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Fluorescence Spectroscopic and Calorimetry Based Approaches to Characterize the Mode of Interaction of Small Molecules with DNA

Amrita Banerjee · Jasdeep Singh · Dipak Dasgupta

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Abstract Ethidium bromide displacement assay by fluorescence is frequently used as a diagnostic tool to identify the intercalation ability of DNA binding small molecules. Here we have demonstrated that the method has pitfalls. We have employed fluorescence, absorbance and label free technique such as isothermal titration calorimetry to probe the limitations. Ethidium bromide, a non-specific intercalator, netropsin, a (A-T) specific minor groove binder, and sanguinarine, a (G-C) specific intercalator, have been used in our experiments to study the association of a ligand with DNA in presence of a competing ligand. Here we have shown that netropsin quenches the fluorescence intensity of an equilibrium mixture of ethidium bromide - calf thymus DNA via displacement of ethidium bromide. Isothermal titration calorimetry results question the accepted interpretation of the observed decrease in fluorescence of bound ethidium bromide in terms of competitive binding of two ligands to DNA. Furthermore, isothermal titration calorimetry experiments and absorbance measurements indicate that the fluorescence change might be due to formation of ternary complex and not displacement of one ligand by another.

Keywords Ethidium bromide displacement assay · DNA · Isothermal titration calorimetry · Competitive binding · Ternary complex

Abbreviations

EtBr	Ethidium bromide
ITC	Isothermal titration calorimetry
Net	Netropsin
ct DNA	Calf thymus DNA
SGR	Sanguinarine

A. Banerjee · D. Dasgupta (✉)
Biophysics Division, Saha Institute of Nuclear Physics, Block-AF, Sector-I, Bidhan Nagar,
Kolkata 700 064 West Bengal, India
e-mail: dipak.dasgupta@saha.ac.in

J. Singh
Kusuma School of Biological Sciences,
Indian Institute of Technology-Delhi, Hauz Khas,
New Delhi 110016, India

Introduction

Displacement of DNA intercalator, ethidium bromide (EtBr), from DNA has been widely employed as a diagnostic tool for the intercalation [1–4] and in some cases as the confirmatory evidence [5–11] for the intercalation ability of DNA binding small molecules including those with therapeutic potential. There are also propositions that those which could not displace EtBr are groove binders [12, 13]. On the other hand, there are reports that suggest that the minor groove binder non-intercalators have the ability to displace ethidium bromide form DNA [14, 15] and synthetic polynucleotides with defined sequences [16]. The experimental parameter monitored for the purpose has been progressive decrease in fluorescence of EtBr in an equilibrium mixture of EtBr and DNA consequent to increase in the input concentration of the ligand under study. The current report is aimed to show that the decrease in fluorescence of EtBr only indicates the ability of the small ligands (or small peptides) to bind DNA via minor groove. We have employed fluorescence, absorbance and a label free technique such as isothermal titration calorimetry (ITC). The third technique has been used because it provides the thermodynamic scenario of the competitive binding of two ligands to DNA. We have used netropsin (Net), a bicationic oligopeptide antibiotic [17], as the competing ligand to bind calf thymus DNA (ct DNA) when it is added to an equilibrium mixture of EtBr and DNA. It binds preferentially to A–T rich regions of minor groove of B-DNA [18, 19]. It binds via the minor groove. Therefore, it has the potential to displace EtBr from its binding site in the minor groove of DNA. Another (G–C) base specific intercalator, sanguinarine (SGR), has been used in the isothermal titration calorimetry studies. We have also examined the competitive binding of the intercalator, EtBr when it is added to

an equilibrium mixture of Net and ct DNA or SGR and ct DNA. The results from the studies are interesting from two perspectives. It proves unequivocally that the ability to replace EtBr as monitored by fluorescence should not be interpreted as the intercalation potential of a small DNA binding ligand. The isothermal titration calorimetry results raise a serious doubt about the accepted interpretation of the observed decrease in fluorescence of EtBr in terms of competitive binding of the two ligands to DNA. Together the results from the study suggest that the above widely used method by means of fluorescence is not appropriate to draw a conclusion regarding the binding mode of the small molecule.

Results and Discussions

Absorbance Spectroscopy

The absorption spectra of EtBr in absence and presence of ct DNA have been shown in Fig. 1(a). There is a decrease in absorbance of EtBr with increasing concentrations of ct DNA and a gradual red shift of EtBr absorption maximum from 480 nm to 521 nm. The marked red shift is in accordance with previous literature reports [20] and is due to a decrease in polarity of the environment of EtBr in EtBr - ct DNA complex compared to free EtBr. The absorption spectra of the EtBr - ct DNA complex was also monitored in presence of Net (Fig. 1b). An increase in absorbance was observed in presence of increasing concentrations of Net with gradual blue shift of the peak. The absorbance maximum of ct DNA bound EtBr shifted to 489 nm from 521 nm in presence of Net. The results of absorbance measurements indicate that A-T specific minor groove binder Net can replace an intercalator EtBr from the A-T sites of the minor groove of DNA when added to an equilibrium mixture of EtBr - ct DNA. However, EtBr remains bound to G-C rich sites of ct DNA. Therefore the absorption maximum of EtBr does not regain its original position in presence of Net. A net red shift of 9 nm exists. This might be due to ternary complex formation with Net bound to A-T rich sites and EtBr bound to G-C rich sites in ct DNA when Net is gradually added to an equilibrium mixture of EtBr- ct DNA.

Fluorescence Spectroscopy

Fluorescence emission spectra of EtBr in presence of ct DNA is shown in Fig. 2a. Enhancement in fluorescence emission intensity and red shift around 600 nm is in accordance with previous reports [21, 22]. The emission spectra of EtBr bound ct DNA in presence of Net is shown in Fig. 2b. Successive addition of Net to EtBr - ct DNA complex resulted in progressive quenching of fluorescence at 603 nm. Non-linear curve fitting analysis of the binding isotherm obtained for EtBr - ct DNA interaction is shown in Fig. 2c. Apparent dissociation constant of EtBr - ct DNA interaction was found to be 11.15±0.09 μM. The apparent dissociation constant of Net - ct DNA interaction obtained by means of fluorescence titration of Net with ct DNA was found to be 5.99±0.9 μM (data not shown). The equilibrium dissociation constant (K_i) of Net - ct DNA interaction in the presence of EtBr was determined from the fitted curve shown in Fig. 2d from the fluorescence spectra shown in Fig. 2b. K_i was found to be 41.87 μM. The value is higher than that obtained from direct titration of Net with ct DNA. The shape of the dependence throws light upon the mechanism of ligand interactions at equilibrium. The steepness of the competitive binding curve may be quantified with a slope factor, often termed Hill slope [23]. A one-site competitive binding curve that obeys the law of mass action has a slope of −1.0. If the curve is shallow the slope factor is a negative fraction. The slope factor is negative because the curve goes downhill. In case of strong competitor the curve is sigmoidal and steep. If there is a ternary complex formation and more than one type of binding sites is present, the curve is shallow and the slope deviates significantly from 1.

By means of fluorescence spectroscopy we have shown that Net, a well-established minor groove binder replaces EtBr

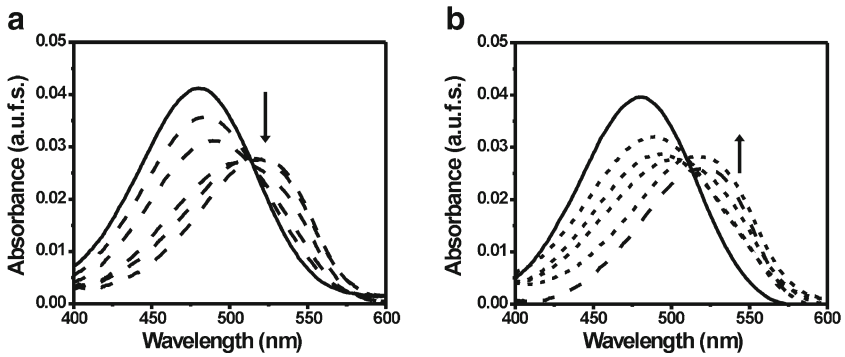

Fig. 1 **a** Absorbance spectra of EtBr (7 μM) in absence (*solid line*) and presence (*dashed line*) of ct DNA (7, 14, 28, 42, 120 μM). Panel **b** represents absorption spectra of free EtBr (7 μM) (*solid line*), equilibrium mixture of EtBr (7 μM) - ct DNA (100 μM) (*dashed line*) and EtBr- ct DNA complex in presence of different concentrations of Net (5, 35, 131, 300 μM) (*short dashed line*)

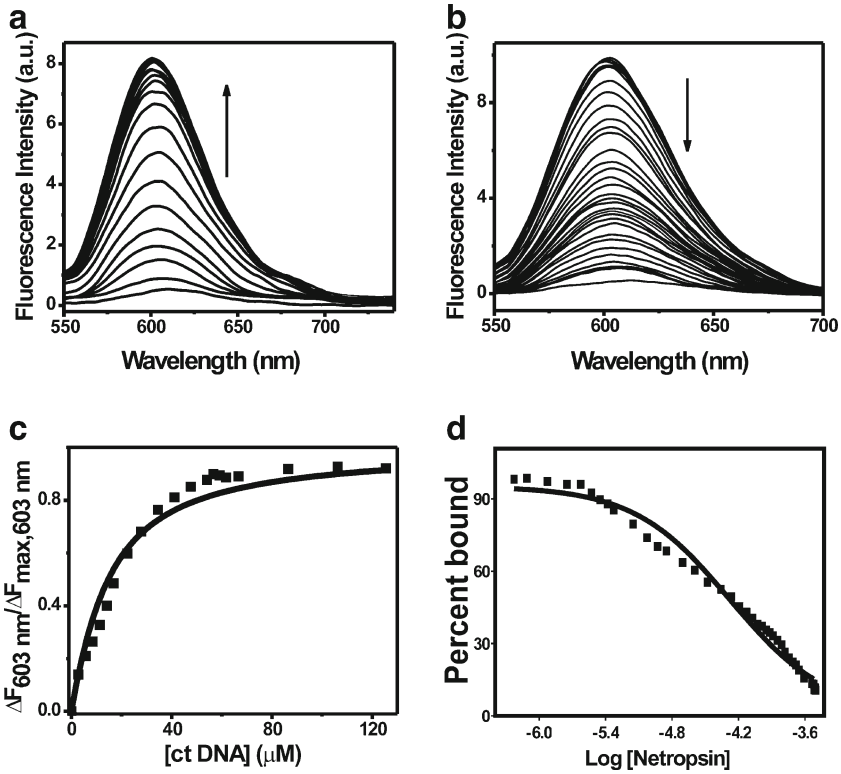

Fig. 2 Fluorescence emission spectra (550–750 nm) for the titration of **a** EtBr with ct DNA, **b** Net with (EtBr – ct DNA). Panel **c** depicts nonlinear curve fitting analysis to determine the apparent dissociation constant (K_d) for EtBr – ct DNA interaction. Panel **d** represents nonlinear regression least square curve fitting analysis using competitive binding model (one site) to determine equilibrium dissociation constant (K_i) of Net – ct DNA binding in the presence of EtBr

from the A-T rich sites of the minor groove of DNA from an equilibrium mixture of EtBr - ct DNA thereby contradicting the notion that groove binders cannot replace the intercalator EtBr from EtBr - DNA complex [12, 13].

Isothermal Titration Calorimetry

To probe the thermodynamic scenario of the interactions we took recourse to ITC. We also examined the competitive binding potential of an intercalator, SGR, to an equilibrium mixture of EtBr and ct DNA. Fluorescence spectroscopy could not be employed for the overlapping excitation spectra of EtBr and SGR. Therefore, label free technique such as ITC was employed.

Energetics of Association of the Ligands, EtBr, Net and SGR, with ct DNA

The titration curves for the binding of EtBr, Net and SGR to ct DNA are shown in Fig. 3(a–c). The thermodynamic parameters estimated from ITC using single site binding model are summarized in Table 1. The results are consistent with previous reports [24, 25]. All interactions with ct DNA were accompanied by a comparable free energy (−7.1 to −7.4 kcal/mol). Enthalpy changes for the binding of EtBr and Net to ct DNA were more or less similar (−9.3 to −9.9 kcal/mol) with negative entropy changes indicating the binding interactions being predominantly enthalpy driven whereas SGR binding to ct DNA was characterized by both favorable enthalpy and entropy contributions.

Competitive Binding Studies

Competitive binding studies of the following ligands to DNA were done for the following systems: Net, EtBr and Net, SGR. The thermogram and the binding isotherm for each system are shown in Fig. 4(a–b). The thermodynamic parameters of competitive binding studies are summarized in Table 1. The binding affinity of Net decreased in presence of EtBr whereas it remained unaltered in presence of SGR. EtBr binds to A–T and G–C sites with comparable affinity [26] whereas Net is A–T specific and SGR is G–C specific. Therefore Net can easily access the vacant A–T sites of ct DNA in the SGR – ct DNA equilibrium mixture as a result of which its affinity for ct DNA remained unaltered even in presence of SGR. The decrease in binding affinity of Net in presence of EtBr might be attributed to the displacement of EtBr from AT sites of ct DNA. Free energy change for the interaction of Net with EtBr – ct DNA equilibrium mixture was less favorable than that for Net – ct DNA interaction. However ΔG for the interaction of Net with SGR – ct DNA equilibrium mixture was similar to that for Net – ct DNA interaction. Decrease in the site size, number of DNA bases bound per ligand molecule, and magnitude of entropy was found to be associated with competitive titrations.

Calorimetric Demonstration of Ternary Complex Formation

Ternary complex formation characterizes the following two systems: EtBr – ct DNA – Net and EtBr – ct DNA – SGR.

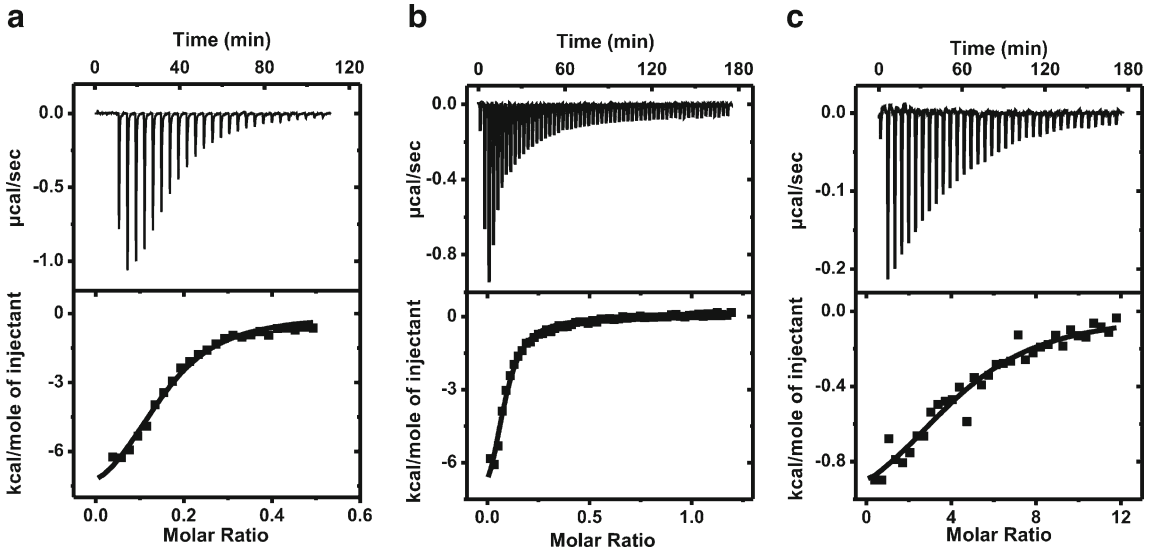

Fig. 3 Representative ITC profiles for the binding of **a** EtBr, **b** Net and **c** SGR with ct DNA at 298 K. Data were fitted using one site model

The thermograms resulting from the addition of EtBr to an equilibrium mixture of i) Net and ct DNA, and ii) SGR and ct DNA, respectively are shown in Fig. 4(c–d). Both of them could be fitted using single site binding model. The resulting thermodynamic parameters are summarized in Table 1. The binding affinity of EtBr for ct DNA decreased in presence of Net and SGR. Free energy change (ΔG) for ternary complex formation was found to be less than that for the binding of EtBr with ct DNA in absence of a second ligand.

To summarize, DNA binding agents, which replace EtBr from EtBr – ct DNA equilibrium mixture and quench the fluorescence intensity of EtBr – ct DNA complex, bind to DNA via minor groove. They should not be characterized as intercalators as had been claimed in earlier reports [1–11]. In fact, ITC experiments provide a better picture than that obtained from simple fluorescence titration. Results from ITC experiments indicate that EtBr, an intercalator, can bind to DNA via the formation of a ternary complex in the presence of (A–T) specific groove binding ligand, Net, or (G–C) specific intercalator, SGR. This is due to the ability of EtBr to bind both (A–T) and (G–C) base sequences. On the other hand, the other set of ITC experiments show that Net could replace the intercalators, EtBr/SGR, from ct DNA, thereby supporting the observation from fluorescence experiments. However, both fluorescence and ITC experiments indicate a substantial decrease in binding free energy (and binding constant) for Net – ct DNA association in presence of EtBr. In contrast, the replacement of SGR by Net is not accompanied with decrease in binding free energy (and binding constant) for Net – ct DNA complex. It may be concluded from these results that displacement of EtBr (or any other intercalator) from DNA by a ligand does not provide any information regarding the binding mode of the small molecule to DNA. It only suggests that the molecule can access the DNA via minor groove. Then, it can either intercalate into DNA bases or simply stay in the minor groove. Furthermore, ITC experiments indicate that the fluorescence change might be also due to formation of ternary complex and not displacement of one ligand by another. The results of absorbance experiments also corroborate the above findings. In conclusion, it may be noted that simple demonstration of the ability of a ligand to cause fluorescence decrease upon addition to an equilibrium mixture

Table 1 Thermodynamic parameters obtained from ITC experiments

System	N (drugs/base)	K_d (μM)	ΔH (kcal/mol)	TΔS (kcal/mol)	ΔG (kcal/mol)
EtBr + ct DNA[a]	0.15±0.01	6.54	−9.32±0.60	−2.20	−7.12
SGR + ct DNA[a]	0.22±0.00	3.95	−5.46±0.28	1.95	−7.41
EtBr + (Net-ct DNA)[a]	0.19±0.02	28.57	−6.59±0.89	−0.35	−6.24
EtBr + (SGR-ct DNA)[a]	0.26±0.02	63.29	−12.68±1.32	−6.92	−5.76
Net + ct DNA[a]	0.08±0.00	5.99	−9.93±0.72	−2.76	−7.17
Net + (EtBr-ct DNA)[b]	0.45±0.02	36.36	−11.20±0.53	−5.11	−6.09
Net + (SGR-ct DNA)[b]	0.39±0.05	5.29	−11.83±0.91	−4.59	−7.24

Data were fitted using [a] One set of sites [b] Competitive binding model

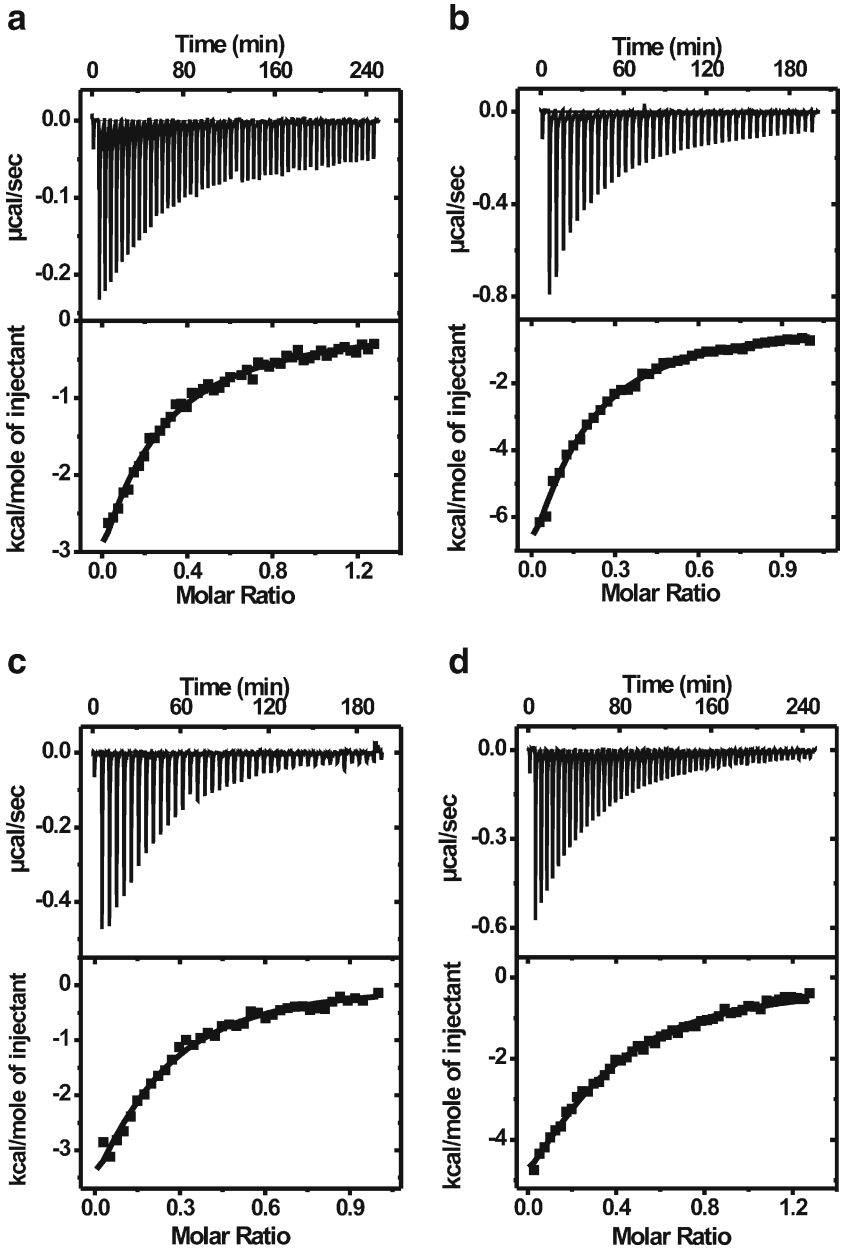

Fig. 4 Representative ITC profiles of **a** Net with EtBr – ct DNA complex, **b** Net with SGR – ct DNA complex; Data were fitted using competitive binding model **c** EtBr with Net – ct DNA complex and **d** EtBr with SGR – ct DNA complex at 298 K. Data were fitted using one site model

of EtBr and DNA is not sufficient to comment upon its mode of interaction with DNA. In fact, our results from the parallel ITC studies raise serious concern about the validity of this approach to judge the mode of interaction of a ligand with DNA.

Materials and Methods

Materials

Calf thymus DNA, ethidium bromide, netropsin, sanguinarine, sodium chloride, trizma base were purchased from Sigma Chemical Corporation, USA. Calf thymus DNA was made free from protein impurities by means of phenol-chloroform extraction method. All buffers were prepared in MilliQ water from Millipore Water System, Millipore, USA and filtered through 0.1 μm syringe filters from Millipore, USA prior to use. All experiments were performed in 5 mM Tris-HCl pH-7.4 plus 15 mM sodium chloride buffer at 298 K.

Methods

Absorbance Measurements

The absorbance measurements were carried out in Cecil 7500 spectrophotometer at 298 K. Stock concentrations of calf thymus DNA, ethidium bromide, netropsin and

sanguinarine were determined using molar extinction coefficient values of $6.6\times10^{3}\,M^{-1}cm^{-1}$ at 260 nm for calf thymus DNA [27], 5,600 $M^{-1}cm^{-1}$ at 480 nm for ethidium bromide [28], 21,500 $M^{-1}cm^{-1}$ at 296 nm for netropsin [17] and 30,700 $M^{-1}cm^{-1}$ at 327 nm for sanguinarine [29]. The absorption spectra of EtBr (7 μM) in absence and presence of different concentrations of ct DNA (7 μM–120 μM) were recorded. The effect of addition of Net to the equilibrium mixture of EtBr – ct DNA ([EtBr]=7 μM, [ct DNA]= 100 μM) was also monitored. The concentration of Net was varied from 5 μM–300 μM.

Fluorescence Measurements

Fluorescence spectra were recorded in a Perkin-Elmer LS55 Luminescence Spectrometer using 1 cm path length quartz cuvette. Excitation and emission slits were fixed at 10 nm each. In case of experiments with EtBr, the excitation and emission wavelengths were 520 nm and 603 nm, respectively. The absorbance of the samples did not exceed 0.05 at the excitation wavelength. Net was added in small aliquots to the cell containing 100 μM ct DNA complexed with 7 μM EtBr. The curve fitting analysis was performed using GraphPad Prism software (GraphPad Software Inc., San Diego, CA) by nonlinear regression least square curve fitting analysis using competitive binding model (one site) [30–32] which employs Eqs. (1) and (2) to determine equilibrium dissociation constant (K_i) of Net – ct DNA binding in the presence of EtBr.

$$Y = Nonspecific + \frac{(Total - Nonspecific)}{1 + 10^{\log[D] - \log(C_{50})}} \tag{1}$$

$$K_i = \frac{C_{50}}{1 + \frac{[EtBr]}{K_d}} \tag{2}$$

where, Y is the normalized emission intensity value measured in the presence of various concentrations of Net, log[D] is the logarithm of the concentration of competitor (Net) plotted on the X-axis. 'Nonspecific' and 'Total' are the normalized emission intensity values in the presence of a saturating concentration of D and in the absence of the competitor; C_{50} is the concentration of Net that reduces the fluorescence intensity of EtBr bound ct DNA by 50 %. Equilibrium dissociation constant K_d for EtBr – ct DNA interaction was determined from an independent experiment under similar conditions.

Isothermal Titration Calorimetry (ITC)

ITC experiments were performed in a VP-ITC MicroCalorimeter (MicroCal Inc., USA). Samples were extensively degassed prior to titration. Stirring speed was maintained at 307 rpm for all experiments. We have used two approaches to study the interaction of ct DNA with small molecules. 1) Single site binding model was employed to analyze the association of the small ligands to ct DNA as given below:

$$A + M \rightleftharpoons AM$$
$$K_A = \frac{[AM]}{[A][M]}$$

where, M is the macromolecule (ct DNA) and A represents the small molecule (such as EtBr/ Net/ SGR). K_A is the equilibrium association constant of the small molecule with ct DNA. Single site binding model was also employed for the analysis of the following equilibrium when one ligand is added to the equilibrium mixture of DNA and the second ligand as given below:

$$\begin{array}{ccc} M + A & \rightleftharpoons & AM \\ + & & + \\ B & & B \\ \uparrow\downarrow & & \uparrow\downarrow \\ BM + A & \rightleftharpoons & AMB \end{array}$$

The following five systems were analyzed using one site binding model:

(a) Association of EtBr with ct DNA: 150 μM ct DNA was loaded into calorimetric cell and titrated against EtBr (single injection of 1 μl followed by 25 injections of 4 μl each from a stock solution of 1 mM).
(b) Association of Net with ct DNA: 150 μM ct DNA was loaded into calorimetric cell and titrated against Net (single injection of 1 μl followed by 56 injections of 5 μl each from a stock solution of 800 μM).

Heat changes corresponding to dilutions of EtBr and Net in buffer served as controls for (a) and (b) respectively.

(c) Association of SGR with ct DNA: 11 μM SGR was loaded into calorimetric cell and titrated with ct DNA (single injection of 1 μl followed by 34 injections of 5 μl each from a stock solution of 1 mM). Dilution of 1 mM ct DNA in buffer served as control.
(d) Formation of ternary complex, EtBr – ct DNA – Net: 150 μM ct DNA pre-incubated with 70 μM of Net and titrated against EtBr (single injection of 1 μl followed by 39 injections of 5 μl each from a stock solution of 1 mM).
(e) Formation of ternary complex, EtBr – ct DNA – SGR: 150 μM ct DNA pre-incubated with 60 μM SGR and titrated against EtBr (single injection of 1 μl followed by 44 injections of 5 μl each from a stock solution of 1 mM).

The isotherms were analyzed using one site binding model of Levenberg-Marquardt non-linear least squares

curve fitting algorithm, using pre-supplied Origin 7.0 software from Origin Lab Corporation, USA, to obtain best-fit values. Equilibrium association constant (K_A), enthalpy (ΔH) and entropy (ΔS) of association were obtained from each isotherm.

2) Competitive binding based upon the following equilibria was analyzed as follows:

$$A + M \rightleftharpoons AM$$
$$K_A = \frac{[AM]}{[A][M]}$$
$$B + M \rightleftharpoons BM$$
$$K_B = \frac{[BM]}{[B][M]}$$

where, A is the strongly binding ligand under investigation and B is the competing ligand. K_A and K_B are the apparent association constants of ligand A and ligand B to the macromolecule M respectively [33]. Total concentration of the competing ligand, $[B]_{total}$ in the cell was maintained such that

$$\frac{K_A}{[B]_{total}K_B} = 10^5 - 10^8 M^{-1}$$

The following systems were analyzed using competitive binding model:

(i) Competitive binding of Net and EtBr with ct DNA: The cell was loaded with 150 μM ct DNA pre-incubated with 60 μM of EtBr and titrated against Net (single injection of 1 μl followed by 49 injections of 5 μl each from a stock solution of 1 mM).

(ii) Competitive binding of Net and SGR with ct DNA: The cell was loaded with 150 μM ct DNA pre-incubated with 60 μM SGR and titrated against Net (single injection of 1 μl followed by 39 injections of 5 μl each from a stock solution of 1 mM).

The resulting isotherms were fitted using competitive binding model supplied by the in-built Origin 7.0 software. Binding stoichiometry (N), equilibrium association constant (K_A), enthalpy of association (ΔH) and the concentration of the competing ligand were supplied prior to fitting using competitive binding model to obtain thermodynamic parameters for the association of one ligand to the macromolecule in the presence of the competing ligand.

The Gibbs free energy was calculated using the Eq. (3)

$$\Delta G = -RT \ln K \quad (3)$$

and the entropy factor was calculated using Eq. (4).

$$\Delta H = \Delta G + T\Delta S \quad (4)$$

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