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Interaction of indomethacin with calf thymus DNA: a multi-spectroscopic, thermodynamic and molecular modelling approach†‡

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Indomethacin belongs to the acetic acid derivative class of non-steroidal anti-inflammatory drugs with diverse pharmacological and biological activities. Understanding the mechanism of interaction of drugs with possible target and off-target biomolecules can prove useful in the development of a rational drug design system. In this paper, we have attempted to ascertain the mode of binding of indomethacin with calf thymus DNA (Ct-DNA) through various biophysical techniques and *in silico* molecular docking. Analysis of the UV-visible absorbance spectra and fluorescence emission profile of indomethacin upon addition of Ct-DNA indicates the formation of a drug–DNA complex. UV-visible absorbance and steady state fluorescence experiments revealed a binding constant on the order of 10^3 L mol^{-1} , which is consistent with those of well-known groove binders. Competitive displacement studies with ethidium bromide, acridine orange and Hoechst 33258 further suggested that indomethacin binds to the minor groove of the Ct-DNA. The above observations were further confirmed by KI induced quenching experiments, DNA melting studies, CD spectral analysis and viscosity measurements. The thermodynamic parameters like spontaneous free energy ($\Delta G < 0$) and large favourable enthalpy ($\Delta H < 0$) obtained from isothermal calorimetry indicated the involvement of hydrogen bonding and van der Waals forces in the binding process. Molecular docking further corroborated the experimental results.

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1 Introduction

Deoxyribonucleic acid (DNA) is an essential genetic material, which plays a key role in cell proliferation, synthesis of proteins and transcription of genetic information in living cells of an organism. Ever since the discovery of the structure of DNA, it has been the prime target for various therapeutically important small molecules that belong to different classes from anticancer drugs to antibiotics.¹ There is growing interest in the binding studies of small molecules with DNA and understanding the drug–DNA interactions. The mode of binding and interference of small molecules with DNA replication and RNA transcription provides greater insight into the drug controlled expression of genes.^{2–4} Such studies are useful in developing sensitive chemical probes of nucleic acid struc-

tures and designing new and promising drugs for clinical use. Drug–DNA binding is generally stabilized through a series of weak interactions such as π -stacking interactions of aromatic heterocyclic groups between base pairs (intercalation), hydrogen bonding and van der Waals interactions of functional groups bound to major or minor grooves without causing any major distortion of the DNA helix.^{5,6} Electrostatic interaction is also a type of non-covalent interaction which takes place out of the groove during drug–DNA binding.⁷ The well-studied three-dimensional structure of DNA, the predictability of their accessible chemical and functional groups and the availability of the genome sequence make DNA an attractive drug target to study. Interestingly, the number of known drugs targeting DNA is still very limited compared to the drugs targeting proteins and a detailed study is needed to explore this field.⁸

Small molecules that have already been approved for a particular treatment may have uncharacterized potential for other targets as well. This has led to the re-screening of these molecules in the past few years. Understanding the nature of interaction of these drugs with off target biomolecules like DNA and protein can characterize the potential of these drugs for other targets as well as to minimize the side effects of these drugs.⁹

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Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most widely used pharmaceutical drugs. They exhibit favourable anti-inflammatory, analgesic and antipyretic properties and are broadly used for the relief of pain and inflammation.¹⁰ Indomethacin (Fig. 1B inset) is an NSAID that belongs to the group called acetic acid derivatives. It is commonly used as a prescription medication to reduce pain, fever, swelling, and stiffness. Indomethacin acts by inhibiting the production of prostaglandins, which are known to cause these symptoms. It has also been widely used for the treatment of rheumatoid arthritis, gout and collagen disease.¹¹ An earlier study reports the interaction of copper complexes of indomethacin with Ct-DNA.¹² Although a lot has been studied about the pharmacological properties of indomethacin, its mode of binding with DNA has still not been elucidated. This study reports the molecular aspects and energetics of indomethacin complexation to DNA. The interaction study of indomethacin and DNA is much needed to reveal how this compound may be further modified to enhance its biological properties.

The present study demonstrated the mode of binding of indomethacin with DNA by using various spectroscopic techniques such as UV-visible absorbance and fluorescence spectroscopy. In order to gain deeper insight into the mode of interaction between indomethacin and DNA, viscosity measurements, circular dichroism (CD) and melting studies were performed. Isothermal titration calorimetry (ITC) was also employed for the measurement of stoichiometry, binding affinity and various thermodynamic parameters of indomethacin–DNA interaction. *In silico* molecular docking further supports the groove binding mode of interaction between indomethacin and DNA.

2 Experimental

2.1 Materials

Indomethacin, calf thymus DNA (Ct-DNA), acridine orange (AO) and Hoechst 33258 were purchased from Sigma-Aldrich, USA. Ethidium bromide (EB) was purchased from HiMedia, India. All reaction mixtures were prepared in 10 mM Tris–HCl buffer (pH 7.2) unless otherwise mentioned. All the other chemicals and solvents were of reagent grade and used without any further purification.

2.2 Sample preparation

A stock solution of indomethacin (10 mM) was prepared in 5% DMSO. Ct-DNA was dissolved in 10 mM Tris–HCl buffer (pH 7.2) at room temperature with occasional stirring to ensure the formation of a homogeneous solution. To check the purity of Ct-DNA solution, the ratio of UV absorbance at 260 to that at 280 nm was determined and was found to be more than 1.8, indicating that Ct-DNA was sufficiently free from protein and no further purification was needed. The concentrations of the DNA solutions were determined by using the average extinction coefficient value of $6600 \text{ M}^{-1} \text{ cm}^{-1}$ of a single nucleotide at 260 nm.

2.3 UV-visible spectroscopy

The UV spectra were recorded with a Shimadzu UV-1800 dual beam UV-visible spectrophotometer (Japan) using a $1 \text{ cm} \times 1 \text{ cm}$ quartz cuvette. The UV-visible spectra of indomethacin and the indomethacin–Ct-DNA complex were recorded in the wavelength range of 240–350 nm. The experiment was carried out in the presence of a fixed concentration of indomethacin

Fig. 1 (A) Interaction of indomethacin with Ct-DNA using UV-visible spectroscopy. UV-visible absorption spectra of indomethacin (50 μM) in the presence of increasing concentrations of Ct-DNA (0–25 μM) in 10 mM Tris–HCl buffer (pH 7.2). The spectra were recorded in the range of 220–350 nm. (B) Double reciprocal plot of $1/(A_0 - A)$ versus $1/C_{\text{DNA}}$. It was found to be linear at 298 K and the value of the constant K was found to be $9.6 \times 10^3 \text{ L mol}^{-1}$. The inset shows the structure of indomethacin.

(50 μM) and titrated with varying concentrations of DNA (0–25 μM). The final volume of the reaction mixture was made to 3 ml by adding 10 mM Tris–HCl buffer (pH 7.2). The same concentrations of DNA solutions without indomethacin were used as the blank to observe the UV-spectra specific to the indomethacin–DNA complex.

2.4 Steady state fluorescence

The fluorescence emission spectra of indomethacin were recorded on a Shimadzu RF-5301PC spectrofluorophotometer (Japan) equipped with a xenon flash lamp using 1.0 cm quartz cells. The spectra of indomethacin were recorded in the range of 300 to 520 nm upon excitation at 281 nm after setting the widths of both the excitation and the emission slits at 5 nm. A change in the fluorescence intensity was observed by titrating a fixed amount of indomethacin (50 μM) with varying concentrations of Ct-DNA from 0 to 40 μM .¹³ The final volume of the reaction mixture was made to 3 ml by adding 10 mM Tris–HCl buffer (pH 7.2). In order to minimize inner filter effects, corrections to the fluorescence spectra were made using the correction factors described earlier.¹⁴ First, the background solvent emission spectrum was corrected using the following equation:

$$F = (1 - 10^{-A_{\text{exc}}})2.303A_{\text{exc}}$$

where A_{exc} is the absorbance of the sample in the same solvent at the excitation wavelength. This corrected background spectrum was then subtracted from the sample emission spectrum to obtain the spectrum $F_0(\bar{\nu})$. Then, the following correction factor was used to correct for the primary and secondary inner filter effects:

$$F_{\text{corr}}(\bar{\nu}) = F_0(\bar{\nu}) \times 10^{(A_{\text{ex}}l_{\text{ex}} + A_{\text{em}}l_{\text{em}})}$$

where $F_{\text{corr}}(\bar{\nu})$ is the corrected fluorescence intensity, A_{ex} is the absorbance at the excitation wavelength, l_{ex} is the penetration depth of the light into the sample, A_{em} is the absorbance over the emission wavelength and l_{em} is the emission path length. According to a report described earlier,¹⁴ $l_{\text{ex}} = 0.44$ cm and $l_{\text{em}} = 0.05$ cm were used for spectrofluorometric experiments.

2.5 Competitive displacement assays

EB displacement assay was performed as reported earlier.¹³ The assay was carried out by adding EB (5 μM) to Ct-DNA (20 μM) solution and this mixture was titrated with varying concentrations of indomethacin from 0 to 80 μM . The EB–Ct-DNA complex was excited at 476 nm and its emission spectra were recorded between 520 nm and 670 nm.

In another set of similar experiments, AO displacement assay was performed. The AO–Ct-DNA complex containing 5 μM AO and 50 μM Ct-DNA was excited at 480 nm and its fluorescence emission spectra were recorded between 500 and

580 nm by titrating the AO–Ct-DNA complex with varying concentrations of indomethacin from 0 to 80 μM .

Hoechst 33258 displacement assay was also performed under similar conditions to EB and AO displacement assays. The Hoechst–Ct-DNA complex containing 5 μM Hoechst and 50 μM Ct-DNA was excited at 343 nm and its fluorescence emission spectra were recorded between 375 and 600 nm by titrating with increasing concentrations of indomethacin from 0 to 70 μM . The final volume of the reaction mixture in all of the above experiments was made to 3 ml by adding 10 mM Tris–HCl buffer (pH 7.2).

2.6 Potassium iodide (KI) quenching method

Iodide quenching experiment was performed in two sets in the presence and absence of Ct-DNA. In one set, a fixed concentration of indomethacin was taken (50 μM) and titration was performed by varying the concentration of KI (0–18 mM). In another set, a similar experiment was performed in the presence of 50 μM of Ct-DNA. The emission spectra in the range of 300–500 nm were recorded upon excitation at 281 nm.

2.7 Effects of ionic strength

The effects of ionic strength on the interaction between indomethacin and Ct-DNA were studied as reported earlier.¹³ The assay was carried out by varying the concentration of NaCl between 0 and 45 μM in a total volume of 3 ml containing 50 μM indomethacin, 50 μM Ct-DNA and 10 mM Tris–HCl (pH 7.2). Excitation was performed at 281 nm and the emission spectra were recorded between 300 and 500 nm.

2.8 DNA melting studies

UV-visible spectroscopy is a simple method for determining the melting temperature of DNA double helices. A DNA melting study was performed in the absence and presence of indomethacin by monitoring the absorbance intensity of Ct-DNA at 260 nm over a wide range of temperatures from 30–100 °C. The samples contained Ct-DNA (50 μM) alone or 50 μM indomethacin and Ct-DNA each in buffer and the sample volume was adjusted to 3 ml using 10 mM Tris–HCl buffer (pH 7.2). The temperature of the sample was monitored with the help of a thermostat attached to the sample holder. The absorbance was recorded at 260 nm and the result was plotted as a function of temperature. The melting temperature (T_m) of DNA was determined as the transition midpoint of the melting curve.

2.9 Viscosity measurement

To further elucidate the binding mode of indomethacin, viscosity measurements were performed using an Ubbelohde viscometer, which was immersed in a thermostat water bath maintained at 25 ± 0.1 °C. Samples were prepared by adding an appropriate amount of indomethacin to Ct-DNA so as to give [indomethacin]/[Ct-DNA] ratios in the range of 0.2–1.

After a thermal equilibrium time of 15 min, the flow times of the samples were repeatedly measured using a digital stopwatch with an accuracy of ± 0.2 s. The data were presented as $(\eta/\eta_0)^{1/3}$ versus the ratio of indomethacin/DNA concentrations, where η is the viscosity of DNA in the presence of indomethacin and η_0 is the viscosity of DNA alone.

2.10 Circular dichroism studies

The CD spectra of Ct-DNA (50 μ M) alone and the indomethacin-Ct-DNA complex were recorded using an Applied Photophysics CD spectrophotometer (model CIRASCAN, U.K.) equipped with a Peltier temperature controller to keep the temperature of the sample constant at 25 $^{\circ}$ C. All the CD spectra were recorded in the far-UV range (230–300 nm) with a scan speed of 200 nm per min and with a spectral band width of 10 nm. The molar ratios of DNA concentration to indomethacin concentration were 1:0, 1:1 and 1:2. Each spectrum was the average of four scans. The background spectrum of the buffer solution (10 mM Tris-HCl, pH 7.2) was subtracted from the spectra of DNA and the indomethacin-DNA complex.

2.11 Isothermal titration calorimetric measurements (ITC)

The thermodynamics of DNA and indomethacin interaction was measured using a model VP-ITC (Microcal Inc., Northampton, MA) at 25 $^{\circ}$ C. Indomethacin, DNA and a reference buffer were degassed thoroughly in a Thermovac for 15 min prior to their loading. Indomethacin (750 μ M) was introduced into the sample cell by means of syringes and the amount of each injection was 10 μ L. Calf thymus DNA (50 μ M) was maintained in the sample cell in 20 mM phosphate buffer (pH 7.2) and the reference cell contained only phosphate buffer. 29 successive injections with an initial delay of 60 s were carried out. The time duration for each injection was set to 20 s and the time interval between two consecutive injections was kept at 180 s. The stirring speed and the reference power were fixed at 307 rpm and 16 μ cal s^{-1} , respectively. The calorimetric data were analyzed by using MicroCal Origin 7.0 software. The other thermodynamic parameters were calculated using the formula:

$$\Delta G = -RT \ln Kb = \Delta H - T\Delta S$$

where T is the absolute temperature (298 K) and $R = 8.315$ J mol^{-1} K^{-1} . Isothermal titration calorimetry allows the measurement of the magnitude of the enthalpy (ΔH), entropy (ΔS) and binding affinity.

2.12 Molecular docking

HEX 8.0.0, a molecular graphics program, was used to study the indomethacin-DNA interaction. It is an interactive molecular graphics program used for calculating and displaying feasible docking modes of DNA.¹⁵ The structure of the B-DNA dodecamer d(CGCGAATTCGCG)₂ (PDB ID: 1BNA) was

downloaded from the Protein Data Bank (<http://www.rcsb.org/pdb>) and the Mol file of indomethacin was obtained from <http://pubchem.ncbi.nlm.nih.gov/>. The Mol file was further converted into PDB format using Avogadro's 1.01 software. The HEX 8.0 program performs docking using spherical polar Fourier correlations. It requires the ligand and the receptor as input in PDB format. The parameters that were used for docking include: FFT mode – 3D, correlation type – shape only, grid dimension – 0.6, ligand range – 180, receptor range – 180, distance range – 40, and twist range – 360. Visualization of the docked poses was carried out by using the PyMol software (DeLano Scientific, San Carlos, CA, USA).

3 Results and discussion

3.1 UV-visible absorption spectroscopy

UV-visible absorption spectroscopy is the most widely used and effective method to explore the structural changes of bio macromolecules in the presence of small molecules. Generally, when a small molecule interacts with DNA and forms a complex, changes in the magnitude of absorbance and in the position of the peak occurs.¹⁶ The shift in the peak position and the magnitude of change are correlated with the strength of the interaction.^{17,18} In order to investigate the interaction between indomethacin and Ct-DNA, the UV-visible absorption spectra of indomethacin in the presence of increasing concentrations of Ct-DNA were measured (Fig. 1A). The absorption spectra showed large hyperchromic shifts (*i.e.*, increase in band intensity), with a peak position at 281 nm upon addition of increasing concentrations of Ct-DNA. Hyperchromism is a spectral feature depicting non-covalent interactions between Ct-DNA and small molecules.^{6,19} This indicated the formation of adducts between Ct-DNA and indomethacin.

The absorption relationship between indomethacin and Ct-DNA is expressed by the double reciprocal equation:²⁰

$$1/A_0 - A = 1/A_0 + 1/(K \times A_0 \times C_{DNA}) \quad (1)$$

where A_0 and A are the absorbances of indomethacin in the absence and presence of Ct-DNA, respectively, K is the binding constant between indomethacin and Ct-DNA and C_{DNA} is the concentration of Ct-DNA. The dependence of $1/(A_0 - A)$ on the reciprocal value of the Ct-DNA concentration ($1/C_{DNA}$) is linear, with a slope equal to the value of $1/KA_0$. The value of $1/A_0$ is fixed on the ordinate. The constant K is the measure of an ordinate $1/A_0$ and a slope $1/KA_0$. From Fig. 1B, it can be seen that the double reciprocal plot of $1/(A_0 - A)$ versus $1/C_{DNA}$ was linear at 298 K. The value of K was obtained from eqn (1) and was found to be 9.6×10^3 L mol^{-1} . It has been reported that the binding constant of well-known intercalators are usually on the order of 10^4 – 10^6 L mol^{-1} . For example, the K value of EB²¹ is equal to 4.3×10^5 L mol^{-1} . Hence, it can be concluded that the binding mode of indomethacin with DNA might be a non-intercalation binding mode.

3.2 Steady-state fluorescence

A steady state fluorescence technique was employed to study the interaction between indomethacin and DNA. The fluorescence emission spectra showed the effect of Ct-DNA on the emission spectrum of indomethacin. The fluorescence emission spectra of indomethacin were studied in all subsequent experiments, as the endogenous fluorescence properties of DNA are poor. The fluorescence intensity of a compound decreases due to a variety of molecular interactions, such as molecular rearrangements, energy transfer, excited-state reactions, and ground-state complex formation.²² The decrease in intensity due to such molecular interactions is called fluorescence quenching. Fig. 2A shows the fluorescence emission spectra of indomethacin with the emission maxima at 373 nm after excitation at 281 nm. Upon subsequent addition of Ct-DNA, there is a gradual quenching of the fluorescence

intensity without any significant change in the emission maxima which is direct evidence of the interaction between indomethacin and Ct-DNA.²³ To explain the results of fluorescence measurements quantitatively, the ratio of the peak fluorescence intensity in the presence and in the absence of Ct-DNA (F/F_0) has been plotted as a function of DNA concentration for indomethacin. The inset in Fig. 2A shows that with subsequent addition of DNA, the fluorescence intensity decreases.

Further, the Stern-Volmer quenching constant (K_{sv}) was used to evaluate the fluorescence quenching efficiency. Quantitative estimation in terms of the fluorescence change was performed using the Stern-Volmer equation.

$$F_0/F = 1 + K_{sv}[Q] \quad (2)$$

Fig. 2 (A) Interaction of indomethacin with Ct-DNA using fluorescence spectroscopy. Fluorescence emission spectra of indomethacin (50 μM) in the presence of increasing concentrations of Ct-DNA (0–40 μM). The excitation wavelength was 281 nm and the emission maxima were obtained at 373 nm. The spectra were recorded in the range of 300–520 nm. The inset shows the variation of fluorescence intensity with an increase in DNA concentration. The arrow shows the decrease in intensity upon increasing the Ct-DNA concentration. (B) Stern-Volmer plot for the interaction of indomethacin with Ct-DNA. The fluorescence intensity quenching was found to be directly proportional to the DNA concentration. A binding constant of $1.1 \times 10^3 \text{ L mol}^{-1}$ was obtained from the slope. (C) Modified Stern-Volmer plot of $\log(F_0 - F)/F$ vs. $\log[\text{Ct-DNA}]$ at 298 K.

where F_0 and F are the fluorescence intensities of indomethacin in the absence and presence of a quencher *i.e.*, Ct-DNA (Q), respectively. $[Q]$ is the concentration of the quencher and K_{sv} is the quenching constant. K_{sv} is considered to be a measure of the efficiency of fluorescence quenching by DNA and was calculated from the slope of Fig. 2B according to eqn (2). It was found to be $1.1 \times 10^3 \text{ L mol}^{-1}$ (Table S1†), which is consistent with the K_{sv} of most groove binders.¹⁵ Thus, indomethacin is suggested to interact with Ct-DNA in a non-covalent manner, possibly through a groove binding mode.

The linear Stern–Volmer plot as obtained in Fig. 2B is suggestive of only one type of binding or quenching process, either static or dynamic quenching which can be differentiated using the following equation:²⁴

$$k_q = K_{sv}/\tau_0 \quad (3)$$

where k_q is the apparent biomolecular quenching rate constant and τ_0 is the fluorescence lifetime of the biomolecule in the absence of a quencher and is around 10^{-8} s .²⁵ The limiting diffusion rate constant of biomolecules is known to be around $2.0 \times 10^{10} \text{ L mol}^{-1} \text{ s}^{-1}$. Thus, if the value of k_q is higher than the limiting diffusion rate constant, the quenching process is static rather than dynamic. In order to determine the type of quenching process, k_q was calculated using eqn (3) and was found to be $1.1 \times 10^{11} \text{ L mol}^{-1} \text{ s}^{-1}$ (Table S1†), which is higher than the limiting diffusion rate constant, indicating the quenching process to be static rather than dynamic.²⁶

3.2.1 Binding equilibrium. For static quenching, the relationship between the fluorescence intensity and the concentration of a quencher can be described by the following equation:

$$\log[(F_0 - F)/F] = \log K + n \log[Q] \quad (4)$$

where K and n are the binding constant and the number of binding sites, respectively. From the plot of $\log(F_0 - F)/F$ vs. $\log[Q]$, the K and n values can be determined from the intercept and slope (Fig. 2C). The value of K was found to be in the range of 10^3 at 298 K (Table S1†) which is in accordance with the value of K obtained in the UV-visible absorbance experiment, further suggesting that the type of interaction might be groove binding.

3.3 Competitive displacement assay

To study the mode of drug–DNA interactions, various well known DNA binding dyes whose binding modes have been established are extensively used. In competitive displacement assays, any small molecule that replaces bound dye from the DNA helix interact with DNA in a similar fashion to the bound dye.²⁷ Thus, the change in fluorescence intensity in the dye–DNA complex on addition of small molecules gives valuable information regarding the mode of interaction which can be easily interpreted. EB is a well-known fluores-

cent probe having a planar structure which binds to DNA in an intercalative fashion.²⁸ EB shows weak fluorescence in aqueous solution but its fluorescence intensity is drastically enhanced due to intercalation within DNA base pairs.²⁹ In this assay, any molecule that binds to DNA in a similar fashion to EB will replace EB from the DNA helix in a competitive manner and result in a decrease in the fluorescence intensity of the EB–DNA complex. With continuous addition of indomethacin to the EB–Ct-DNA system, no significant decrease in the fluorescence intensity was observed, which is possibly due to non-displacement of EB from the EB–Ct-DNA complex by the drug (Fig. 3A). This suggested that indomethacin does not replace EB from the DNA helix, further suggesting that indomethacin binds to DNA in a non-intercalative manner.

To further elucidate the intercalative mode of binding of indomethacin with Ct-DNA, acridine orange (AO) displacement assay was carried out. AO, having a planar aromatic structure, is also a classical intercalating dye with fluorescence properties.³⁰ The fluorescence properties of AO–Ct-DNA is remarkably stronger compared to AO alone. However, in the presence of any other intercalating molecule, a decrease in the fluorescence intensity of the AO–DNA system will be observed due to competitive displacement of AO from the DNA helix. The emission spectra of the AO–DNA system is shown in Fig. 3B. No significant quenching in fluorescence intensity of the AO–DNA system was observed upon subsequent addition of indomethacin, indicating that indomethacin is not able to replace AO from DNA, thus confirming the non-intercalative binding of indomethacin.

In another set of experiments, Hoechst 33258, a fluorescent minor groove binding dye, was used to study competitive displacement by groove binders.³¹ Due to quenching by solvent molecules, Hoechst 33258 produces weak fluorescence in Tris–HCl buffer. However, the fluorescence intensity of Hoechst is significantly enhanced when the dye binds to DNA.³² Any drug that binds to DNA *via* a groove binding mode will replace Hoechst from the minor groove of the DNA double helix resulting in the quenching of the fluorescence intensity of the Hoechst–DNA system. On subsequent addition of indomethacin to the Hoechst–DNA system, a significant decrease in the fluorescence intensity of the Hoechst–Ct-DNA system was observed, suggesting that the Hoechst dye is replaced by indomethacin from the groove of DNA (Fig. 3C). These results show that indomethacin interacts with DNA through a groove binding mode rather than intercalation.

The quenching of EB, AO and Hoechst bound to Ct-DNA by indomethacin was calculated in terms of K_{sv} from the Stern–Volmer plot, as shown in Fig. 3D with the help of eqn (2). The K_{sv} values for Hoechst, AO and EB are found to be $3 \times 10^3 \text{ L mol}^{-1}$, $1.09 \times 10^3 \text{ L mol}^{-1}$, $0.156 \times 10^3 \text{ L mol}^{-1}$, respectively, as mentioned in Table 1. It is evident that the K_{sv} value is much higher for Hoechst as compared to those for EB and AO. This suggests that Hoechst from Ct-DNA was replaced by indomethacin to a much greater extent than EB and AO. Thus, from this assay it is concluded that indomethacin binds to Ct-DNA in the minor groove.

Fig. 3 Competitive displacement assays. (A) Fluorescence titration of the EB-Ct-DNA complex with indomethacin. The EB-Ct-DNA complex was excited at 476 nm and its emission spectra were recorded from 520–670 nm. (B) Fluorescence titration of the AO-Ct-DNA complex with indomethacin. The AO-Ct-DNA complex was excited at 480 nm and its emission spectra were recorded from 500–580 nm. (C) Fluorescence spectra of the Hoechst-Ct-DNA complex with indomethacin. The Hoechst-Ct-DNA complex was excited at 343 nm and its emission spectra were recorded from 370–600 nm. (D) Stern–Volmer plot for the quenching of fluorescence intensity of different fluorescent dye–Ct-DNA systems by successive addition of indomethacin.

3.4 Iodide quenching studies

Iodide ion quenching experiments further provide great help in deducing the binding interaction of a drug with DNA.³³ Iodide ions are well known fluorescence quenchers for small fluorescent molecules. Being negatively charged, these ions

can effectively quench the fluorescence of small molecules in an aqueous medium. However, due to the negatively charged phosphates present in the DNA backbone, the negatively charged iodide ions are repelled. Therefore, an intercalatively bound drug molecule is well protected from being quenched by the approach of any anionic quencher. The electrostatic and groove binders provide much less protection for the fluorophore as they are exposed to the external environment and are comparatively approachable for iodide ions as compared to intercalators even in the presence of DNA.³³ The relative accessibility of small molecules to anionic quencher in the presence of DNA and in free solution was studied by calculating K_{sv} using Stern–Volmer eqn (2). Fig. 4 shows the Stern–Volmer plot for fluorescence quenching of indomethacin by KI in the absence and presence of Ct-DNA, and the calculated K_{sv} values from the Stern–Volmer equation are

Table 1 Comparison of the K_{sv} values for the quenching of fluorescence intensity by displacement of different fluorescent dyes from Ct-DNA by indomethacin

Dye	K_{sv} (L mol^{-1})	R	S.D.
Hoechst	3.00×10^3	0.99995	0.0834
AO	1.09×10^3	0.99997	0.0272
EB	0.156×10^3	0.99999	0.0041

R is the correlation coefficient and S.D. is the standard deviation.

Fig. 4 KI quenching studies. Stern-Volmer plot of F_0/F vs. $[KI]$ for the fluorescence quenching of indomethacin ($50 \mu\text{M}$) by successive addition of KI (0 – 18 mM) in the absence and presence of $50 \mu\text{M}$ Ct-DNA. The quenching constants were calculated in both cases and the difference in K_{sv} values was further used to determine the binding mode of indomethacin with DNA. In the figure, the red line and black line denote the Stern-Volmer plot with DNA and without DNA, respectively.

summarized in Table 2. The slopes of the plots of (F_0/F) versus $[KI]$ yield the values of K_{sv} (Fig. 4). The K_{sv} values of free indomethacin and in the presence of DNA and iodide anion were 1.6 and 1.3 L mol^{-1} , respectively. It is clearly evident from the very little decrease in the K_{sv} value of indomethacin bound to DNA that the drug molecules have not intercalated into the base pairs of the DNA, which indicated that indomethacin could be partly protected. Thus, it can be concluded that the groove binding mode of interaction occurs between indomethacin and Ct-DNA.

3.5 Effect of ionic strength

Studying the effect of ionic strength on the interaction of small molecules with DNA is a resourceful method to differentiate the binding mode. It is apparent that both groove binding and intercalative binding are closely related to the DNA double helix, but electrostatic binding can take place from outside the helix. NaCl was used to study the role of electrostatic interactions in indomethacin–DNA binding. In the presence of double helical DNA, Na^+ partly neutralizes the negative charges of the DNA phosphate backbone

resulting in reduced electrostatic repulsion between them. The electrostatic attraction between the small molecule and the DNA surface is weakened by the addition of Na^+ . The effect of the ionic strength on the fluorescence spectra of the indomethacin–Ct-DNA system was observed *via* the addition of NaCl (Fig. 5A). On addition of NaCl, no significant change in the fluorescence spectra was observed. The relative extent of the fluorescence intensity of Ct-DNA bound indomethacin was demonstrated as a function of NaCl concentration (Fig. 5B). This suggests that there may be negligible electrostatic interactions between indomethacin and Ct-DNA.¹⁸

3.6 DNA melting studies

The double-helical structure of DNA is remarkably stable due to hydrogen bonding and base stacking interactions. With increasing temperature, the double helix dissociates into single strands due to the weakening of various binding forces.³⁴ The temperature at which half of the DNA double helix is denatured into single stranded DNA is known as the melting temperature (T_m), and is strongly related to the stability of the double-helical structure.³⁴ Interactions of small molecules with DNA are known to influence T_m . The intercalative mode of binding of small molecules can further stabilize the DNA double helical structure and increases the T_m by about 5 – $8 \text{ }^\circ\text{C}$. But non-intercalative binding such as groove binding or electrostatic binding causes less or no significant increase in T_m .³⁵ The value of T_m for Ct-DNA in the absence and presence of indomethacin was determined by monitoring the absorbance at 260 nm as a function of temperature ranging from $30 \text{ }^\circ\text{C}$ to $100 \text{ }^\circ\text{C}$. For each monitored transition, the T_m of the assay solution was determined as the transition midpoint of the melting curve (Fig. 6). Here $A_{30^\circ\text{C}}$ is the absorbance at $30 \text{ }^\circ\text{C}$ and A is the absorbance at increasing temperatures ranging from $30 \text{ }^\circ\text{C}$ to $100 \text{ }^\circ\text{C}$. Under the experimental conditions, the value of T_m for Ct-DNA alone was $66.3 \pm 1 \text{ }^\circ\text{C}$, while in the presence of indomethacin it was found to be $68.2 \pm 1 \text{ }^\circ\text{C}$. The very small change observed suggests the absence of the intercalation mode of binding. The slight increase in T_m is presumably due to the change in the conformation of DNA as a result of groove binding of indomethacin with DNA.

3.7 Viscosity measurements

To further confirm the interaction between indomethacin and DNA, viscosity measurements were carried out. This technique is regarded as the least ambiguous and most reliable tool to determine the binding modes of small molecules with DNA.³⁶ In the case of binding of classical intercalators to DNA, the length of the DNA helix is increased due to separation of base pairs resulting in increased viscosity of DNA. On the other hand, under the same conditions, small molecules that bind exclusively in the DNA grooves or interacts in an electrostatic manner typically cause negligible or a slight change in the viscosity of the DNA solution.³⁷ A viscosity plot of $(\eta/\eta_0)^{1/3}$ versus $[\text{indomethacin}]/[\text{DNA}]$ was obtained to study

Table 2 Parameters obtained in KI quenching studies in the absence and presence of Ct-DNA

Drug	Indomethacin	R	S.D.
$K_{sv} (\text{L mol}^{-1})$ in buffer	1.6	0.9989	0.0874
$K_{sv} (\text{L mol}^{-1})$ in drug–DNA complex	1.3	0.9980	0.0963
Relative reduction in K_{sv} (%)	19

Fig. 5 Role of ionic strength. (A) Fluorescence emission intensity plot of the indomethacin-DNA complex with increasing concentration of NaCl (0–45 mM). The excitation wavelength was 281 nm. The fluorescence emission spectra were recorded in the range of 300–500 nm. (B) The variation of fluorescence intensity with increasing concentration of NaCl.

any change in the viscosity of the Ct-DNA solution in the presence of indomethacin. As shown in Fig. 7A, with continuous addition of indomethacin to the Ct-DNA solution, the viscosity of the Ct-DNA solution remains the same. This confirms that indomethacin binds to DNA *via* a groove binding mode and hence does not intercalate into the DNA helix.³⁸

3.8 Circular dichroism studies

CD spectroscopy is a sensitive technique which detects any conformational changes in DNA upon addition of a ligand. It

has been used extensively for analysis of changes in secondary structures of polypeptides, proteins and DNA upon interaction with ligands. The intrinsic CD spectral behaviour is altered when the structure of the DNA is affected in non-covalent DNA-drug interactions.³⁹ The observed CD spectrum of Ct-DNA consists of two major peaks at 245 nm (negative) and 275 nm (positive) that are assigned to the right handed helicity of B-DNA and base pair stacking, respectively. These bands are considered to be highly sensitive toward the interaction of small molecules with DNA.⁴⁰ In the case of groove binding or non-intercalative binding, very little or no perturbation of the base stacking and helicity bands is observed, whereas in the presence of intercalators, the intensities of both the bands are perturbed.⁴¹ The CD spectrum of DNA in the presence and absence of indomethacin is shown in Fig. 7B. As is evident from the figure, with increasing concentration of indomethacin, there is a negligible change in the spectra of both the bands. Thus, it is established that indomethacin binds to Ct-DNA primarily in a groove binding mode. From the above results of UV-visible absorption spectroscopy, fluorescence spectroscopy, viscosity measurements and CD spectroscopic studies, we can conclude that indomethacin binds to DNA primarily in a groove binding mode.

Fig. 6 Effect of indomethacin on the melting temperature of Ct-DNA. Melting curves of Ct-DNA (50 μM) in the absence (■) and presence (●) of indomethacin (50 μM), where $A/A_{30^\circ\text{C}}$ represents the ratio between the absorbance of Ct-DNA with increasing temperature (30–100 $^\circ\text{C}$) and absorbance at 30 $^\circ\text{C}$. Absorbance was measured at 260 nm.

3.9 Isothermal titration calorimetry

ITC is a sensitive technique that has emerged as a powerful tool for studying the thermodynamic as well as kinetic aspects of complexation in biological systems. The validation of various thermodynamic parameters of DNA-indomethacin was further investigated from ITC studies. It not only provides information about thermodynamic quantities such as enthalpy change (ΔH), entropy change during binding (ΔS) and Gibb's free energy change (ΔG) but also determines the

Fig. 7 (A) Effect of increasing the concentration of indomethacin on the viscosity of Ct-DNA. Concentration of Ct-DNA was kept constant (50 μM) while varying the concentration of indomethacin. Data represent the mean $\pm$ SD of three experiments. (B) Effect of indomethacin on the CD spectra of Ct-DNA. CD spectra of Ct-DNA (50 μM) in 10 mM Tris-HCl (pH 7.2) with varying concentrations of indomethacin (0–100 μM). Each spectrum was obtained at 25 $^{\circ}\text{C}$ with a 10 mm path length cell.

binding affinity (K) and the number of binding sites (n).⁴² The ITC profiles of the binding of indomethacin with DNA are depicted in Fig. 8 after baseline correction. The measurements of various thermodynamic quantities are described in Table S2.†

The negative value of ΔH confirms that the formation of the Ct-DNA–drug complex is exothermic. The thermodynamic parameters, binding constant and stoichiometry of the drug–Ct-DNA complex were predicted by fitting the integrated heats according to an independent binding model. The negative values of ΔH and ΔS indicated the role of hydrogen bond and Van der Waals interactions in the binding of indomethacin to Ct-DNA.⁴³ The large negative enthalpy changes along with calculated favourable free energy changes from the indomethacin–Ct-DNA binding constant suggest that the binding was enthalpy driven. It is a well-known fact that interactions in the case of intercalative binding and groove binding are entropically and enthalpically driven, respectively.⁴⁴ The negative value of ΔG revealed the spontaneous process of the interaction between the drug and Ct-DNA. The value of n being close to unity reveals that there is a single binding site for indomethacin per nucleotide of Ct-DNA.

3.10 Molecular docking

The molecular docking technique is an attractive tool to decipher the drug–DNA interactions in rational drug design and also to gain insight into the mechanistic study. Although the crystal structure of the complex can represent specific details of the interactions, general observations which can corroborate the experimental results may be obtained from docking studies. In order to predict the best fit orientation of the

Fig. 8 (A) Representative isothermal calorimetry profiles of the Ct-DNA and indomethacin. The upper panel represents each heat burst curve as a result of single injection of indomethacin into the Ct-DNA solution. (B) The panel presents the corresponding normalized heat signals versus the molar ratio. The solid line represents the calculated fit of data and the points represent the experimental injection heat.

Fig. 9 Different poses of the molecular docked structure of indomethacin complexed with DNA. (A) Surface representation showing minor groove binding of indomethacin to the GC region of the dodecamer duplex of the sequence (CGCGAATTCGCG)₂ (PDB ID: 1BNA). (B) Stereoview of the docked conformation of indomethacin with DNA showing two hydrogen bonds (shown as yellow dotted lines) between O-1 of indomethacin and the tenth and sixteenth guanine of the A chain of DNA. (C) The distance of the hydrogen bond between O-1 and the tenth guanine of DNA (N $\cdots$ H: 2.7 Å) and between O-1 and the sixteenth guanine of DNA (N $\cdots$ H: 3.3 Å). The relative binding energy of the complex system was found to be ≈ 4.652 kcal M $^{-1}$.

Further, competitive molecular docking was performed using Hoechst and EB. This also reveals that indomethacin binds to the minor groove of DNA near Hoechst and does not bind to DNA by intercalation (Fig. 10A and B). Thus, we conclude that there is a mutual coherence between spectroscopic techniques and molecular modelling, which can substantiate our experimental results and at the same time provide further evidence of a groove mode of binding of indomethacin with DNA.

4 Conclusion

We have studied the mode of interactions between indomethacin and DNA using various approaches and confirmed the groove mode of binding. Through the analysis of UV absorbance spectra and steady state fluorescence, formation of an indomethacin–Ct-DNA complex was confirmed. The quenching constant was found to be in the range of 10^3 L mol^{-1} , which is consistent with those of the well-known groove binders. Iodide induced quenching and competitive displacement assays with EB, AO and Hoechst 33258 revealed that indomethacin interacts with DNA through a groove mode of binding and not through intercalation. CD spectral analysis and viscosity measurements further confirmed our results. The ionic strength experiment didn't show any significant result. However, the possibility of electrostatic interactions between indomethacin and DNA cannot be completely ruled out. ITC results confirmed that the reaction is spontaneous and exothermic. *In silico* molecular docking results

Fig. 10 Competitive molecular docked structures. (A) Indomethacin (green) competitively binds to the minor groove of DNA where Hoechst (pink) is bound. (B) Ethidium bromide (red) intercalates into the DNA double helix while indomethacin (green) binds to the minor groove.

further revealed the groove binding mode of interaction between indomethacin and Ct-DNA. Our results provide valuable information regarding drug-DNA interaction which can be used for the development of potential probes for DNA structures as well as further understanding the pharmacological effects of other related compounds.

Notes

The authors declare that there is no conflict of interest in this work.

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