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DNA-Binding Studies of Benzimidazo[2,1-a]Ellipticine Derivatives

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Abstract

The DNA-binding mode of three new benzimidazo[2,1-a]ellipticine derivatives (compound 1, 2 and 3) with CT-DNA was investigated by absorption spectroscopy, EB-DNA displacement, circular dichroism, thermal denaturation and viscosity measurements. Results indicated that these compounds intercalate into the base pairs of CT-DNA. The effect of ionic strength on the fluorescence property of the system indicated the presence of electrostatic interaction *via* phosphate backbone of DNA helix. The intrinsic binding constant values suggested that compound 3 has significantly greater DNA binding propensity. These compounds promote the cleavage of plasmid pBR322 DNA upon irradiation at 365 nm. These results may be useful for the design of benzimidazo[2,1-a]ellipticine derivatives with desired binding characteristics and useful to better understand the DNA binding mode of ellipticine.

Keywords: *Benzimidazo[2,1-a]ellipticine; DNA-binding; pBR322 cleavage; DNA helix*

Introduction

Despite of many therapeutic successes, cancer is a most common disease and frequent cause of death over the world. DNA is the molecular target of many anticancer drugs in clinical use and development. Small molecule inhibitors can interact with DNA *via* covalent or non-covalent interactions. In general, most drugs have three distinct modes of non-covalent interactions with DNA:

- DNA-intercalative association in which a planar aromatic moiety slides between the DNA base pairs,
- DNA groove binding through a combination of hydrophobic, electrostatic and hydrogen bonding interactions and
- External binding by electrostatic attraction [1,2]. Their associative interactions with the DNA molecule can cause dramatic changes in the physiological functions of DNA [3-5].

Therefore, understanding the interactions of small molecules with DNA is of significance in the rational design of more powerful and selective anticancer agents. This can be achieved by systematically modifying the structural features of the lead compound and analyzing the subsequent changes in the DNA binding properties [6-8]. Ellipticine is a natural plant alkaloid originally isolated from *Ochrosia elliptica* of the *Apocynaceae* family that has been found to be a potent antitumour agent [9]. Some of its more soluble derivatives, that have different DNA-binding affinities, shows antitumour and cytotoxic effects and exhibit promising results in the treatment of breast cancer metastasis, kidney sarcomas, brain tumours and myeloblastic leukaemia [10]. Although a multimodal mechanism of action on DNA is accepted, the effects of ellipticine derivatives are considered to be mainly based on DNA intercalation and/or the inhibition of topoisomerase II [11]. Unfortunately, the exact mechanism remains unclear as a realistic binding mode has not been fully depicted to date; however, many experimental data suggest that ellipticine unwinds DNA by intercalative binding in an orientation parallel to base pairs [12].

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Compounds containing benzimidazole core systems and their fused heterocyclic variants have attracted considerable attention from medicinal chemists because of their wide range of biological activities, such as anxiolytic, antibacterial/antifungal, antineoplastic, anticancer, DNA intercalator etc [13-17]. The cyano and amidino-substituted derivatives of styryl-2-benzimidazoles, benzimidazo[1,2-a]quinolines, benzimidazo[1,2-c]quinazolines showed that these compounds have excellent DNA intercalation properties [18]. In this study, we characterized the DNA binding mode of benzimidazo[2,1-a]ellipticine derivatives so as to gain better understanding of their interactions with the DNA molecule [19].

Materials and Methods

CT-DNA was purchased from SRL (India) and plasmid DNA pBR322 was purchased from Genei (India), all chemicals were of analytical grade and used without further purification. All experiments involving the interaction of the compounds with DNA was carried out in Tris-HCl buffer (pH 7.2) containing 5 mM Tris-HCl and 50 mM NaCl prepared with Ultra-pure MilliQ water (Millipore). Compounds were dissolved in DMSO at 1 mM as a stock. The stock DMSO solutions were kept at room temperature and freshly diluted with Tris-HCl buffer to the desired concentration just before use. The concentrations of compound solutions were determined spectrophotometrically.

A solution of CT-DNA in the Tris-HCl buffer gave a ratio of UV absorbance at 260 nm and 280 nm of >1.85, indicating that the DNA was free of protein [20]. The DNA concentration per nucleotide was determined by absorption spectroscopy using the molar absorption coefficient (6600 M^{-1} cm^{-1}) at 260 nm [21]. The DNA solution was stored for a short period of time at 4°C if not used immediately [22]. The following spectrometric measurements were performed at 25°C in a quartz cuvette of 1 cm path length and the sample solution was incubated for 10 min beforehand.

Electronic absorption titration

Absorption titration was performed at a fixed compound concentration (10 µM) with various concentrations (0 µM-100 µM) of CT-DNA. The absorption spectra were recorded on a Shimadzu UV-1800 spectrophotometer. The absorbance due to DNA at the measured wavelength was nullified.

Fluorescence spectroscopic studies

EB displacement measurement: CT-DNA (10 µM) was pretreated with EB (10 µM) for 30 min at 25°C. Small aliquots of concentrated compound solution were added into the pretreated solution at various final concentrations (0 µM-100 µM). The samples were excited at 480 nm and the emission spectra were observed between 550 nm to 750 nm on a fluoromax-4 spectrofluorometer. The slit width Ex/Em was 5 nm/5 nm.

Effect of ionic strength

CT-DNA (20 µM) was pretreated with compound (10 µM) for 30 min at 25°C. Small aliquots of a concentrated NaCl solution were added into the pretreated solution at various final concentrations (0 M-0.4 M). The corresponding fluorescence spectra were recorded by exciting the samples at 325 nm and the emission was observed between 400 nm-625 nm. The slit width Ex/Em was 5 nm/5 nm.

Circular dichroism titration

Circular Dichroism (CD) spectra were recorded on a Jasco J-810 spectropolarimeter. CD titration was first performed at a fixed CT-DNA concentration (50 µM) with various concentrations (0 µM-50 µM) of the compounds using the instrument parameters of 230 nm-320 nm wavelength, scan speed 50 nm/min, 1 nm bandwidth, 100 millidegree sensitivity and 1 s response time, with an average of three scans. From sample, the buffer and compound background were subtracted.

Viscosity experiment

Viscosity measurements were carried out using an Ubbelodhe viscometer, immersed in a thermostatic water-bath that maintained at a constant temperature at 25.0°C ± 0.1°C. DNA samples approximately 200 base pairs in average length were prepared by sonication of CT-DNA in order to minimize complexities arising from DNA flexibility [23]. The compounds (1 µM-80 µM) were titrated with the DNA solution (100 µM). The flow time of each sample was measured by a digital stopwatch for three times and the average result was considered.

Thermal denaturation studies

Thermal denaturation studies were carried out with a Perkin-Elmer Lambda 35 spectrophotometer equipped with a Peltier temperature-controlling programmer (± 0.1°C). The absorbance at 260 nm was continuously monitored for solutions of CT-DNA (30 µM) in the absence and presence of the compound (10 µM). The temperature of the solution was increased by

1°C/min. Data were presented as A/A_0 versus temperature, where A_0, A are the initial and observed absorbance at 260 nm, respectively.

DNA cleavage study

For the gel electrophoresis experiments, supercoiled pBR322 DNA (0.2 µg) was treated with compound in Tris-HCl buffer and the solution were then irradiated at room temperature with a UV lamp (365 nm, 10 W). A loading buffer containing 25% bromophenol blue, 30% glycerol was added and electrophoresis was carried out at 70 V for 90 min in tris-acetic acid-EDTA buffer using 0.8% agarose gel containing 1.0 µg/mL EB and photographed under UV light.

Results and Discussion

Three benzimidazo[2,1-a]ellipticine derivatives 1-3 were chosen for the DNA binding mode studies, which were prepared as described previously (FIG. 1-3) [24].

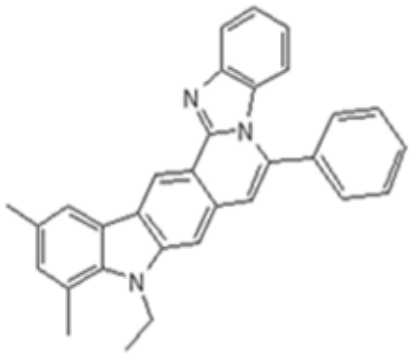

FIG 1. **Schematic structure of compounds benzimidazo[2,1-a]ellipticine.**

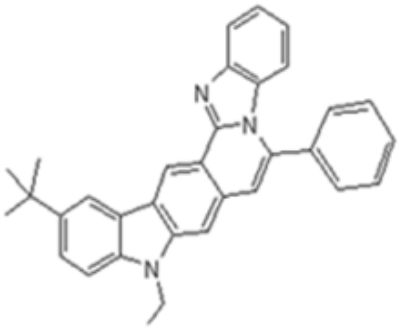

FIG 2. **Schematic structure of benzimidazo[2,1-b]ellipticine.**

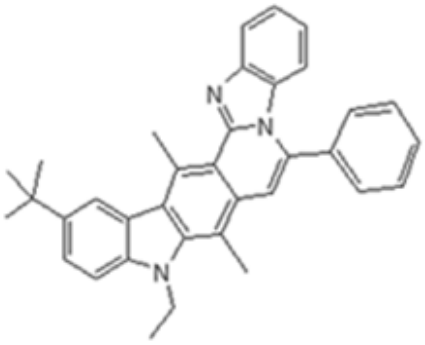

FIG 3. **Schematic structure of benzimidazo[2,1-c]ellipticine.**

Electronic absorption titration

The electronic absorption spectroscopy is one of the most useful techniques in DNA-binding studies [25]. FIG. 4-6 shows the absorption spectra of compounds 1, 2 and 3 in absence and presence of increasing amounts of CT-DNA.

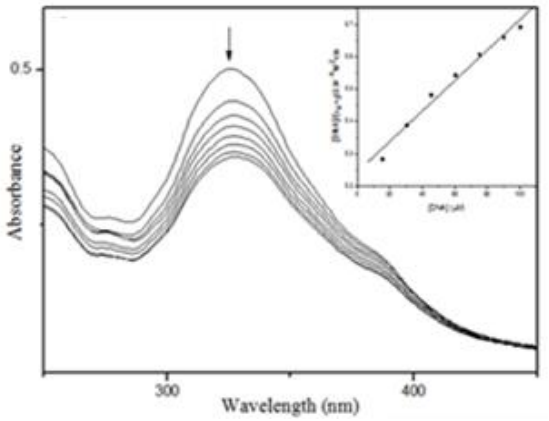

FIG 4. **Absorption spectra of compound of benzimidazo[2,1-a]ellipticine (10 µM) in the absence and presence of increasing amounts of CT-DNA (0 µM-100 µM) at 25°C in 50 mM NaCl and 5 mM Tris-HCl buffer (pH 7.2). The arrow shows the absorbance changing upon increasing the DNA concentration. Inset shows the plot of (DNA)/(ε_a-ε_f) *vs.* (DNA) for the titration of DNA to the compounds.**

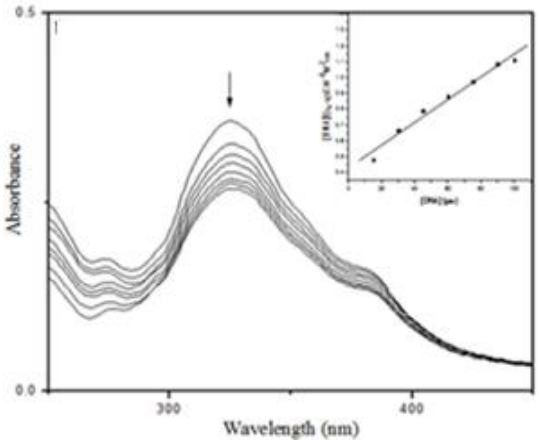

FIG 5. **Absorption spectra of compound of benzimidazo[2,1-b]ellipticine (10 µM) in the absence and presence of increasing amounts of CT-DNA (0 µM-100 µM) at 25°C in 50 mM NaCl and 5 mM Tris-HCl buffer (pH 7.2). The arrow shows the absorbance changing upon increasing the DNA concentration. Inset shows the plot of (DNA)/(ε_a-ε_f) *vs.* (DNA) for the titration of DNA to the compounds.**

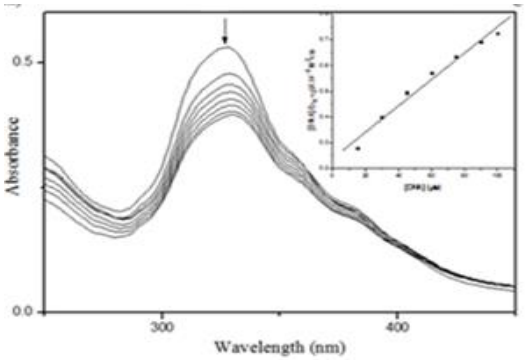

FIG 6. **Absorption spectra of compound of benzimidazo[2,1-c]ellipticine (10 µM) in the absence and presence of increasing amounts of CT-DNA (0-100 µM) at 25°C in 50 mM NaCl and 5 mM Tris-HCl buffer (pH 7.2). The arrow shows the absorbance changing upon increasing the DNA concentration. Inset shows the plot of (DNA)/(ε_a-ε_f) *vs.* (DNA) for the titration of DNA to the compounds.**

Addition of increasing amounts of CT-DNA results in hypochromic and bathochromic shifts in the UV spectra of three compounds. These spectral characteristics suggest that the compounds mostly bind to DNA by intercalation. Intercalation usually results in hypochromism and bathochromism due to the intercalative mode involving a strong stacking interaction between an aromatic chromophore and the base pairs of DNA [26]. After intercalating the base pairs of DNA, the π^* orbital of the intercalated ligand can couple with the π orbital of the base pairs, thus decreasing the π^* transition energy and resulting in the bathochromism. On the other hand, the coupling π orbital is partially filled by electrons, thus decreasing the transition probabilities and concomitantly resulting in hypochromism [27]. From the absorption titration data, the intrinsic binding constant K_b was determined using Wolfe-Shimer equation [28].

$$(DNA)/(€_a-€_f)=(DNA)/(€_b-€_f)+1/K_b\,(€_b-€_f)$$

Where ε_a, ε_f and ε_b correspond to A_{obsd}/(compound), the extinction coefficient of the free compound and the extinction coefficient of the compound in the fully bound form, respectively. A plot of (DNA)/(ε_a-ε_f) *versus* (DNA) gives the K_b as the ratio of the slope to the intercept. Intrinsic binding constants K_b of 1.933×10^4 M^{-1}, 1.68×10^4 M^{-1} and 2.146×10^4 M^{-1} were measured for compounds 1, 2 and 3, respectively. Based on the measurement of the spectroscopic properties and binding constants, the DNA-binding affinity of compounds follow the order of 3>1>2. The results are due to increasing the substitutions on carbazole moiety of benzimidazo[2,1-a]ellipticine. Upon increasing the substitutions, hydrophobicity increases, leading to a greater DNA-binding affinity for compound 3.

Fluorescence spectroscopic studies

EB displacement measurement: The molecular fluorophore, EB, is known to show intense fluorescence light when bound to DNA, due to its strong intercalation between the adjacent DNA base pairs [29,30]. In earlier literature, it was reported that the fluorescent light of EB-DNA system can be reduced by the addition of a second molecule indicating the competition of second molecule with EB in binding to DNA. The emission spectra of EB bound to DNA in the absence and presence of compound (FIG. 7-10).

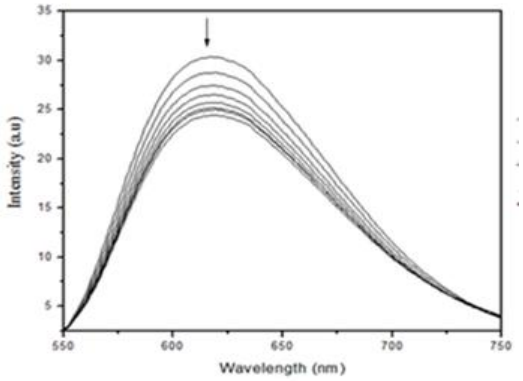

FIG 7. **Emission spectrum of EB bound to DNA in the presence of compound benzimidazo[2,1-a]ellipticine at 25°C ((EB)=10 µM, (DNA)=10 µM, (compound)=0-100 µM, ex=480 nm). The arrow shows the intensity change upon increasing compound concentrations.**

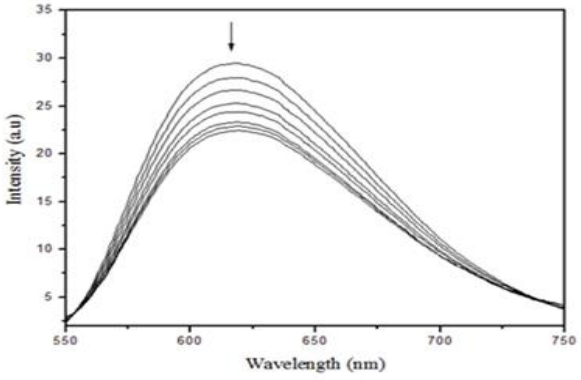

FIG 8. **Emission spectrum of EB bound to DNA in the presence of compound benzimidazo[2,1-b]ellipticine at 25°C ((EB)=10 µM, (DNA)=10 µM, (compound)=0-100 µM, ex=480 nm). The arrow shows the intensity change upon increasing compound concentrations.**

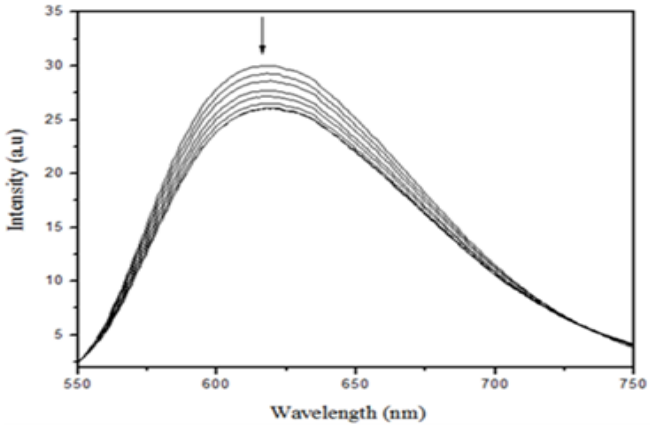

FIG 9. **Emission spectrum of EB bound to DNA in the presence of compound benzimidazo[2,1-c]ellipticine at 25°C ((EB)=10 µM, (DNA)=10 µM, (compound)=0 µM-100 µM, ex=480 nm). The arrow shows the intensity change upon increasing compound concentrations.**

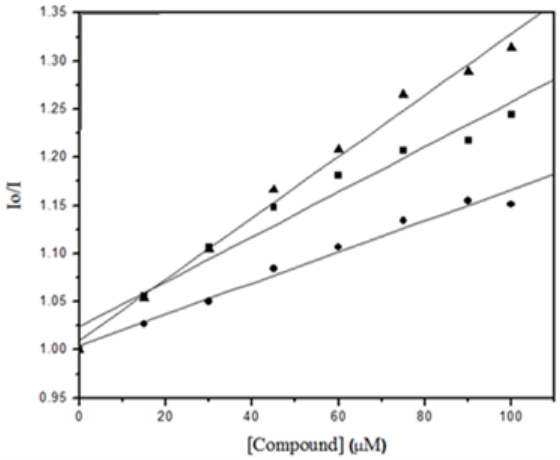

FIG 10. **Plot of I0/I *vs.* (Compound) for the titration of the compounds 1-3 to DNA-EB system.**

The addition of the compound to DNA pretreated with EB causes an appreciable reduction in fluorescence intensity, indicating that these compounds compete with EB to bind with DNA. Therefore, the effective EB displacement by three compounds may be associated with their intercalative DNA binding mode. The quenching of EB bound to DNA by the compounds is in agreement with the classical Stern-Volmer equation [31].

$$I_0/I=1+K_{sv}(Q)$$

Where I_o and I represent the fluorescence intensities in the absence and presence of quencher, respectively, K_{sv} is the linear Stern-Volmer quenching constant and (Q) is the concentration of the compound. Linear Stern-Volmer quenching constant (K_{sv}) of 2.33×10^3 M^{-1}, 1.62×10^3 M^{-1} and 3.19×10^3 M^{-1} were determined for compounds 1, 2 and 3, respectively. These results further confirm the effect of the substitution on the DNA-binding behavior of the compounds.

Effect of ionic strength

Monitoring the spectral changes with different ionic strength is an efficient method for distinguishing the binding modes between small molecules and CT-DNA. Since DNA is an anionic poly-electrolyte with phosphate groups, the addition of cation would weaken the electrostatic interaction between DNA and molecules due to the competition for phosphate groups [32,33]. The effect of ionic strength on compound-DNA binding was studied (FIG. 11-14).

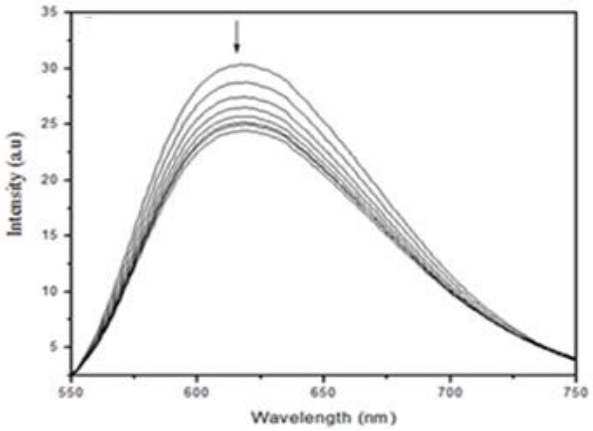

FIG 11. **Emission spectrum of compound benzimidazo[2,1-a]ellipticine bound to DNA in the presence of NaCl ((compound)=10 µM, (DNA)=20 µM, (NaCl)=0-0.4 M, ex=325 nm) at 25 °C. The arrow shows the intensity change upon increasing NaCl concentrations.**

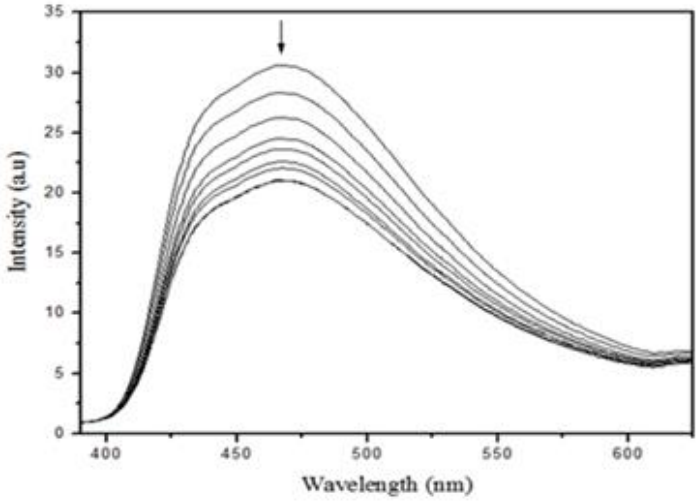

FIG 12. **Emission spectrum of compound benzimidazo[2,1-b]ellipticine bound to DNA in the presence of NaCl ((compound)=10 µM, (DNA)=20 µM, (NaCl)=0-0.4 M, ex=325 nm) at 25°C. The arrow shows the intensity change upon increasing NaCl concentrations.**

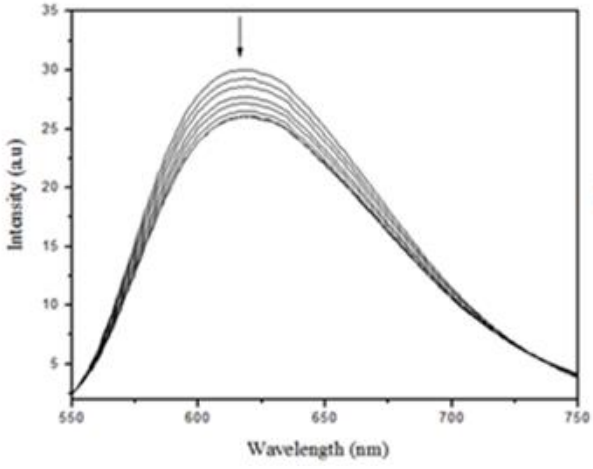

FIG 13. **Emission spectrum of compound benzimidazo[2,1-c]ellipticine bound to DNA in the presence of NaCl ((compound)=10 µM, (DNA)=20 µM, (NaCl)=0-0.4 M, ex=325 nm) at 25°C. The arrow shows the intensity change upon increasing NaCl concentrations.**

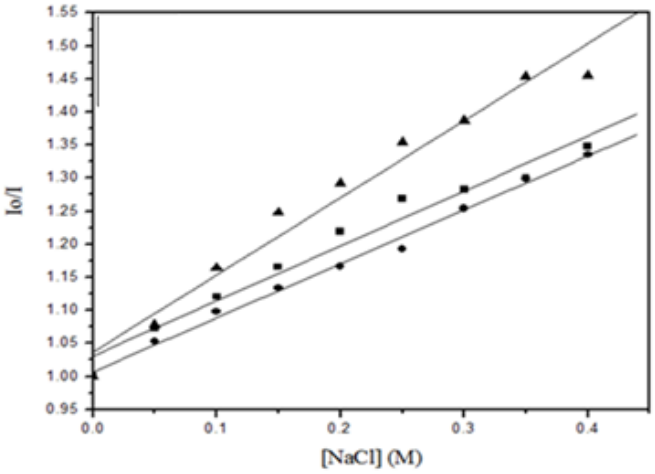

FIG 14. **Plots of emission intensity I0/I *vs.* (NaCl).**

Linear Stern-Volmer quenching constant (K_{sv}) of 0.833 × 10^3 M^{-1}, 0.817 × 10^3 M^{-1} and 1.17 × 10^3 M^{-1} were determined for compounds 1, 2 and 3 respectively. This result displayed a strong dependence of fluorescence intensity on ionic strength. Furthermore, compound 3 displays stronger electrostatic interaction in comparison to compounds 1 and 2. This is in accordance with our other DNA binding assays. This result indicated that the mode of binding of compounds is partially electrostatic *via* DNA phosphate backbone. This electrostatic interaction may be stabilizing the DNA-compound complex.

Circular dichroism titration

CD spectral techniques give us useful information on how the conformation of DNA is influenced by the binding of small molecules to DNA. The observed CD spectrum of CT-DNA consists of a positive band at 277 nm due to base stacking and a negative band at 245 nm due to helicity, which is characteristic of DNA in the right-handed B form [34]. These bands are sensitive towards binding of any small molecule or drug and hence can be exploited to investigate the binding of small molecules to DNA [35]. Simple groove binding and electrostatic interactions of small molecules lead to no perturbation or marginal perturbations in these two CD bands of B-DNA. On the addition of compound to a solution of the CT-DNA, significant changes in its CD spectrum. The negative band shifted to higher wavelength, whereas the positive signal disappeared gradually, such changes are likely to result from structural alterations induced by the compound upon binding to CT-DNA (FIG. 15-17) [36].

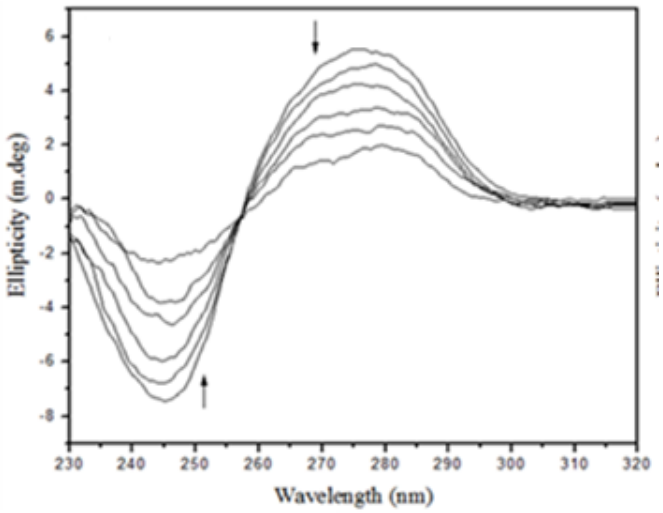

FIG 15. **CD spectra of CT-DNA (50 µM) titrated with compound benzimidazo[2,1-a]ellipticine (0-50 µM) at 25°C over the wavelength range 230-320 nm.**

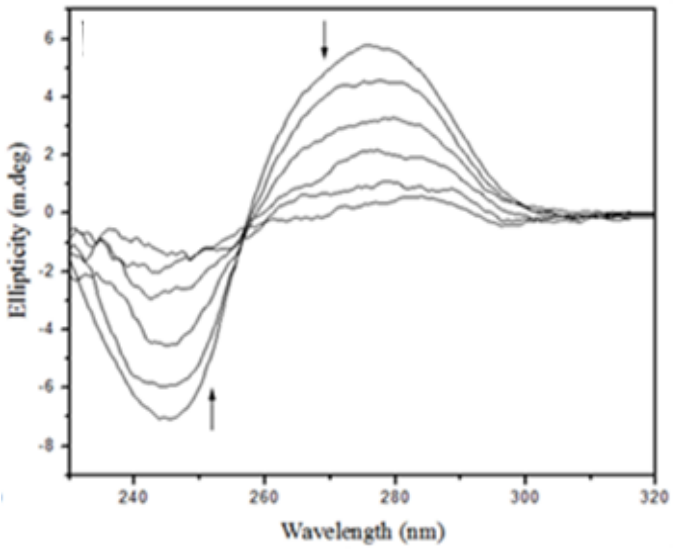

FIG 16. **CD spectra of CT-DNA (50 µM) titrated with compound benzimidazo[2,1-b]ellipticine (0-50 µM) at 25°C over the wavelength range 230 nm-320 nm.**

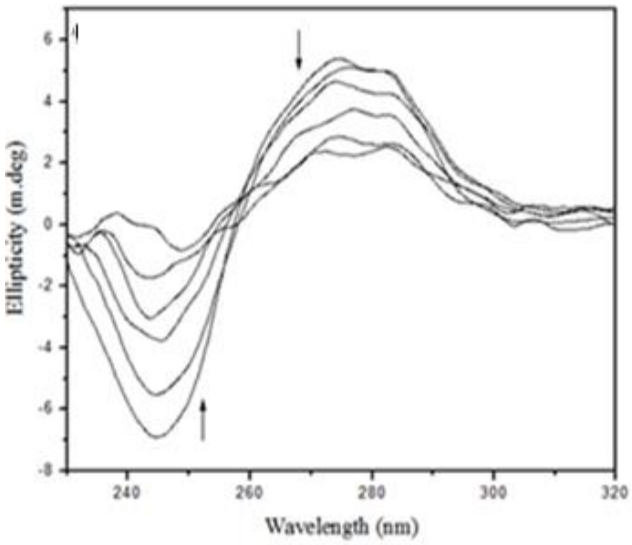

FIG 17. **CD spectra of CT-DNA (50 µM) titrated with compound benzimidazo[2,1-c]ellipticine (0-50 µM) at 25°C over the wavelength range 230-320 nm.**

Viscosity experiment

Viscosity measurements are regarded as the least ambiguous and the most critical test of a DNA binding model in solution and provide strong evidence for intercalative DNA binding mode [37]. To throw further light on the DNA binding mode, viscosity measurements were carried out for compounds 1, 2 and 3 [38]. A classical intercalation model results in the lengthening of the DNA helix because base pairs become separated to accommodate the binding molecule, leading to an increase in the viscosity of CT-DNA.

In contrast, a partial and/or non-classical intercalation of molecule could bend (or kink) the DNA helix, reducing its effective length and concomitantly, its viscosity [39]. The effect of compounds on the CT-DNA data are presented as $(\eta/\eta_0)^{1/3}$ *vs.* binding ratio where η and η_0 are the viscosity of DNA in the presence and absence of complex, respectively (FIG. 18) [40].

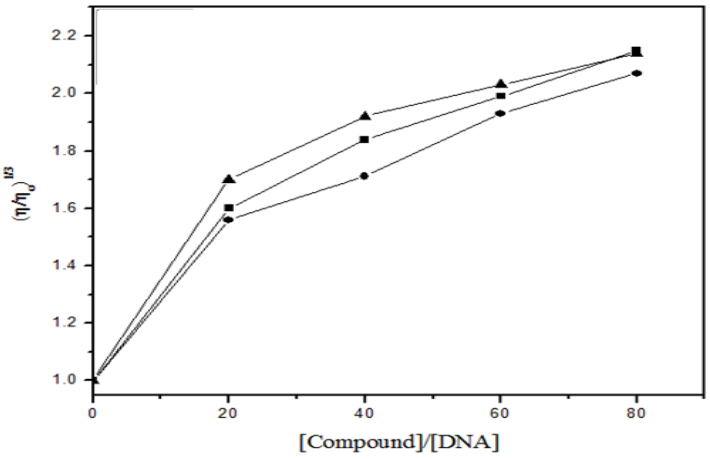

FIG 18. **Effect of increasing amounts of compound 1-3 on the relative viscosity of CT-DNA at 25.0 ± 0.1°C. (DNA)=100 µM, r=(compound)/(DNA)=0-0.8.**

Viscosity values were calculated from the observed flow time of DNA containing solutions corrected from the flow time of buffer alone (t_0), $\eta=t-t_0$ [41-44]. Result reveals that the relative viscosity of DNA increased steadily following the order of 3>1>2 with an increasing amount of the compounds. The increased degree of viscosity may depend on the affinity of the compounds to DNA. These results suggest that the compounds intercalate between the base pairs of DNA, the difference in binding strength of the compounds is caused by the structural modification in molecule.

Thermal denaturation studies

Thermal behaviors of DNA in the presence of compounds can give an insight into their conformational changes when temperature is raised and offer information about the interaction strength of compounds with DNA. It is well known that when the temperature in the solution increases, the double-stranded DNA gradually dissociates to single strands and generates a hyperchromic effect on the absorption spectra of DNA bases (λ_{max}=260 nm). In order to identify this transition process, the melting temperature Tm, which is defined as the temperature where half of the total base pairs is unbound, is usually introduced. According to the literature, the intercalation of natural or synthesized organic intercalators generally results in a considerable stabilization of the DNA duplex with a corresponding increase in melting temperature (T_m) [45]. Here, the thermal denaturation experiment carried out for DNA in the absence of the compound revealed a Tm of 76°C under our experimental conditions. However, with the addition of compound, the Tm of the DNA increased to 77.1°C for compound 1,76.8°C for compound 2 and 78.4°C for compound 3, respectively following the order of 3>1>2 (FIG. 19). These results further confirm the order of binding strength of these compounds.

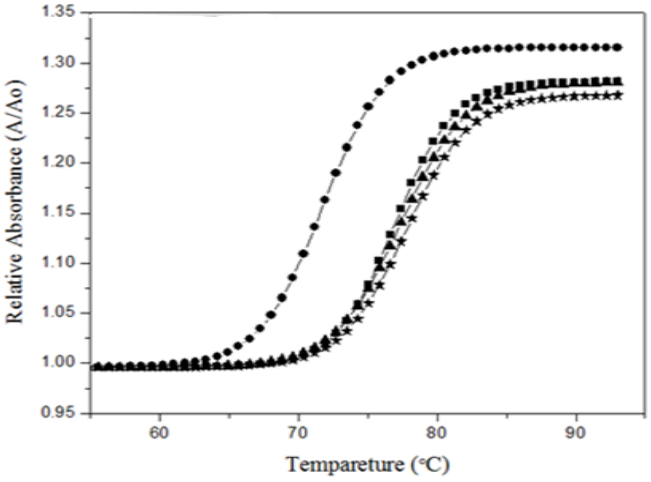

FIG 19. **Melting temperature curves of DNA in the absence and presence of compound 1, 2 and 3 (Compound)=10 µM and (DNA)=30 µM.**

Cleavage of plasmid pBR322 DNA

It is known that DNA cleavage is controlled by relaxation of super-coiled circular conformation of pBR322 DNA to nicked circular and/or linear conformations. When electrophoresis is applied to circular plasmid DNA, a fast migration will be observed for DNA of closed circular conformations (Form I). If one strand is cleaved, the super-coil will relax to produce a slower-moving nicked conformation (Form II). If both strands are cleaved, a linear conformation (Form III) will be generated that migrates in between the forms I and II. The photo-cleavage of pBR322 DNA in the absence and presence of three compounds were carried out in FIG. 20-22.

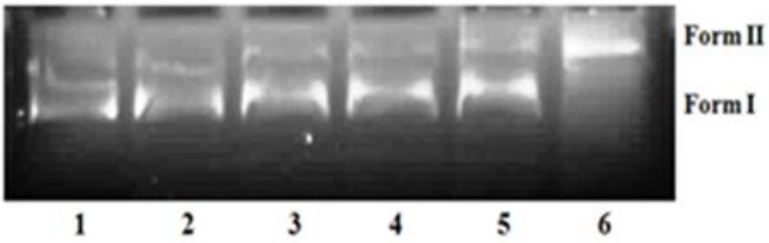

FIG 20. **Photo activated cleavage of supercoiled plasmid DNA, pBR322 (0.2 µg) by increasing the concentration of compound benzimidazo[2,1-a]ellipticine. Lane 1: DNA control; Lane 2: DNA+10 µM; Lane 3: DNA+20 µM; Lane 4: DNA+30 µM; Lane 5: DNA+40 µM; Lane 6: DNA+50 µM.**

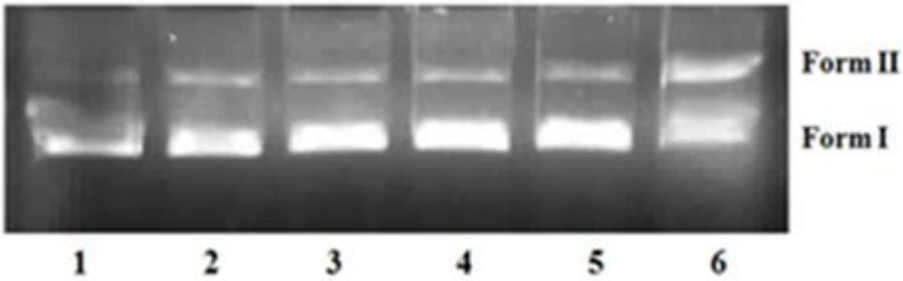

FIG 21. **Photo activated cleavage of supercoiled plasmid DNA, pBR322 (0.2 µg) by increasing the concentration of compound benzimidazo[2,1-b]ellipticine. Lane 1: DNA control; Lane 2: DNA+10 µM; Lane 3: DNA+20 µM; Lane 4: DNA+30 µM; Lane 5: DNA+40 µM; Lane 6: DNA+50 µM.**

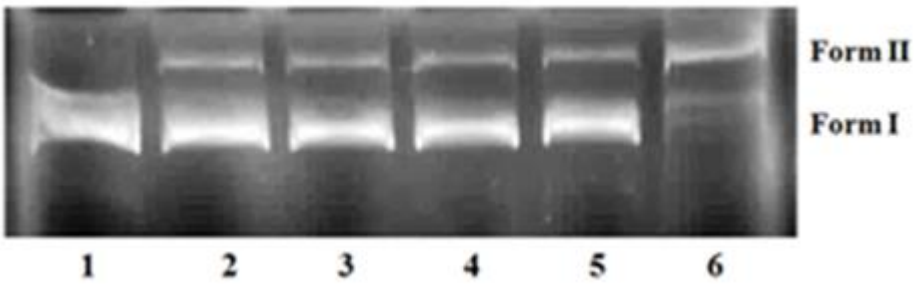

FIG 22. **Photo activated cleavage of supercoiled plasmid DNA, pBR322 (0.2 µg) by increasing the concentration of compound benzimidazo[2,1-c]ellipticine. Lane 1: DNA control; Lane 2: DNA+10 µM; Lane 3: DNA+20 µM; Lane 4: DNA+30 µM; Lane 5: DNA+40 µM; Lane 6: DNA+50 µM.**

Conclusion

Almost no DNA cleavage was observed for the control, in which compound was absent (lane 0). With increasing concentrations of the three compounds (lanes 1-5), the amount of form I of pBR322 DNA diminishes gradually, whereas form II increases. Under comparable experimental conditions, compounds 3 and 1 exhibit more effective DNA cleavage activity than compound 2.

This may be related to the different binding affinities of these compounds to DNA. In summary, three compounds are able to perform an efficient cleavage of pBR322 DNA. All results were obtained from experiments that were performed at least in triplicate. The interaction of all the ligands and their complexes with plasmid DNA and their restriction endonuclease reactions by BamHI and HindIII enzymes were studied by agarose gel electrophoresis.

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Conflict of Interest

The authors are declaring that we have no conflicts of interest.

Compliance with ethical standards

This article does not contain any studies involving human participants or animals performed by any of the authors.

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