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ORIGINAL ARTICLE

DNA Interaction Studies and In Vitro Cytotoxicity of Newly Synthesized Steroidal Imidazolidinones

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Abstract New steroidal imidazolidinone derivatives (**7**–**9**) were synthesized after reacting steroidal thiosemicarbazones with oxalyl chloride in absolute ethanol. After characterization by spectral and analytical data, the interaction studies of compounds (**7**–**9**) with DNA were carried out by UV–vis, fluorescence spectroscopy, circular dichroism, molecular docking and gel electrophoresis. The compounds bind to DNA preferentially through electrostatic and hydrophobic interactions with K_b; 2.31×10^4 M^{-1}, 2.57×10^4 M^{-1} and 2.16×10^4 M^{-1}, respectively indicating the higher binding affinity of compound **8** towards DNA. Gel electrophoresis demonstrated that the compounds **7**–**9** show strong interaction during the cleavage activity with pBR322 DNA. The docking study suggested the intercalation of imidazolidinone moiety of steroid derivative in minor groove of DNA. During in vitro cytotoxicity, compounds **7**–**9** revealed potential toxicity against the different human cancer cells (MTT assay). Apoptotic degradation of DNA in presence of compounds **7**–**9** was analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining (comet assay). FACS analysis shows that the compound **8** bring about cell cycle arrest at 7 μM concentration.

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Keywords Imidazolidinone · UV–vis and fluorescence · CD · MTT assay · Comet assay

Introduction

Medicinal chemistry has gained enormous popularity for its role in drug discovery over the past two decades. The number of natural products and their semi synthetic analogs have been discovered and evaluated for their role in the treatment of various fatal diseases such as cancer, diabetes, microbial infections and cardiovascular diseases [1]. However because of the drug resistance and drug tolerance problems, there is always scope for the design and development of new and modified analogs as more efficient drug candidates. This has been done through the rational design and synthesis of receptor based lead molecules which still remains an open area. Natural products have extensively been used as starting tools for the design and synthesis of lead therapeutic scaffolds.

Substituted imidazolidine derivatives are considered central substructures of many compounds exhibiting biological and pharmacological properties [2, 3]. They exhibit different biological activities such as nitric-oxide synthase inhibition, anti-inflammatory, anti-parasitic, antifungal, antidepressant, antitubercular, anticancer and antiviral activities [4–11]. Hence, imidazole moiety is found in numerous drugs, such as anti-inflammatory agents, kinase inhibitors, antagonists of CB1 cannabinoid and glucagon receptors and antibacterial agents [12].

In the field of molecular biology and drug development, the cleaving agents of nucleic acid have attracted extensive attention due to their potential applications [13]. The phosphodiester bonds of DNA are extremely stable and the half life of DNA for hydrolysis is estimated to be around 200 million years under uncatalyzed physiological conditions [14]. Metal

complexes have been widely investigated as cleaving agents of nucleic acids and are found to be reasonably efficient [15], but their use in pharmacy is restricted because of serious issues over the lability and toxicity produced due to free radical generation of some transition metals during the redox processes [16]. To overcome these limitations of lability and toxicity, Gobel and co-workers [17] put forward the concept of 'metal-free cleaving agents' which are being applied to active phosphodiesters like 'nucleic acid mimic' and RNA.

Literature reveals that several number of imidazolidinone derivatives have shown potential binding to DNA. In most of the intercalation complexes, the substituted imidazolidinone derivatives are located in the major or minor grooves with the imidazolidinone chromophore sandwiched between base pairs. These compounds allow base-specific groove binding by the NH or C=O groups [18]. The ability of these intercalators to direct functionality into either of the grooves of nucleic acids makes them good candidates for selective ligands which are able to provide molecular recognition [18]. The planar imidazolidinone scaffold is being an important pharmacophore and potent fluorescent ligand intercalating between DNA base pairs and is often used in syntheses of antitumor DNA-targeting drugs [19]. Metal free imidazolidinone compounds are also able to inhibit topoisomerase I and II enzymes, render DNA damage, disrupt DNA repair and replication, and induce cell death without showing toxicity towards the normal cells [20, 21]. It has also been found that the damage of DNA by these compounds is usually strengthened by the oxidative stress [21]. The cytotoxicity of most imidazolidinone-based drugs is based on their ability to suppress topoisomerase activity [22]. There are two possibilities for an intercalator to influence the topoisomerase activity and thereby suppress the proliferation of the cell: (a) by intercalation; the binding site of the topoisomerase is occupied and formation of the complex between the enzyme and the DNA is hindered; (b) a ternary complex between DNA, intercalator and topoisomerase may be formed which is significantly more stable than the DNA-topoisomerase complex. The stability of the ternary complex may lead to an enhanced lifetime of the cleaved DNA, i.e., the re-ligation of the strands cannot take place and the strand breaks remain permanent. Thus, the topoisomerase acts as an endogeneous poison and may induce apoptosis [23]. In continuation of our previous work [24] herein, we report the synthesis of new steroidal imidazolidinones as metal free DNA binding agents. The presence of -NH or -CO groups in the molecules can cooperatively participate in the interaction with DNA via hydrogen bonding. A computer aided molecular docking study was carried out to validate the specific binding mode of the newly synthesized compounds. Furthermore, these compounds have also been screened for in vitro cytotoxicity as well as genotoxicity.

Experimental

Material and Chemicals

Chemicals were purchased from Merck and Sigma-Aldrich as 'synthesis grade'. Melting points are recorded in degrees Celsius on a Kofler apparatus. The IR spectra were recorded on KBr pellets with Pye Unicam SP3-100 Spectrophotometer and values are given in cm^{-1}. ^{1}H 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 (TLC) plates were coated with silica gel G and exposed to iodine vapours to check the homogeneity as well as the progress of reaction. Sodium sulphate (anhydrous) was used as a drying agent. Super coiled pBR322 DNA was purchased from GeNei (India) while as double-stranded calf thymus DNA, purchased from Sigma, was dissolved in a 0.1 M Tris-buffer. The purity of DNA was verified by monitoring the ratio of absorbance at 260 nm to that at 280 nm, which was in the range 1.8–1.9. The concentration of the DNA was determined spectrophotometrically using ϵ_{260}=6600 M^{-1} cm^{-1} [25]. The human cancer cell lines used for the test were A545, MCF-7, HeLa, HL-60, SW480, HepG2, HT-29 and A549 and were obtained from National Cancer Institute (NCI), biological testing branch, Federick Research and Development Centre, USA.

General Method for the Synthesis of Steroidal Thiosemicarbazones (4–6)

The steroidal thiosemicarbazones (**4**–**6**) were synthesized by a literature method [26] which involves the refluxing of an equimolar solution of steroidal ketones (**1**–**3**) and thiosemicarbazide in ethanol in the presence of few drops of HCl for 5 h. After cooling, the compounds were filtered and recrystallized from methanol.

General method for the synthesis of [2′-thioxoimidazolidin-4′, 5′-dione-1-yl]-6-imino-5α-cholestane derivatives (7–9)

To a solution of steroidal thiosemicarbazone (**4**–**6**) (1.5 mmol) in absolute ethanol (25 mL), an equimolar amount of oxalyl chloride was added. The reaction mixture was refluxed for 2 h. The progress and completion of the reaction was monitored by thin layer chromatography. After completion of reaction the excess solvent was removed to three fourths of the original volume under reduced pressure. The reaction mixture was then taken in diethyl ether, washed with water and dried over anhydrous sodium sulphate. Evaporation of solvents and recrystallization from methanol afforded respective product (**7**–**9**).

3β-Acetoxy [2′-thioxoimidazolidin-4′, 5′-dione-1-yl]-6-imino-5α-cholestane (7)

White powder; yield: 78 %. m.p. 136–138 °C; Anal. Calcd for $C_{32}H_{49}N_3O_4S$: C, 67.21, H, 8.64, N, 7.35, S, 5.61 found: C, 67.04, H, 8.42, N, 7.19, S, 5.50; IR (KBr) ν cm^{-1}: 3325 (NH), 1714 (O*CO*CH_3), 1692 (C=O), 1678 (*CO*NH), 1646 (C=N), 1268 (C=S), 1080 (C-O), 1021 (C-N); ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 8.4 (s, 1H, N*H*, exchangeable with D_2O), 4.7 (m, 1H, $C_3\alpha$-*H*, *W½*=15 Hz), 2.03 (s, 3H, OCOC*H*$_3$), 1.18 (s, 3H, C_{10}-C*H*$_3$), 0.70 (s, 3H, C_{13}-C*H*$_3$), 0.97 and 0.83 (other methyl protons). ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 186, 173.2, 171.8, 167.2, 155.3, 70, 46, 44, 42, 39, 35, 26, 24, 22, 20, 19, 18; ESI MS: m/z 571 [$M^{+\cdot}$].

3β-Chloro [2′-thioxoimidazolidin-4′, 5′-dione-1-yl]-6-imino-5α-cholestane (8)

White powder; yield: 77 %. m.p. 146–148 °C; Anal. Calcd for $C_{30}H_{46}ClN_3O_2S$: C, 65.73, H, 8.46, N, 7.66, S, 5.85 found: C, 65.61, H, 8.35, N, 7.52, S, 5.67; IR (KBr) ν cm^{-1}: 3317 (NH), 1696 (C=O), 1673 (*CO*NH), 1654 (C=N), 1278 (C=S), 1031 (C-N), 741 (C-Cl); ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 8.1 (s, 1H, N*H*, exchangeable with D_2O), 3.9 (m, 1H, $C_3\alpha$-*H*, *W½*=17 Hz), 1.17 (s, 3H, C_{10}-C*H*$_3$), 0.71 (s, 3H, C_{13}-C*H*$_3$), 0.98 and 0.80 (other methyl protons). ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 184, 171.5, 165.2, 156.3, 50, 45, 43, 42.2, 39, 35, 26, 24, 22, 20, 19, 18; ESI MS: m/z 547/549 [$M^{+\cdot}$].

[2′-Thioxoimidazolidin-4′, 5′-dione-1-yl]-6-imino-5α-cholestane (9)

Yellow powder; yield: 75 %. m.p. 146–148 °C; Anal. Calcd for $C_{30}H_{47}N_3O_2S$: C, 70.13, H, 9.22, N, 8.18, S, 6.24 found: C, 69.93, H, 9.07, N, 8.11, S, 6.07; IR (KBr) ν cm^{-1}: 3327 (NH), 1694 (C=O), 1670 (*CO*NH), 1651 (C=N), 1276 (C=S), 1033 (C-N); ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 7.9 (s, 1H, N*H*, exchangeable with D_2O), 1.17 (s, 3H, C_{10}-C*H*$_3$), 0.71 (s, 3H, C_{13}-C*H*$_3$), 0.98 and 0.80 (other methyl protons). ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 183, 171.2, 164.7, 157.3, 45, 43, 42.2, 39, 35, 26, 25, 24, 22, 20, 19, 18; ESI MS: m/z 513 [$M^{+\cdot}$].

DNA Binding Experiments

Electronic Absorption and Fluorescence Spectroscopy

The absorption and emission spectroscopy was done as per the standard methods and practices reported in the literature [27]. The UV-visible spectra for DNA-steroid interactions were obtained using an Agilent 8453 spectrophotometer while as fluorescence measurements were carried out with a JASCO spectrofluorimeter (FP 6200). Solutions of DNA and steroid were scanned in a 1 cm quartz cuvette. To eliminate the absorbance of the DNA while measuring the absorption spectra, an equal amount of DNA was added to both the compound solution and the reference solution.

Circular Dichroism (CD) Measurements

CD measurements [28] were recorded on a JASCO (J-810) spectropolarimeter by keeping the concentration of DNA constant (5×10^{-5} M) while varying the steroid concentration (ri=[Compound] / [DNA] = ri=0.6, 0.12, 0.20). The optical chamber of the 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.

Gel Electrophoresis

Two concentrations (100 and 200 μM) of compounds and 3 μL of 0.5 μg/μL of pUC19 DNA was loaded with 2 μL of EtBr into 1 % agarose gel. The final volume of the reaction mixture for all the wells of gel was 10 μL. The DNA band was visualised under UV transilluminator.

Molecular Docking

The rigid molecular docking studies were performed using HEX 6.1 software [29]. The compound **8** was taken for 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, 3.1 GHz based machine running MS Windows XP as the operating system. First of all the water molecules were deleted. The DNA was enclosed in a box with number of grid points in x × y × z directions, 76×78×120 and a grid spacing of 0.375 Å. All calculations were carried out on an Intel CORE i5, 3.1 GHz based machine running MS Windows XP as the operating system. Visualization of the docked pose have been done using PyMol molecular graphics program [30].

In Vitro Anticancer Activity

Cell culture and conditions Human cancer cell lines SW480 (colon adenocarcinoma cells) / ATCC (CCL-228), HeLa (cervical cancer cells) / ATCC (CCL-2), MCF-7 (breast cancer cells) / ATCC (HTB-22), HepG2 (hepatic carcinoma cells) / ATCC (CRL-8065) and HL-60 (Leukaemia cells) / ATCC (CCL-240) were taken for the study. SW480, HL-60 and HepG2 cells were grown in RPMI 1640 supplemented with 10 % foetal bovine serum (FBS), 10 U penicillin and 100 μg/mL streptomycin at 37 °C with 5 % CO_2 in a humidified atmosphere. HeLa and MCF7 cells were grown in Dulbecco's

modified Eagle's medium (DMEM) supplanted with FCS and antibiotics as described above for RPMI 1640.

Cell viability assay (MTT) The anticancer activity in vitro was measured using the MTT assay [31]. 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 and 5-Fluorouracil at various concentrations for 48 h. The concentration of DMSO was always kept below 1.25 %, which was found to be non-toxic to the cells. Twenty μl MTT solution was 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. The absorbance of the wells was read with a microplate reader (Bio-Rad Instruments) at 570 nm.

Data analysis Cell survival was calculated using the formula: Survival (%) = [(absorbance of treated cells -absorbance of culture medium) / (absorbance of untreated cells - absorbance of culture medium)]×100 [32]. The experiment was done in triplicate and the inhibitory concentration (IC) values were calculated from a dose response curve. IC_{50} values 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 %.

Comet Assay

To assess the genotoxic effect of the steroidal imidazolidinones, comet assay [33] was performed in A545 cells. A545 (1×10^6) cells were treated with definite concentrations of steroidal imidazolidinones and 5-Fluorouracil (5-Fu) for 24 h. The cells were then washed and 200 μL of cell suspension in low melting Agarose (LMA) was layered on to the labelled slides precoated with Agarose (1.5 %). The slides were placed on ice for 10 min and submerged in lysis buffer (2.5 % NaCl, 100 mM EDTA, 10 mM Tris, 10 % DMSO and 1 % Troton X-100) at pH 10 at 4 °C for more than 1 h. The slides were then equilibrated in alkaline buffer (30 mM NaOH, 1 mM EDTA) at pH 13 at 4 °C, electrophoresed at 0.86 V/cm at 4 °C, neutralized, washed and dried. At the time of image capturing, the slides were stained with ethidium bromide (EtBr, 150 μL 1×) and cover slips were placed over them. For visualization of DNA-damage, EtBr stained slides were observed under 209 objectives of a fluorescent microscope (Olympus BX-51, Japan). The images of 50–100 randomly selected cells were captured per slide using a CCD camera.

Analysis of DNA Content by FACS/ Cell Cycle Arrest

When cell density in a culture flask reached 75–85 % confluence, they were trypsinized and seeded in 6-well plates at a density of $5–8\times10^5$ cells/well and grown for 24 h. Four and 7 μM of compound **8**, were added to the cells and grown for 24 h. After the respective experimental period, the cells were trypsinized and collected in a falcon tube. After washing the cells with PBS, they were fixed by gently adding ice-cold 70 % ethanol with simultaneous vortexing and left overnight at 4 °C. On the day of analysis, samples were centrifuged for 10 min at 1500 RPM. The supernatant was discarded, and the pellets were resuspended in PBS. This step was repeated again to remove ethanol. The cells were then resuspended in PBS containing 0.5 % Triton X-100, 0.1 mg/ml RNase and 40 mg/mL propidium iodide in a dark room. Triton-X and RNAase were added to permeabilize the cell membrane and eliminate RNA. After 30 min incubation at 37 °C, the cells were analysed on a flow cytometer, equipped with an air-cooled argon laser providing 15 mW at 488 nm with standard filter setup. Ten thousand events were collected and the percentages of each cell cycle phases were analysed using Cell quest Pro software (Becton Dickinson, USA).

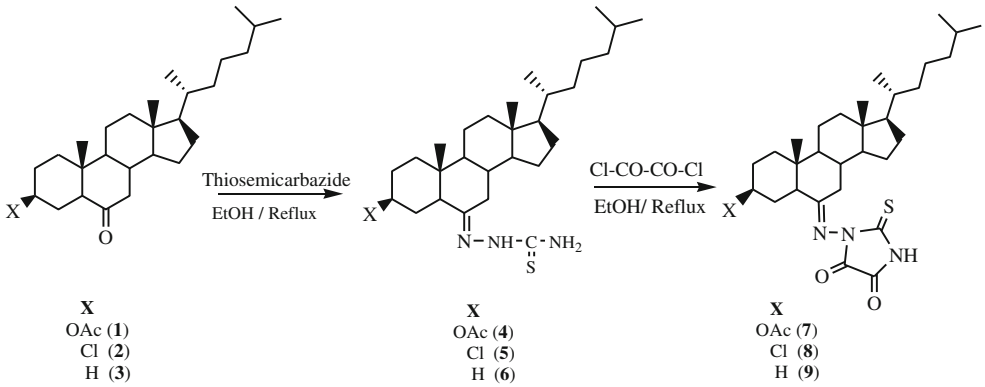
Results and Discussion

Chemistry

The intensive research on heterosteroids has focused in recent years on the development of new bioactive heterocyclic molecules [34], with the aim of obtaining new derivatives that may be of value in designing potentially anticancer active and DNA binding agents. Hence we have envisioned the convenient and an efficient synthesis of steroidal imidazolidinones (**7**–**9**) from corresponding steroidal thiosemicarbazones (**4**–**6**) and oxalyl chloride (Scheme 1). The reaction does not demand rigorously dried solvents, reagents or inert atmosphere but simply refluxing steroidal thiosemicarbazones and oxalyl chloride in absolute ethanol. The mechanism for the formation of compounds (**7**–**9**) involve the nucleophilic attack of the two nitrogen atoms of thiosemicarbazone on the two carbonyl groups, making both the chlorine atoms to leave which leads the formation of corresponding products.

The characterization studies for structural elucidation of steroidal imidazolidinones **7**–**9** included IR, ^{1}H NMR, ^{13}C NMR and MS. In the IR spectra, the strong absorption bands in the ranges 3317–3327, 1692–1694 and 1670–1678 cm^{-1} were attributed to the NH, CO, *CO*NH groups, respectively while absorption bands at 1646–1654 and 1268–1276 cm^{-1} confirmed the presence of the C=N and C=S group in compounds **7**–**9**. In ^{1}H NMR study, a singlet in the range δ 8.4−7.9

Scheme 1 Showing the pathway for the formation of steroidal imidazolidinones (**7**–**9**)



confirmed the presence of N*H* group in compounds **7**–**9**. In ^{13}C NMR study, the signals at δ 183–186, 164–167, 171–173, 155–157 confirmed the presence of the *C*=S, *C*ONH, *C*=O, *C*=N groups, respectively, in compounds **7**–**9**. Finally, the presence of distinct molecular ion peaks [$M^{+\cdot}$] at m/z 571, 547/549, 513, respectively in the MS spectra also proved the formation of compounds **7**–**9**. This strategy can also be applied to diverse thiosemicarbazones; in that way imidazolidinones may also allow further modifications on the substituted heterocyclic systems.

DNA Binding Studies

Electronic Absorption Titration

The binding activities of DNA-heterocycles have been a clue of paramount importance for understanding the mechanism of effective chemotherapeutic drugs. Absorption titration is usually used to determine the binding strength and the mode of DNA binding with small molecules [35]. Here in the UV–vis spectra of compounds (**7**–**9**) with increasing concentrations of CT DNA is shown in Fig. 1 which exhibited potential absorption bands at 290 nm, attributed to the π-π* or intraligand transitions. Upon the addition of an increasing concentration of DNA (0.70–4.24×10^{-5} M) to the compounds (**7**–**9**) in a 2 % DMSO/ 5 mM Tris HCl/50 mM NaCl buffer solution, there was an increase in the absorption intensity of the intraligand absorption band (hyperchromism), without any shift of the position of the band. The hyperchromic effect is due to the electrostatic binding of the compound with the DNA base pairs by involving the hydrogen-bonding interaction between coordinated –C=S with functional groups positioned on the edge of DNA bases, thereby causing the maximum exposure of base pairs to the light that leads to the increase in the absorption intensity hence the hyperchromism occurs [36]. These spectral studies reveal that compounds (**7**–**9**) exhibited higher binding propensity with DNA and interact presumably by electrostatic interaction via the phosphate backbone of the DNA double helix together with the hydrophobic interaction. The hydrophobic interaction with DNA replaces the water molecules in the DNA grooves, leading to an enhancement of the entropy and to the stabilization of the DNA-bound compound [37]. In order to further compare the binding strength of the compounds, their intrinsic binding constant (K_b) were determined from the following Eq. (1) [38].

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

Where, [DNA] is the concentration of DNA, ε_a, ε_f and ε_b are apparent extinction coefficients A_{obs}/[M], the extinction coefficient for free compound and the extinction coefficient for compound in the fully bound form, respectively. In the plots of [DNA] / ε_a–ε_f versus [DNA], K_b is given by the ratio of the slope to the intercept.

The intrinsic binding constants for compounds (**7**–**9**) were found to be 2.31×10^4 M^{-1}, 2.57×10^4 M^{-1} and 2.16×10^4 M^{-1}, respectively hence the binding affinity follows the order **8**> **7**>**9**.

To confirm the better absorption behaviour of compounds (**7**–**9**), the absorption intensity of the compounds was compared under the same concentration of DNA and it was found that the absorbance of the compounds (**7**–**9**) was very slightly different from each other and more clearly, the absorbance shown by the compounds (**7**–**9**) followed the order **8**>**7**>**9**.

Fluorescence Spectroscopy

The variety of molecular interactions such as excited state reactions, molecular rearrangements, energy transfer and collision are studied by fluorescent quenching techniques [39]. Here the fluorescence quenching experiments were undertaken to study the interaction of synthesized compounds (**7**–**9**) with CT DNA. The emission spectra of compounds (**7**–**9**) displayed intense luminescence at 358 nm at room temperature in the absence of DNA when excited at 290 nm. On addition of increasing concentration of DNA (0.70×10^{-5} to 4.24×10^{-5} M) to the fixed amount of compounds (1×10^{-4} M), the emission intensity appreciably increases as shown in Fig. 2. The increase in the emission intensity is

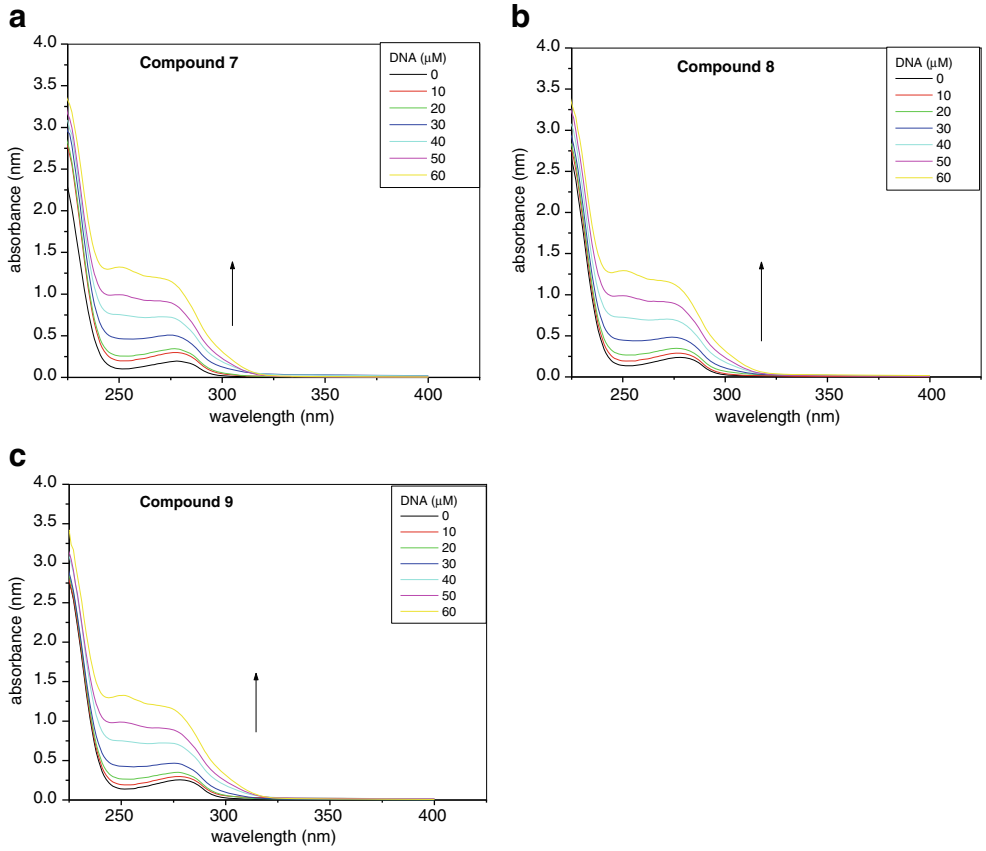


Fig. 1 Variation of UV–vis absorption for steroidal imidiazolidinones (**7**–**9**) with increase in the concentration of Calf thymus DNA

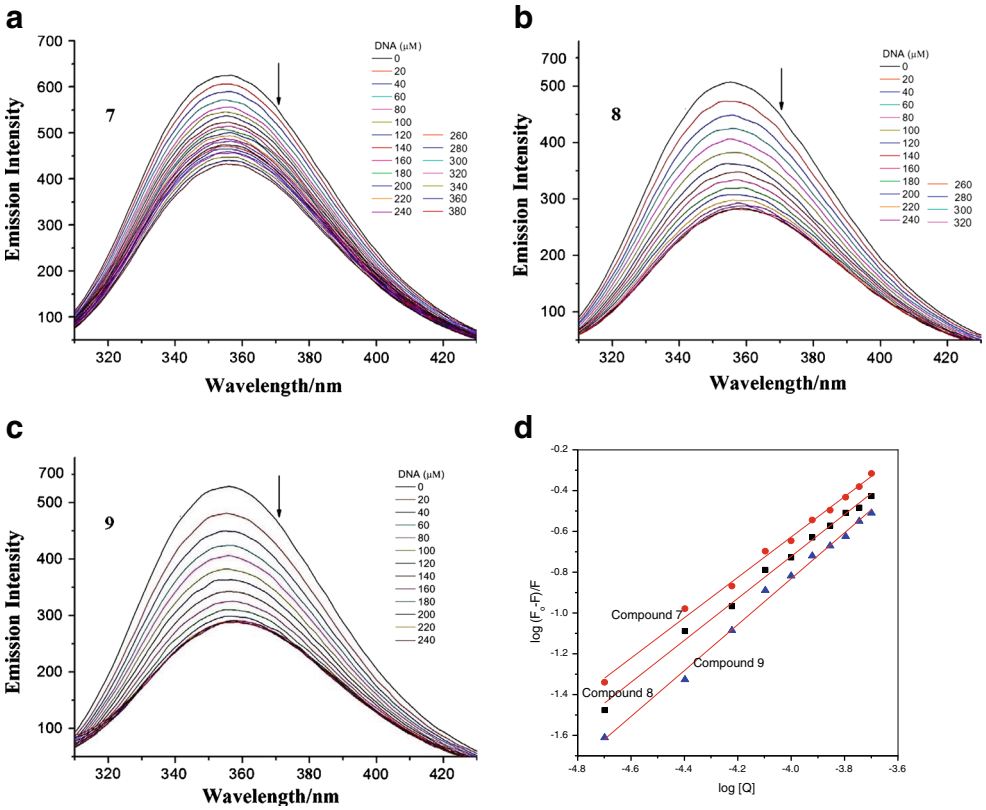


Fig. 2 Fluorescence titration of compounds (**7**–**9**) with the Calf thymus DNA. Fluorescence intensity decreases with subsequent addition of DNA solution. **a**, **b**, and **c**, represent fluorescence quenching of compounds **7**–**9** due to the addition of DNA, respectively. **d** represent variation of $(F_o - F)/F$ with log concentration of DNA

largely due to the extent to which the molecule is inserted into the hydrophobic environment of DNA minor groove. Also the hydrophobicity of DNA helix overcomes the solvent accessibility to the hydrophobic environment inside it and the compound mobility is restricted at the binding site, ultimately leading to a decrease in the vibrational mode of relaxation [40]. Furthermore, the binding of compound to the DNA helix could decrease the collisional frequency of solvent molecules with the compound, leading to the emission enhancement of the compound. To compare the binding affinity of compounds to DNA quantitatively, the binding constant 'K' and binding site number 'n' were calculated by using Scatchard Eqs. (2) and (3) [41].

$$C_F = C_T(F/F_o - P)(1-P) \quad (2)$$

$$r/c = K(n-r) \quad (3)$$

Where, C_F is the concentration of free compound, C_T is the total concentration of compound; F and F_o are fluorescence intensities in the presence and absence of DNA, respectively. P is the ratio of observed fluorescence quantum yield of the bound compound to that of the free compound. The value P was obtained as the intercept by extrapolating from a plot of F/F_o versus 1/[DNA], r denotes the ratio of $C_B = (C_T - C_F)$ to the DNA concentration, 'c' is the free compound concentration and 'n' is the binding site number.

The binding constants for compounds **7**–**9** were calculated to be 3.1×10^4 M^{-1}, 3.6×10^4 M^{-1} and 2.8×10^4 M^{-1}, respectively. The number of steroidal imidazolidinones per DNA 'n' calculated for (**7**–**9**) was found to be 1.12, 1.25 and 1.03, respectively indicating that compound **8** has higher DNA binding propensity in agreement with the absorption titration experiment. Also in order to make sure the fluorescence intensity of compounds (**7**–**9**), the fluorescence intensity of the compounds was compared under the same concentration of DNA and it was found that the fluorescence of the compounds (**7**–**9**) was closely overlapping with each other and more specifically the fluorescence shown by the compounds (**7**–**9**) followed the order **8**>**7**>**9**.

When small molecules bind independently to a set of equivalent sites on a biomacromolecule by static quenching, the equilibrium between free and bound molecules is given by the equation:

$$\log\frac{F_o - F}{F} = \log K + n\log[Q] \quad (4)$$

where K and n are the binding constant and the number of binding sites, respectively. From a plot of $(F_o - F)/F$ vs. log [Q] Fig. 2d, the binding constant K and the binding sites n can be obtained from the intercept and the slope. As the values of n were approximately equal to around 1, which indicated the existence of just one main binding site on DNA for compounds (**7**–**9**). Recently Ruiz et al. [42] have reported the incorporation of steroidal moiety in rhodium and iridium complexes, enhances their antitumor activities. These metal-steroidal complexes also have binding constants in a range of 10^3 M^{-1}. Haramane (HM), photosensitizer interact with DNA through intercalative mechanism and the binding constant (9×10^3 M^{-1}) is found to be on the same order of magnitude as that are in our case [43].

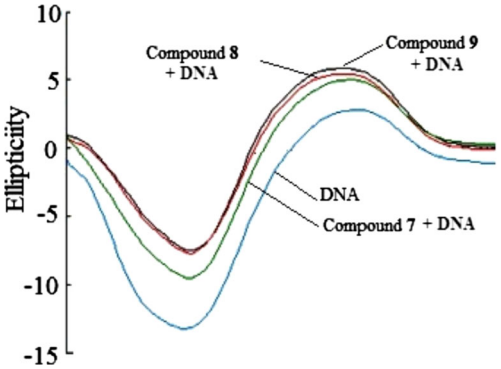


Fig. 3 CD spectra of CT DNA (*blue*), in the presence of compound **7** (*green*), compound **8** (*red*) and compound **9** (*black*)

CD Spectra

Since any particular variation of DNA conformation sensitizes the CD signal hence the CD spectroscopy is a quite sensitive technique to investigate the changes in DNA morphology and to determine mode of drug-DNA interactions [44]. In CD spectrum of B-DNA, the positive band is due to the base stacking (277 nm), while the negative one (245 nm) corresponds to the right-handed helicity [45]. In this study, the CD spectra of B-DNA were recorded in the presence of different molar ratios of the steroid compounds (**7**–**9**). The changes in the CD signals of B-DNA, as observed on the interaction with the compounds can be assigned to the corresponding changes in DNA morphology [46]. Classical intercalative molecules tend to enhance the intensities of bands due to 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.

Also 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

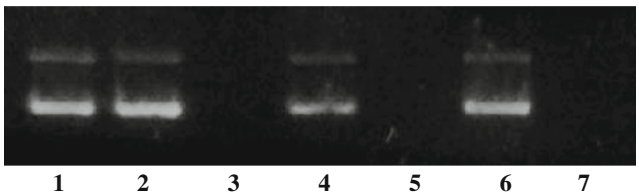


Fig. 4 Agrose gel electrophoresis of reaction mixtures containing DNA and compound **7**–**9**. The concentration of plasmid DNA was 75 mg/L. *Lane 1*, DNA only; *lane 2, 3* DNA and **7** (100, 200 μ M); *lane 4, 5* DNA and **8** (100, 200 μ M); *lane 6, 7* DNA and **9** (100, 200 μ M)

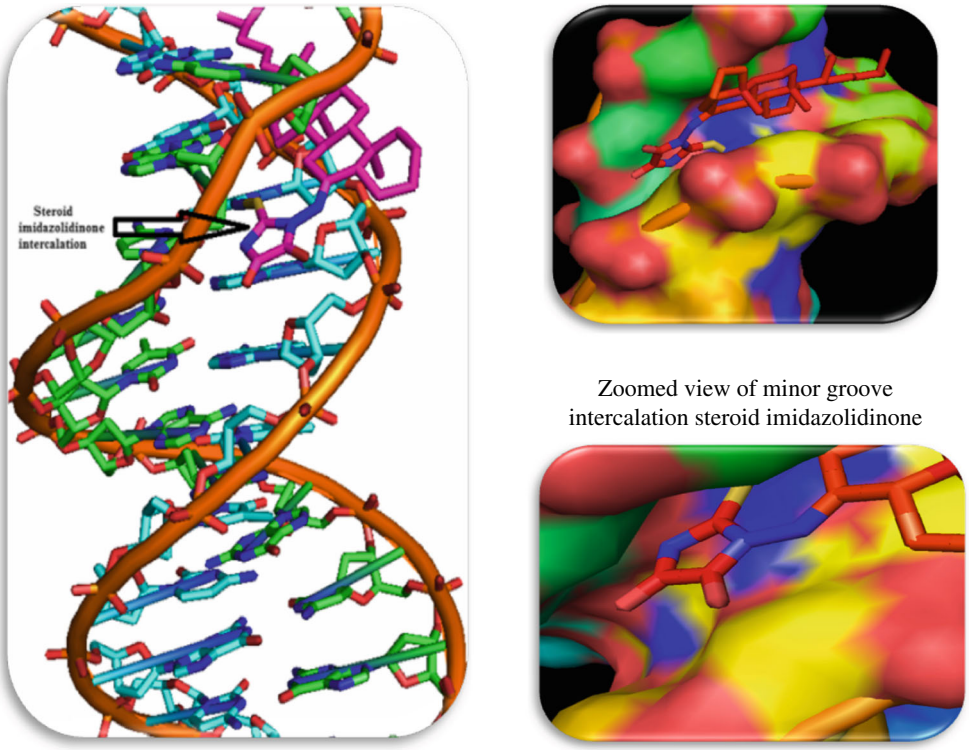


Fig. 5 Docked model of DNA-steroidal imidazolidinone. The N, S and the O termini of the imidazolidinone moiety of compound are shown as blue, yellow and red sticks, respectively. **a**, **b** and **c** show minimum energy poses of DNA-Steroidal imidazolidinone complex

[44]. The CD spectra of DNA alone and in the presence of the compounds (**7**–**9**) are illustrated in Fig. 3. The CD spectra of DNA with different values of the compound exhibit an increase in the positive peak and decrease in the negative band. These CD changes represent more stacking of DNA base pairs due to the hydrophobic interaction in groove region and decrease in the helicity of DNA by unwinding it. These compounds are assisting the electrostatic attraction with the DNA strand which can be followed by partial intercalation of their planar moieties between the base pairs in minor groove, allowing the interaction; therefore at the site of intercalation, DNA double helix can be unwound [47].

Gel Electrophoresis Assay

All the compounds (**7**–**9**) were examined for their binding abilities with DNA by agarose gel electrophoresis. In the Fig. 4, lane 1 contains DNA only, lane 2, 4, 6 contains DNA and 100 μM of compounds **7**–**9** and lane 3, 5, 7 contain DNA and 200 μM of compounds **7**–**9**. At low concentration, the compounds (**7**–**9**) do not show any appreciable change in the band intensity of DNA (lane 2, 4, 6). However, at higher concentrations band intensity gets diminished in compounds **7**–**9** (lane 3, 5, 7). Thus electrophoretic pattern demonstrate that all the compounds interact with DNA. The loss in the band intensity at higher concentration may be assumed due to the intercalation of the compounds **7**–**9** to DNA, which in turn results in the displacement of EtBr. In case of lane 2, 4, 6 the concentration of the compounds is not sufficient for the displacement of EtBr. This conclusion was further supported from the fluorescence studies, which also gave evidence for interaction of the compounds with DNA.

Table 1 The IC_{50} values shown by compounds **4**–**14** and 5-Fu against given cancer cell lines

Comp.	IC_{50} (μM)							
	Lung A545	Breast MCF-7	Cervical HeLa	Leukaemia HL-60	Colon SW480	Hepatic HepG2	Colon HT-29	Lung A549
7	9.55	11.46	14.21	9.51	12.18	11.93	11.85	9.37
8	7.53	7.41	19.36	10.17	9.23	12.62	10.63	12.43
9	11.87	12.31	17.12	12.29	10.36	12.33	14.12	11.69
5-FU	11.46	10.34	10.32	9.45	9.02	11.31	9.79	12.05

5-FU 5-Fluorouracil

Molecular Docking

To understand steroidal–DNA interaction, the molecular docking technique is an attractive tool to get insight of the mechanistic study, by placing a molecule into the binding site of the target specific region of the DNA. In our experiment, rigid molecular docking (two interacting molecules were treated as rigid bodies) studies were performed with HEX 6.1 software [29] to predict the binding modes of compounds with a DNA duplex of sequence $d(CGCAAATTTCGC)_2$ dodecamer (PDB ID: 1BNA), and provide an energetically favourable docked structures (DNA–compound **8** shown in Fig. 8). It is evident from the figure that these type of compounds gets attached with DNA through minor groove and their imidazolidinone moiety shows intercalation between the nucleotide base pairs.

The docked steroidal imidazolidinone-DNA complex is shown in Fig. 5 depicts that the sulphur (of C=S of the imidazolidinone ring) forms a hydrogen bond with the 5th nitrogen of 3rd guanine of DNA. In this configuration, the group at 3β-axial position (i.e., X-moiety) remains inclined towards the phosphodiester bond of DNA and the possibility of H- bonding cannot be ruled out. Since the changes in accessible surface area of interacting residues show a preferential binding of compound between G-C base pairs and bends the DNA slightly in such a way that a part of the molecule comes between the two base pairs of the minor groove of DNA helix. The resulting relative binding energy of docked steroidal imidazolidinone–DNA complexes was found to be −309.38 to −342.71 kJ mol^{-1}. This value is consistent with the high binding constant obtained from spectroscopic techniques.

In Vitro Cytotoxicity

The anticancer activity in vitro was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay in 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 [31]. The data reported in Table 1 suggests that the compounds **7**–**9** depicted potential cytotoxicity against given cell lines by showing effective IC_{50}. The compound **7** depicted IC_{50}=9.55 μM, 9.51 μM and 9.37 μM against A545, HL-60 and A549, respectively. The compound **8** also depicted IC_{50}= 7.53 μM and 7.41 μM, 9.23 μM against A545, SW480 and MCF-7, respectively. The compound **9** showed IC_{50}>10 μM against all cancer cell lines.

The overall anticancer activity of the compounds **7**–**9** may be attributed due to the more hydrophilicity and more bioavailability of steroidal imidazolidinones. From the Table 1 it is clear that compound **7** showed IC_{50} (9.55, 9.37 μM) more effective than that of 5-Fu (11.46, 12.67 μM) against A545 and A549 cell lines, respectively. The compound **8** also showed potential IC_{50} (7.53, 7.41 μM) more effective than that of 5-Fu (11.46, 10.31 μM) against A545 and MCF-7 cell lines, respectively.

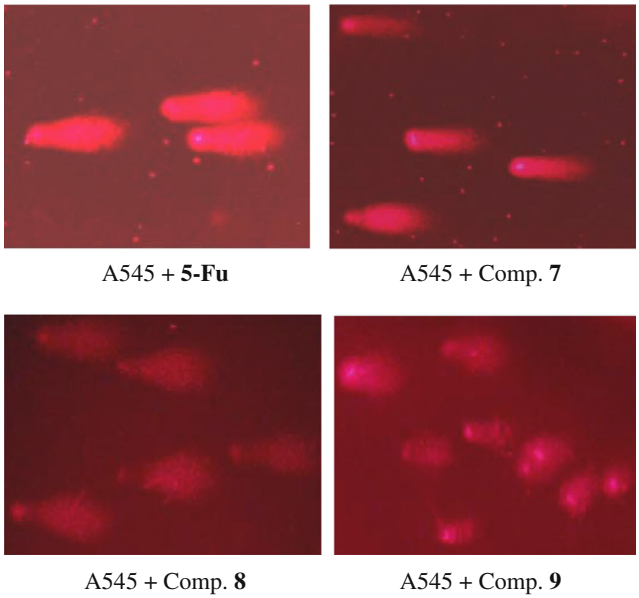


Fig. 6 Detection of DNA damage in A545 cells. Treated cells (24 h) were layered over agarose gel, lysed, electrophoresed in alkaline buffer and stained with propidium iodide

Comet Assay

In the comet assay [33], the images of A545 cells treated with 5-Fu and compounds **7**–**9** showed the formation of comets. There was increase in tail length when treated with compounds **7**–**9** (Fig. 6). Compound **8** and **7** showed maximum apoptotic DNA damage among the three steroidal imidazolidinones and even more apoptotic DNA damage than

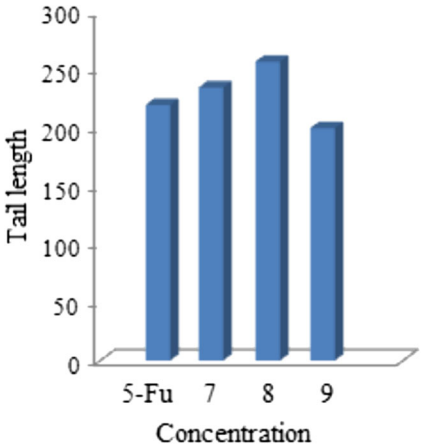


Fig. 7 Diagram comparing the effect of steroidal imidazolidinones on the tail length in comet assay. Graph showing the maximum effect of compound **8** on the tail length in comet assay

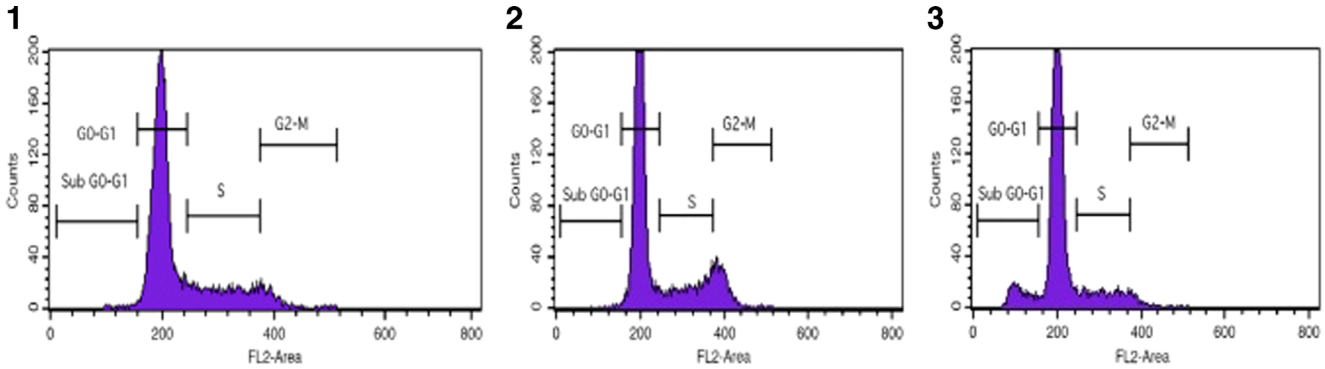


Fig. 8 FACS analysis of DNA from control and compound **8** treated A545 cells. **(1)** Control for **8**, **(2)** 4 μM **8**, **(3)** 7 μM **8**

the extent of 5-Fu. The DNA damage suggested that compounds induced dose-dependent fragmentation of chromosomal DNA leading to apoptosis. The images of comet assay for control, cells treated with 5-Fu (11.46 μM), **7** (9.55 μM), **8** (7.53 μM) and **9** (11.87 μM) are shown in Fig. 6. Slides were analysed for parameter like tail length, using image analyzer CASP software version 1.2.2. The results of the assay for tail length are shown in Fig. 7.

Fluorescence-Activated Cell Sorting (FACS) Analysis of Cell Cycle Arrest

FACS analysis of the control A545 cells show that 77.2 % of the cells are in G_0/G_1 phase, which is the growth phase before replication; 20.6 % of the cells are in the S phase, which is the DNA synthesis phase where DNA replication occurs and 2.2 % are in the G2/M phase where cell division occurs. Steroid imidazolidinone concentration dependent cell cycle arrest is shown in Fig. 8. It can be clearly seen from the figure that in the presence of 4 μM of compound **8**, the S phase of cell cycle shows marginal reduction. It is important to note that in the presence of 4 μM of the steroid imidazolidinone, no change in sub G_0/G_1 phase, which is a marker for induction of apoptosis, is observed in the cell cycle. This indicates that 4 μM of the compound **8** do not bring about apoptosis of A545 cells. In the presence of 7 μM of compound **8** however, the S phase decreases to 13.8 % and the G_2/M phase decreases to 1.04 %. More importantly, at this concentration of the steroid imidazolidinone, cell cycle analysis also shows 8.9 % of sub G_0/G_1 phase. Appearance of G_0/G_1 phase in the cell cycle clearly indicates that 7 μM of the compound **8** bring about apoptosis of A545 cells.

Conclusion

In summary the development and operationally designed simple strategy for the better synthesis of steroidal imidazolidinones was successful. Absorption and fluorescence studies reveal the stabilization of the energy levels of the complexes in presence of DNA. The DNA cleavage and molecular docking studies undertaken in the present work are in total agreement with the primary intercalative mode of binding, although the van der Waals and other types of interactions can also be argued. From in vitro cytotoxicity screening, it is clear that steroidal imidazolidinones were found to be potential cytotoxic agents in comparison with standard drug, 5-Flu. Potential apoptic, genotoxic nature and cell arrest behaviour of compounds was depicted by MTT assay, comet assay and FACS analysis, respectively. Hence, the present study has shown that these synthesized compounds can be used as template for future development through modification and derivatization to design more potent and selective cytotoxic agents.

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References

1. Newman DJ, Cragg GM (2007) Natural products as sources of new drugs over the last 25 years. J Nat Prod 70:461–477
2. Bellina F, Cauteruccio S, Rossi R (2007) Synthesis and biological activity of vicinal diaryl substituted 1H-inidazoles. Tetrahedron 63: 4571–4624
3. Bolani M, Gonzalez M (2005) Imidazole and benzimidazole derivatives as chemotherapeutic agents. Mini-Rev Med Chem 5:409–424
4. Salerno L, Sorrenti V, Guerrera F (1999) N-Substituted-imidazoles as inhibitors of nitric oxide synthase: a preliminary screening. Pharmazie 54:685–690
5. Mukherjee A, Kumar S, Seth M, Bhaduri AP (1989) Synthesis of 1-methyl-4-nitro-5- substituted imidazole and substituted imidazolothiazole derivatives as possible antiparasitic agents. Indian J Chem 28B:391–396
6. Ayhan-Kilcigil G, Altanlar N (2006) Synthesis and antifungal properties of some benzimidazole derivatives. Turk J Chem 30:223–228
7. Hadizadeh FH, Hosseinzadeh V, Shariaty M, Kazemi SJ (2008) Synthesis and antidepressant activity of N-substituted imidazole-5-caboxamides in forced swimming test model. Pharm Res 7:29–33

8. Shingalapur RV, Hosamani KM, Keri RS (2010) Synthesis and evaluation of antimicrobial and anti-tubercular activity of 2-styryl benzimidazoles. Eur J Med Chem 45:3320–3328
9. Ozkay Y, Iskar I, Incesu Z, Akalin GE (2010) Synthesis of 2-substituted-N-[4-(1- methyl- 4, 5- diphenyl-1H-imidazole-2-yl) phenyl] acetamide derivatives and evaluation of their anticancer activity. Eur J Med Chem 45:3320–3328
10. Tonelli M, Simone M, Tasso B, Novelli F, Biodo V (2010) Antiviral activity of benzimidazole derivatives. II. Antiviral activity of 2-phenylbenzimidazole derivatives. Bioorg Med Chem 18:2937–2953
11. Bhandari K, Srinivas N, Marrapu VK (2010) Synthesis of substituted aryloxy alkyl and aryloxy aryl alkyl imidazoles as antileishmanial agents. Bioorg Med Chem Lett 20:291–293
12. Nshimyumukizaa P, Berge EVD, Delest B, Mijatovic T, Kiss R, Marchand- Brynaert J, Robiette R (2010) Synthesis and biological evaluation of novel imidazole-containing macrocycles. Tetrahedron 66:4515–4520
13. Burger RM (1998) Cleavage of nucleic acids by bleomycin. Chem Rev 98:1153–1169
14. Sigman DS, Mazumder A, Perrin DM (1993) Chemical nucleases. Chem Rev 93:2295–2316
15. Jin Y, Cowan JA (2005) DNA cleavage by copper-ATCUN complexes. Factors influencing cleavage mechanism and linearization of dsDNA. J Am Chem Soc 127:8408–8415
16. Smith J, Ariga K, Anslyn EV (1993) Enhanced imidazole-catalyzed RNA cleavage induced by a bis-alkyl guanidinium receptor. J Am Chem Soc 115:362–364
17. Scheffer U, Strick A, Ludwig V, Peter S, Kalden E, Gobel MW (2005) Metal-free catalysts for the hydrolysis of RNA derived from guanidines, 2-aminopyridines, and 2-aminobenzimidazoles. J Am Chem Soc 127:2211–2217
18. Carlson CB, Beal PA (2000) Solid-phase synthesis of acridine-based threading intercalator peptides. Bioorg Med Chem Lett 10: 1979–1982
19. Kapuriya N, Kapuriya K, Zhang X, Chou T-C, Kakadiya R, Wu Y-T, Tsai T-H, Chen Y-T, Lee T-C, Shah A, Naliapara Y, Su T-L (2008) Synthesis and biological activity of stable and potent antitumor agents, aniline nitrogen mustards linked to 9-anilino acridines via a urea linkage. Bioorg Med Chem 16:5413–5423
20. Bacherikov VA, Chang J-Y, Lin Y-W, Chen C-H, Pan W-Y, Dong H, Lee R-Z, Chou T-C, Su T-L (2005) Synthesis and antitumor activity of 5-(9-acridinylamino) anisidine derivatives. Bioorg Med Chem 23:6513–6520
21. Blasiak J, Gloc E, Drzewoski J, Wozniak K, Zadrozny M, Skorski T, Pertynski T (2003) Free radical scavangers can differentially modulate the genotoxicity of amasacrinein normal and cancer cells. Mutat Res 535:25–34
22. Janovec L, Kozurkova M, Sabolova D, Ungvarsky J, Paulikova H, Plsikova J, Vantosa Z, Imrich J (2011) Cytotoxic 3,6-bis((imidazolidionone)imino)acridines: synthesis, DNA binding and molecular modelling. Bioorg Med Chem 19:1790–1801
23. Pommier Y (2013) Drugging topoisomerases: lessons and challenges. ACS Chem Biol 8:82–95
24. Dar AM, Ishrat U, Yaseen Z, Shamsuzzaman, Gatoo MA (2015) In vitro cytotoxcity and interaction of new steroidal oxadiazinanones with calf thymus DNA using molecular docking, gel electrophoresis and spectroscopic techniques. J Photochem Photobiol B Biol 148:340–350
25. Son GS (1998) Binding mode of norfloxacin to calf thymus DNA. J Am Chem Soc 120:6451–6457
26. Shamsuzzaman, Salim A, Aslam M, Naqvi F (1997) Synthesis of spiro-1′,2′,4′-triazolidine- 3′-thiones. Synth Commun 27:2171–2175
27. Reicmann ME, Rice SA, Thomas CA, Doty P (1954) A further examination of the molecular weight and size of deoxy pentose nucleic acid. J Am Chem Soc 76:3047–3053
28. Pasternack RF (2003) Circular dichroism and the interactions of water soluble porphyrins with DNA-a mini review. Chirality 15:329–332
29. Mustard D, Ritchie DW (2005) Docking essential dynamics eigen structures. Proteins: Struct Funct Bioinf 60:269–274
30. Delano WL (2002) The PyMOL molecular graphics system. DeLano Scientific, San Carlos
31. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63
32. Saxena HO, Faridi U, Kumar JK, Luqman S, Darokar MP, Shanker K, Chanotiya CS, Gupta MM, Negi AS (2007) Synthesis of chalcone derivatives on steroidal framework and their anticancer activities. Steroids 72:892–900
33. Singh NP (2000) Microgels for estimation of DNA-strand breaks, DNA protein crosslinks and apoptosis. Mutat Res 455:111–127
34. Kaminskyy D, Bednarczyk-Cwynar B, Vasylenko O, Kazakova O, Zimenkovsky B, Zaprutko L, Lesyk R (2012) Synthesis of new potential anticancer agents based on 4-thiazolidinone and oleanane scaffolds. Med Chem Res 21:3568–3580
35. Wang BD, Yang ZY, Crewdson P, Wang DQ (2007) Synthesis, crystal structure and DNA -binding studies of the Ln(III) complex with 6- hydroxychromone-3-carbaldehyde benzoyl hydrazone. J Inorg Biochem 101:1492–1504
36. Shahabadi N, Kashanian S, Khosravi M, Mahdavi M (2010) Multispectroscopic DNA interaction studies of a water-soluble nickel(II) complex containing different dinitrogen aromatic ligands. Transit Met Chem 35:699–705
37. Berk AJ (2000) TBP-like factors come into focous. Cell 103:5–8
38. Wolfe A, Shimer GH, Meehan T, Wolfe A, Shimer GH, Meehan T (1987) Polycyclic aromatic hydrocarbons physically intercalate into duplex regions of denatured DNA. Biochemistry 26:6392–6396
39. Eftink MR (1991) Fluorescence quenching reaction: probing biological macromolecular structures, biophysical and biochemical aspects of fluorescence spectroscopy. Plenum Press, New York
40. Tan LF, Chao H, Zhen KC, Fei JJ, Wang F, Zhou YF, Ji LN (2007) Effects of the ancillary ligands of polypyridyl ruthenium(II) complexes on ther DNA binding and photocleavage behaviours. Polyhedron 26:5458–5468
41. Scatchard G (1949) The attractions of proteins for small molecules and ions. Ann N Y Acad Sci 51:660–672
42. Ruiz J, Rodríguez V, Cutillas N, Samper KG, Capdevila M, Palacios Ò, Espinosa A (2012) Novel C, N-Chelate rhodium(III) and Iradium(III) antitumor complexes incorporating a lipophilic steroidal conjugate and their interaction with DNA. Dalton Trans 41:12847–12856
43. Paul BK, Guchhait N (2011) Exploring the strength, mode, dynamics, and kinetics of binding interaction of a cationic biological photosensitizer with DNA: Implication on disassociation of the drug-DNA complex via detergent sequestration. J Phys Chem B 115: 11938–11949
44. Nejat Dehkordi M, Bordbar AK, Lincoln P, Mirkhani V (2012) Spectroscopic study on the interaction of ct-DNA with manganese salen complex containing triphenyl phodphonium groups. Spectrochim Acta A 90:50–54
45. Maheswari PU, Palaniandavar M (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. J Inorg Biochem 98:219–230
46. Lincoln P, Tuite E, Nordén B (1997) Short circuiting the molecular wire-coperative binding of Δ-$[Ru(Phen)_2dppz]^{2+}$ and Δ-$[Rh(phi)_2Bipy]^{3+}$ to DNA. J Am Chem Soc 119:1454–1455
47. Lepre C, Lippard S, Eckstein F (1990) In: Lilley DMJ (ed) Nucleic acids and molecular biology, vol 4. Springer, Germany, pp 9–38