aligarh Muslim University, for providing necessary research facilities.

6. REFERENCES

1. A. H. Bandey, I. M. Zargar, B. A. Ganaie, (2011) Synthesis and antimicrobial studies of chalconyl pregnenolones, ***Steroids***, **76:** 1358-1362.
2. R. K. Dubey, S. Oparil, B. Imthurn, E. K. Jackson, (2002) Sex hormones and hypertension, ***Cardiovascular Research***, **53:** 688-708.
3. K. A. Latham, A. Zamora, H. Drought, S. Subramanian, A. Matejuk, H. Offner, E. F. Roslonie, (2003), Estradiol treatment redirects the isotype of the autoantibody response and prevents the development of autoimmune arthritis, ***Journal of Immunology***, **171:** 5820-5827.
4. I. H. Lone, K. Z. Khan, B. I. Fozdar, F. Hussain, (2013) Synthesis antimicrobial and antioxidant studies of new oximes of steroidal chalcones, ***Steroids***, **78:** 945-950.
5. B. Ding, N. Yin, Y. Liu, J. C. Garcia, R. Evanson, T. Orsak, M. Fan, G. Turin, P. B. Savage, (2004) Origins of cell selectivity of cationic steroid antibiotics, ***Journal of the American Chemical Society***, **126:** 13642-13648.
6. N. Siddiqui, S. N. Pandeya, S. A. Khan, J. Stables, A. Rana, M. Alam, M. F. Arshad, M. A. Bhat, (2007) Synthesis and anticonvulsant activity of sulfonamide derivatives-hydrophobic domain, ***Bioorganic and Medicinal Chemistry Letters***, **17:** 255-259.
7. T. Wang, J. F. Kadow, N. A. Meanwell, (2006) ***Benzothiazole Antiviral Agents, Google Patents***.
8. L. Katz, (1953) Antituberculous compounds. III. Benzothiazole and benzoxazole derivatives, ***Journal of the American Chemical Society***, **75(3)**: 712-714.
9. A. Burger, S. N. Sawhney, (1968) Antimalarials. III. Benzothiazole amino alcohols, ***Journal of Medicinal Chemistry***, **11(2)**: 270-273.
10. R. D. Haugwitz, R. G. Angel, G. A. Jacobs, B. V. Maurer, V. L. Narayanan, L. R. Cruthers, J. Szanto, (1982) Antiparasitic agents. 5. Synthesis and anthelmintic activities of novel 2-heteroaromaticsubstituted isothiocyanatobenzoxazoles and benzothiazoles, ***Journal of Medicinal Chemistry***, **25(8)**: 969-974.
11. W. P. Hu, Y. K. Chen, C. C. Liao, H. S. Yu, Y. M. Tsai, S. M. Huang, F. Y. Tsai, H. C. Shen, L. S. Chang, J. J. Wang, (2010) Synthesis, and biological evaluation of 2-(4-aminophenyl) benzothiazole derivatives as photosensitizing agents, ***Bioorganic and Medicinal Chemistry***, **18(16)**: 6197-6207.
12. M. S. Yar, Z. H. Ansari, (2009) Synthesis and *in vivo* diuretic activity of biphenyl benzothiazole-2-carboxamide derivatives, ***Acta Poloniae Pharmaceutica***, **66(4)**: 387-392.

13. S. Shafi, M. M. Alam, N. Mulakayala, C. Mulakayala, G. Vanaja, A. M. Kalle, R. Pallu, M. S. Alam, (2012) Synthesis of novel 2-mercapto benzothiazole and 1, 2, 3-triazole based bis-heterocycles: Their anti-inflammatory and anti-nociceptive activities, ***European Journal of Medicinal Chemistry***, **49:** 324-333.
14. M. Chhabra, S. Sinha, S. Banerjee, P. Paira, (2016) An efficient green synthesis of 2-aryl benzothiazole analogues as potent antibacterial and anticancer agents, ***Bioorganic and Medicinal Chemistry Letters***, **26:** 213-217.
15. P. K. Sahu, P. K. Sahu, P. Samadhiya, P. L. Sahu, D. D. Agarwal, (2016) POM analyses and evaluation of *in vitro* antimicrobial, antitumor activity of 4H-pyrimido [2, 1-b] benzothiazole derivatives, ***Medicinal Chemistry Research***, **25(8)**: 1551-1563.
16. H. Moreno-Díaz, R. Villalobos-Molina, R. Ortiz-Andrade, D. Díaz-Coutiño, J. L. Medina-Franco, S. P. Webster, M. Binnie, S. Estrada-Soto, M. Ibarra-Barajas, I. León-Rivera, G. Navarrete-Vázquez, (2008) Antidiabetic activity of N-(6-substituted-1, 3-benzothiazol-2-yl) benzenesulfonamides, ***Bioorganic and Medicinal Chemistry Letters***, **18(9)**: 2871-2877.
17. P. C. Sharma, A. Sinhmar, A. Sharma, H. Rajak, D. P. Pathak, (2013) Medicinal significance of benzothiazole scaffold: An insight view, ***Journal of Enzyme Inhibition and Medicinal Chemistry***, **28(2)**: 240-266.
18. (a) A. M. Dar, B. Rah, S. Mir, R. Nabi, Shamsuzzaman, M. A. Gatoo, A. Mashrai, Y. Khan, (2018) DNA binding, artificial nuclease activity and cytotoxic studies of newly synthesized steroidal pyrimidines, ***International Journal of Biological Macromolecules***, **111:** 52-61. (b) S. Mir, A. M. Dar, B. A. Dar, (2019) Synthetic strategies of benzothiazines: A mini review, ***Mini-Reviews in Organic Chemistry***, **16:** 1-10.
19. E. C. Anagnostopoulos, F. L. Fieser, (1954) Nitration of unsaturated steroids, ***Journal of the American Chemical Society***, **76:** 532-536.
20. M. E. Reicmann, S. A. Rice, C. A. Thomas, P. Doty, (1954) A further examination of the molecular weight and size of deoxy pentose nucleic acid, ***Journal of the American Chemical Society***, **76:** 3047-3053.
21. Shamsuzzaman, A. M. Dar, Y. Khan, (2013) Synthesis and biological studies of steroidal pyran based derivatives, ***Journal of Photochemistry and Photobiology B: Biology***, **129:** 36-47.
22. D. Mustard, D. W. Ritchie, (2005) Docking essential dynamics Eigen structures, ***Proteins: Structure, Function, and Bioinformatics***, **60:** 269-274.
23. T. Mosmann, (1983) Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays, ***Journal of Immunological Methods***, **65:** 55-63.
24. G. Scatchard, (1949) The attractions of proteins for small molecules and ions, ***Annals of the New York Academy of Sciences***, 51: 660-672.
25. P. U. Maheswari, M. Palaniandavar, (2004) DNA binding and cleavage properties of certain tetrammine ruthenium (II) complexes of modified 1, 10-phenonthrolines-effect of hydrogen bonding on DNA-binding affinity, ***Journal of Inorganic Biochemistry***, **98:** 219-230.
26. P. Lincoln, E. Tuite, B. Nordén, (1997) Short circuiting the molecular wire-coperative binding of Δ-[Ru(Phen)2d ppz]$^{2+}$ and Δ-[Rh(phi)2Bipy]$^{3+}$ to DNA, ***Journal of the American Chemical Society***, **119:** 1454-1455.
27. D.M. Nejat, A. K. Bordbar, P. Lincoln, V. Mirkhani, (2012) Spectroscopic study on the interaction of ct-DNA with manganese salen complex containing triphenyl phosphonium groups, ***Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy***, **90:** 50-54.

***Bibliographical Sketch**

Dr. Ayaz Mahmood Dar is presently working as an Assistant Professor in Chemistry in Department of Higher Education, J&K. He studied B. Sc Hons., M. Sc and Ph. D courses from Aligarh Muslim University, Aligarh. He also qualified NET-JRF conducted by CSIR, India. During research, he has published the research work in almost 50 Journals of international repute. He has also written 05 books for undergraduate courses and has also presented papers in almost 20 international conferences.