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Comprehensive exploration of the anticancer activities of procaine and its binding with calf thymus DNA: Multi spectroscopic and molecular modelling study

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Materials

Sodium salt of ct-DNA (D1501, Type I, fibers) and Procaine hydrochloride (99%) were purchased from Sigma, USA. Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) was of analytical grade and also obtained from Sigma. Minimum essential medium (MEM), fetal bovine serum (FBS), trypsin/ EDTA and penicillin-streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). Trypan blue, phosphate buffered saline (PBS), dimethyl sulfoxide (DMSO), ethidium bromide, acridine orange**,** doxorubicin and Annexin-V FITC apoptosis detection kit were obtained from Sigma-Aldrich (St Louis, MO, USA). Cell Titer 96® Non-radioactive cell proliferation assay kit was obtained from Promega (Madison, WI, USA). Culture wares and other consumables used in this study were procured from Nunc, Denmark.

Stock solution of ct-DNA was prepared by dissolving 1 mg of ct-DNA per milliliter of Tris–HCl buffer of 10 mM and pH-7.4. The stock solution was kept at 4 °C for 24 h and stirred at the frequent intervals to ensure the homogeneity of ct-DNA solution. Solutions of ct-DNA gave a ratio of UV absorbance at 260 and 280 nm, A_{260}/A_{280} of 1.8–1.9, indicating that the ct-DNA was

sufficiently free of protein. Final concentration of the ct-DNA stock solution was measured spectrophotometrically using extinction coefficient of 6600 cm^{-1} $mol^{-1}dm^{-3}$ [16]. Tris-HCl buffer was filtered through a 0.45 μm Millipore Millex-HV PVDF filter and pH was measured by using Mettler-Toledo pH meter (model S20).

General procedure for binding measurements

UV–Visible spectra in the range of 220 to 340 nm were recorded on Perkin-Elmer Lambda 45 Spectrophotometer equipped with autosampler and water-bath with temperature controller. Quartz cuvettes of 1 cm path length were used for the measurements. Increasing concentration of procaine was titrated against 30 $x10^{-6}$ mol dm^{-3} of ct-DNA. A fixed amount of procaine (0-35 $x10^{-6}$ mol dm^{-3}) was taken in the blank and baseline was corrected before each measurement. Therefore, the obtained spectra could be considered as difference spectra.

Fluorescence measurements were performed on Hitachi spectrofluorometer (Model F 7000) equipped with a PC and programmable temperature controller. The fluorescence spectra were collected at 15, 25 and 35 °C with a fluorescence cell of path length 1 cm. The excitation and emission slits were set at 10 nm. Though, ct-DNA displays very small absorbance at the excitation and emission wavelengths of procaine (290 nm and 355 nm, respectively), inner filter effect was corrected for the fluorescence data used for calculations of quenching, binding and thermodynamic parameters by using following equation:

$$F_{corr} = F_{obs} \times 10^{(A_{exi}+A_{emi})/2} \quad \text{(S1)}$$

where, F_{corr} and F_{obs} are the corrected and observed fluorescence emission intensities, respectively, A_{exi} and A_{emi} are the absorbance at the excitation and emission wavelengths, respectively.

The competitive interactions between fluorescence probes (EtBr and DAPI) and procaine with ct-DNA were performed by successively adding an appropriate amount of procaine solution to the quartz cuvette containing a fixed concentration of EtBr–ct-DNA or DAPI–ct-DNA complex solution. The concentrations of EtBr, DAPI and ct-DNA were kept at 80.0 $x10^{-6}$ mol dm^{-3}, 12.5 $x10^{-6}$ mol dm^{-3}, and 10.0 $x10^{-6}$ mol dm^{-3}, respectively [1]. After these solutions were mixed sufficiently and stood for 5 min, the fluorescence emission spectra were recorded with excitation wavelengths at 480 nm and 341 nm, respectively.

DNA melting study was performed in absence and presence procaine by monitoring the absorbance intensity at 260 nm over a wide range of temperature ranging from 25–100 °C. ct-DNA (30 $x10^{-6}$ mol dm^{-3}) was taken alone or in presence of 30 $x10^{-6}$ mol dm^{-3} procaine in 10 $x10^{-3}$ mol dm^{-3} tris-HCl buffer of pH 7.4. The temperature of the sample was monitored with the help of thermostat attached with sample holder. The absorbance was recorded at 260 nm and the result is plotted as the function of temperature. The melting temperature (T_m) of ct-DNA was determined as the transition midpoint of melting curve.

The circular dichroism studies of ct-DNA in presence of procaine were carried out with JASCO J-815 spectropolarimeter equipped with a Peltier-type temperature controller. The instrument was calibrated with d-10-camphorsulfonic acid. All the CD spectra were collected in a cell of 0.2 mm path-length. The scan speed was 100 nm/min and response time of 1 s for all measurements. Each spectrum was the average of 2 scans.

The viscosity of the DNA solution was measured at 25 °C. a 50 mL solution of 30 $x10^{-6}$ mol dm^{-3} ct-DNA was transferred to the Ostwald viscometer and the flow time was measured. Then, the solutions containing the mixtures of ct-DNA and procaine in various ratios were checked for the flow time. Each point measured was the average of at least three readings. The

data were presented as the relative specific viscosity $(\eta/\eta_0)^{1/3}$ versus r_i, η is the specific viscosity of ct-DNA in the presence of the procaine and η_0 is the specific viscosity of ct-DNA alone.

Computational studies

The geometries of procaine and DNA bases were optimized at DFT/ BP RI by ORCA [2]. Autodock 4.2.3 Program was used to perform docking calculations of DNA with procaine [3]. Five different conformer of DNA were taken from RSCB protein databank and the structure of procaine was obtained from pubchem. In the blind docking calculations, a grid box of 90×90×90 Å with spacing of 0.357 Å was used to enclose DNA and procaine. The Lamarckian Genetic Algorithm method was used as the searching algorithm. Then the GA population size, the maximum number of energy evaluation, and the number of GA runs were set at 150, 2,500,000, and 100, respectively. The other AutoDock parameters were set to default. In addition, discovery studio visualizer software [4] and pymol [5] were used to analyse the predicated binding mode.

General procedure for anticancer activities

Cell culture and treatments

The MCF-7 human breast adenocarcinoma cell line was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were maintained in MEM with 15% FBS and 1% penicillin/ streptomycin in a completely humidified atmosphere with 95% air and 5% CO_2 at 37 ^oC. Cells were grown as adherent monolayers in T-25 and T-75 culture flasks. The exponentially growing cells at 90-95% confluence were harvested using 0.25% trypsin/ EDTA solution and sub-cultured into 6-well or 96-well plates according to the experimental requirements. The viability of the cells was determined by staining with trypan blue. The cells were counted using a cell counter (Bio Rad TC20 automated cell counter) and diluted in medium

at a density of 1×10^5 cells/ ml to be used throughout the experiments. A stock solution of procaine and doxorubicin was prepared in Millipore water (w/v) and was then diluted in cell culture medium to obtain the desired concentrations for cell treatment.

Cytotoxicity assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay [36] with modification was used to analyze the cytotoxic activity procaine. A CellTitre 96® non-radioactive cell proliferations assay kit (Promega, Madison, WI, USA) was used following the manufacturer's instructions. Briefly, the MCF-7 cells (1×10^4 cells/ well) were grown overnight in 96-well flat bottom cell culture plates, and were then exposed to six different concentrations of two-fold dilutions of 50 $x10^{-6}$ mol dm^{-3} procaine and doxorubicin (50, 25, 12.5, 6.25, 3.12 and 1.56 $x10^{-6}$ mol dm^{-3}) for 24 hrs. A negative control (untreated) was also maintained for comparison. In addition, another group of experiment was setup for combination treatment using two-fold dilution of procaine (50 $x10^{-6}$ mol dm^{-3}) supplemented with 5 $x10^{-6}$ mol dm^{-3} doxorubicin for same duration. After the completion of the desired treatment, 15 µl of MTT reagent, provided in the kit, was added to each well and further incubated for 3 hrs at 37 °C. Finally, the medium with MTT solution was removed, and 200 µl of solubilization solution was added to each well and further incubated for 30 min by occasional vortexing. The optical density (OD) of each well was measured at 550 nm by using a Synergy microplate reader (BioTek, Winooski, VA, USA). Results were generated from three independent experiments and each experiment was performed in triplicate. The percentage of cytotoxicity compared to the untreated cells was estimated in order to determine the IC_{50} value (the concentration at which 50% cell proliferation is inhibited).

Morphological changes analysis

Cells were seeded in a 6-well plate at a density of 1×10^5 cells per well and allowed to grow overnight. Morphological changes were observed to determine the alterations induced by procaine and doxorubicin alone or in combination in MCF-7 cells treated with 5 $x10^{-6}$ mol dm^{-3} for 24 hrs. After the end of the incubation period, cells were washed with PBS (pH- 7.4) and observed under a phase contrast inverted microscope equipped with a digital camera (Olympus IX51, Tokyo, Japan) at 100× magnification.

Apoptotic morphological changes by acridine orange–ethidium bromide dual staining method

Acridine orange and Ethidium bromide dual staining method was adopted to differentiate between condensed apoptotic or necrotic nuclei from normal cells. Cells were seeded on a cover-slip-loaded 6-well plate at a density of 1×10^5 cells per well and allowed to grow overnight. Then the cells were exposed to 5 $x10^{-6}$ mol dm^{-3} procaine and doxorubicin alone or in combination for 24 hrs. After end of treatment, cells were washed twice using PBS to remove the remaining media. Then, cells were stained by adding equal volumes of AO and PI (20 μg/mL in PBS). Finally, after washing with PBS, the stained cells were mounted onto a microscope slide in mounting medium and images were collected using appropriate filter settings in a compound microscope (Olympus BX41, Japan) fitted with fluorescence attachment and CCD camera. Quantification of apoptotic and necrotic cells based on the uptake of acridine orange and propidium iodide in more than 300 cells was performed. The criteria for identification were as follows: green intact nucleus, viable cells; dense green areas of chromatin condensation in the nucleus, apoptosis; orange intact nucleus, necrosis.

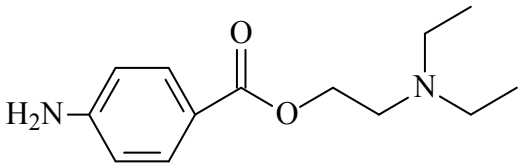
Annexin V- FITC apoptosis assay

Apoptosis was measured using fluorescence microscopy by analyzing the translocation of phosphatidylserine on the outer membrane of apoptotic cells. An annexin-V FITC apoptosis

detection Kit was used for the differentiation of early apoptotic, late apoptotic and necrotic cells. Briefly, MCF-7 cells were grown and exposed to the test compounds on a cover-slip-loaded 6-well plate at a density of 1×10^5 cells per well as mentioned above. After end of treatment, cells were washed twice using PBS to remove the remaining media. Then cells were stained with 5 µl each of annexin V-FITC and PI for 20 min at room temperature in dark. Finally, after washing with PBS, the stained cells were mounted onto a microscope slide in mounting medium and images were collected using appropriate filter settings in a compound microscope (Olympus BX41, Japan) fitted with fluorescence attachment and CCD camera.

Statistical analysis

All experiments were carried out with three independent replicates and values are presented as mean ± standard error of mean (SEM). Data were statistically analyzed using the Student's *t*-test for comparison between the means applying a significance level of $P < 0.05$.



Scheme S1. Structural formula of procaine.

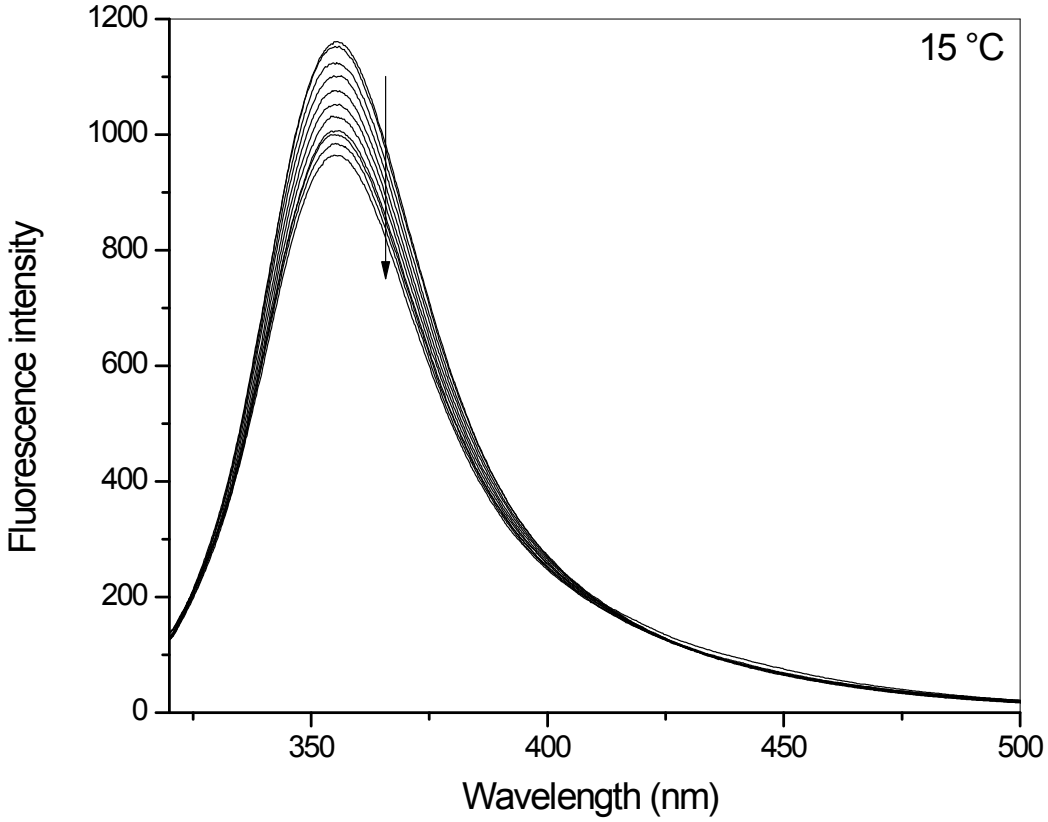


Fig. S1. Fluorescence emission spectra of procaine (30 x 10^{-6} M L^{-1}) in the presence of increasing amount of ct-DNA (0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 x 10^{-6} M L^{-1}) at 15 °C.

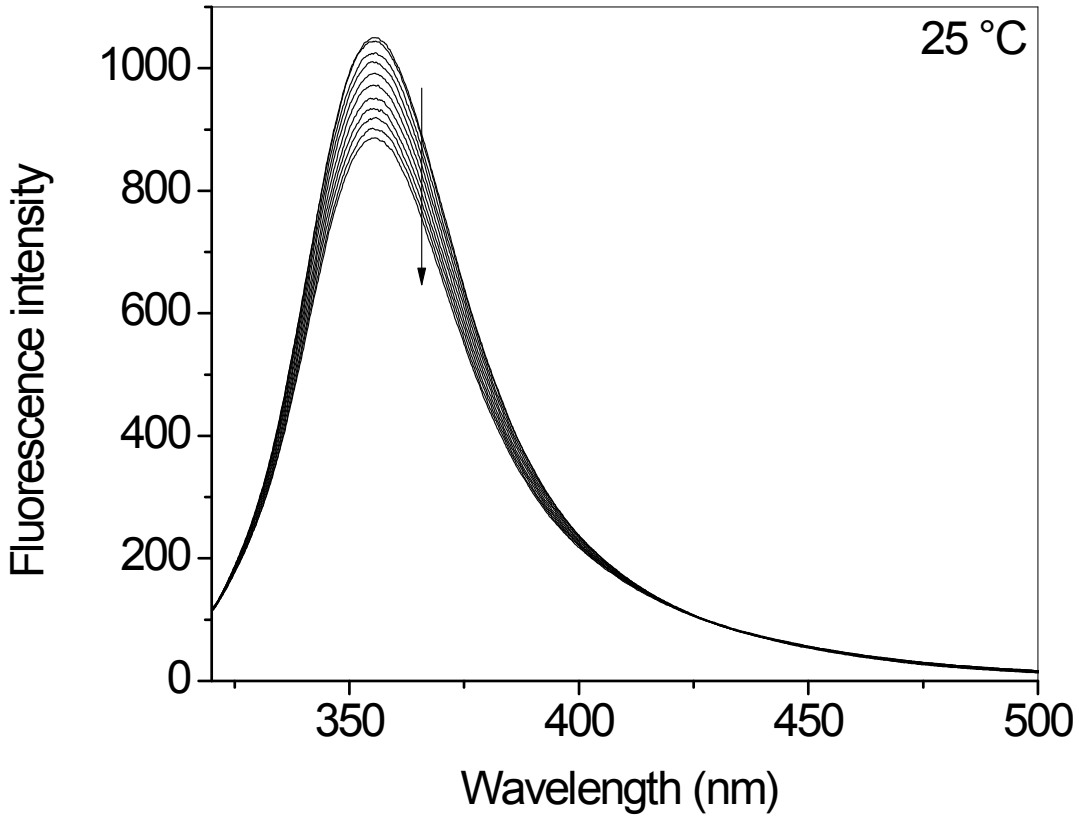


Fig. S2. Fluorescence emission spectra of procaine (30 x 10^{-6} M L^{-1}) in the presence of increasing amount of ct-DNA (0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 x 10^{-6} M L^{-1}) at 25 °C.

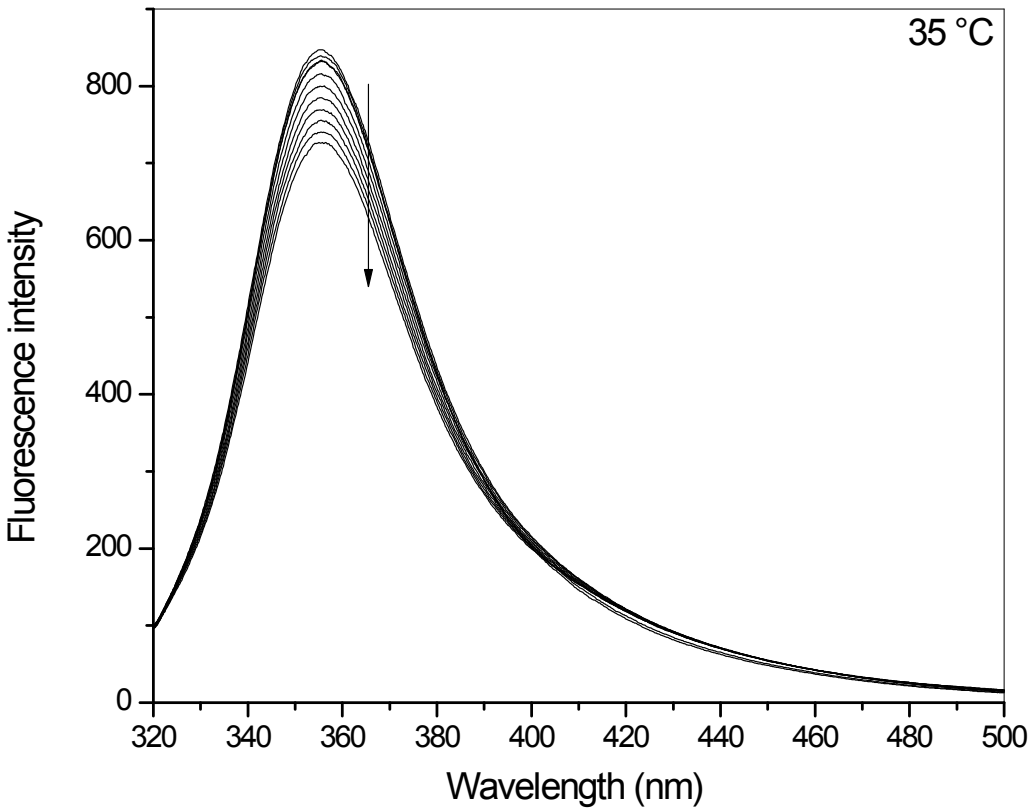


Fig. S3. Fluorescence emission spectra of procaine (30 x 10^{-6} M L^{-1}) in the presence of increasing amount of ct-DNA (0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 x 10^{-6} M L^{-1}) at 35 °C.

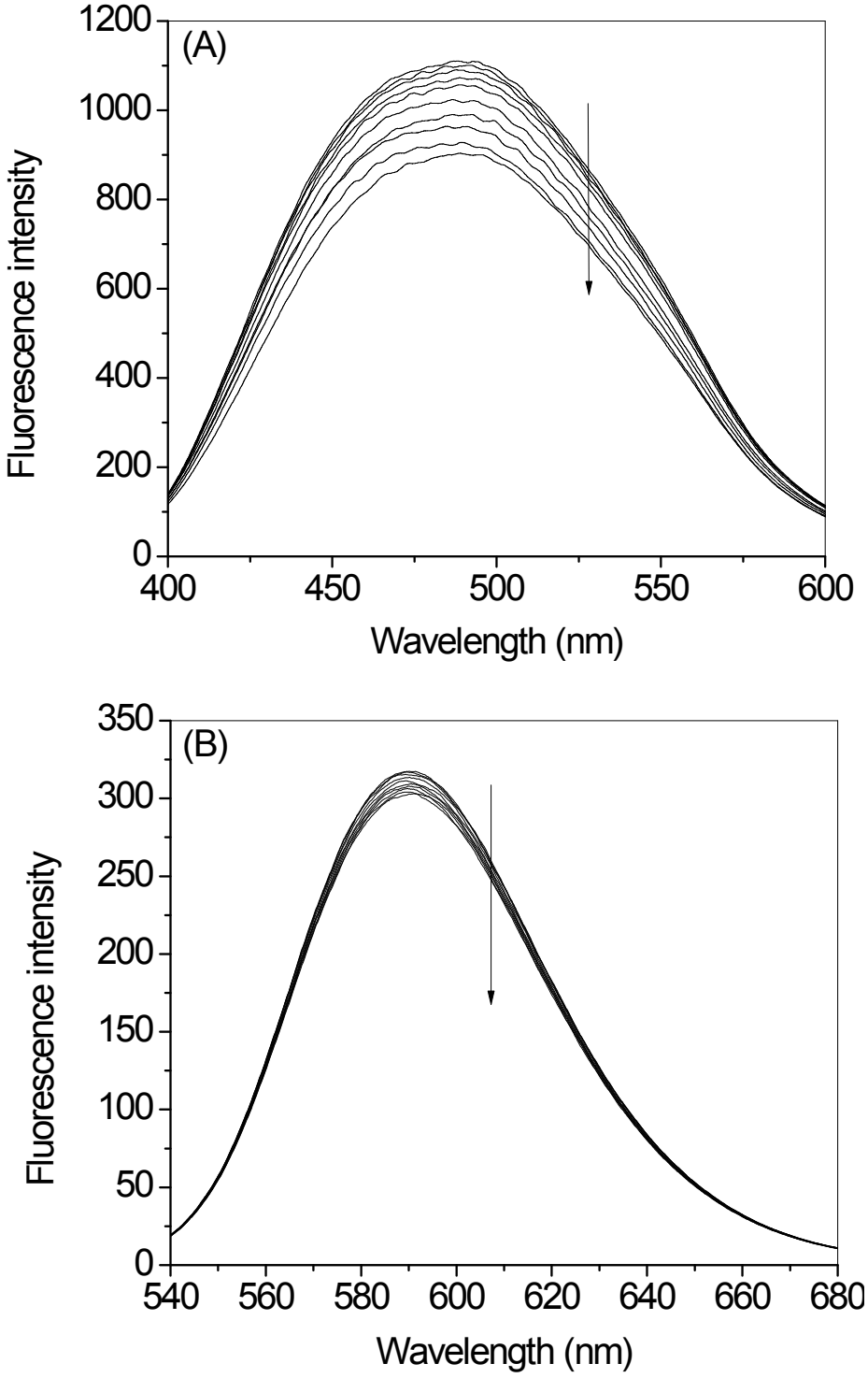


Fig. S4. Competitive displacement assays between procaine and EB/DAPI (A) Fluorescence titration of ct-DNA and DAPI with procaine. (B) Fluorescence titration of EB and ct-DNA with procaine. [procaine] = (0, 5, 10, 15, 20, 25, 30, 35, 40, 50 x 10^{-6} M).

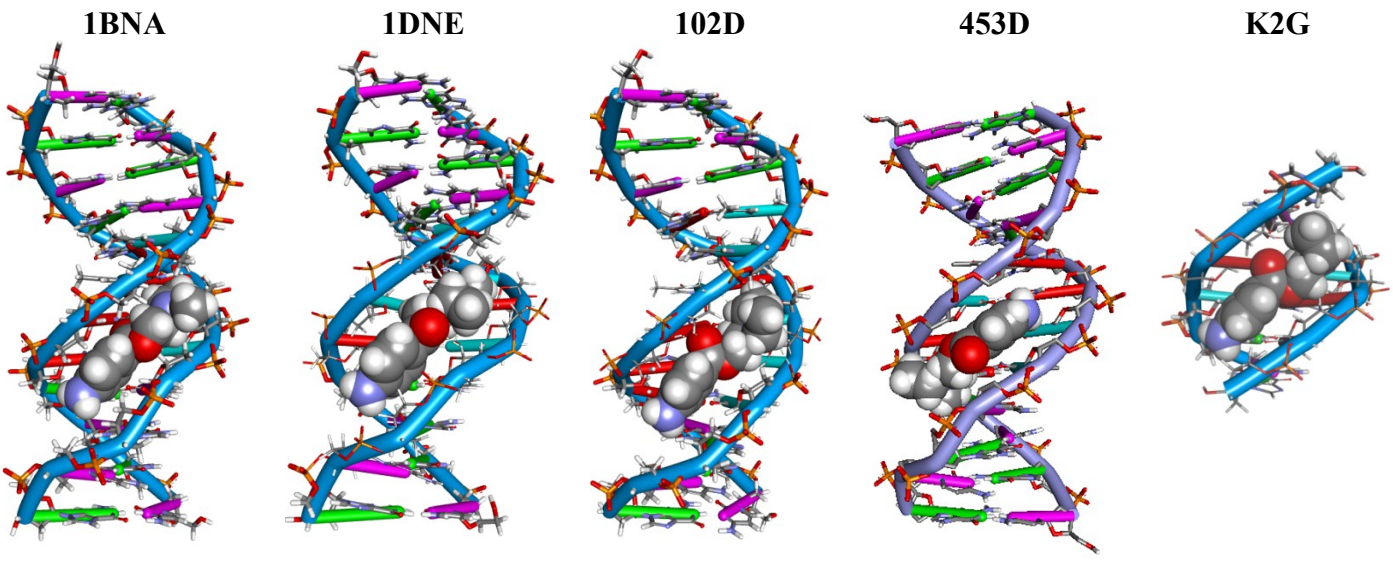


Fig. S5. Molecular docking of procaine bound to B-form DNAs.

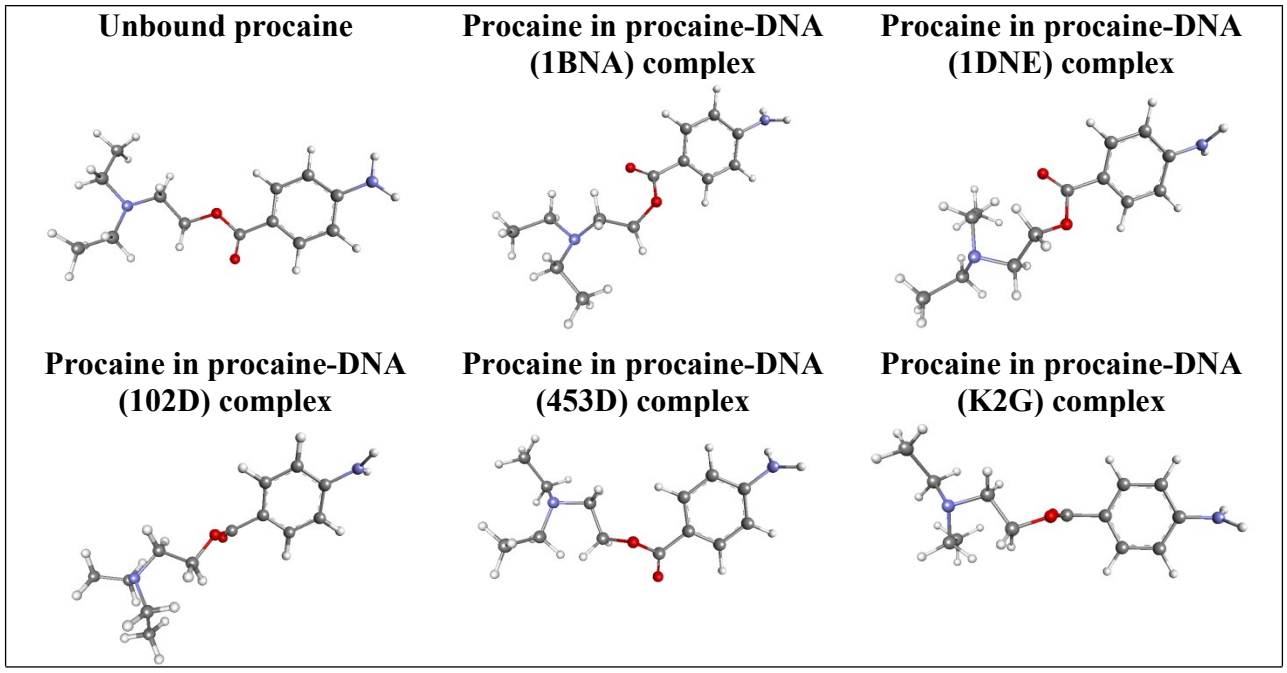


Fig. S6. The conformation of procaine in procaine–DNA complexes.

Table S1 Non-covalent interactions between procaine and various DNA conformers obtained through molecular docking

	Nucleotide	Type of interaction	Distance
1BNA	Thy8	hydrogen bond	3.78
	Cyt9	hydrogen bond	2.56
	Gua10	Pi-anion	4.71
	Ade18	hydrogen bond	1.92
	Thy19	hydrogen bond	2.27
1DNE	Ade7	Pi-alkyl	4.64
	Thy8	hydrogen bond	2.94
		hydrogen bond	3.52
		hydrogen bond	2.89
	Gua10	Pi anion	3.82
	Cyt11	hydrogen bond	1.94
		hydrogen bond	3.09
	Ade19	Pi-alkyl	4.52
		Pi-alkyl	4.95
		hydrogen bond	3.18
102D	Thy8	hydrogen bond	3.78
		hydrogen bond	2.71
		Acceptor-acceptor	2.87
	Ade18	hydrogen bond	3.11
	Thy19	Acceptor-acceptor	2.52
453d	Thy7	hydrogen bond	2.17
	Thy8	hydrogen bond	2.71
	Cyt9	hydrogen bond	3.56
		hydrogen bond	3.52
	ADE17	Pi-alkyl	4.88
	Thy20	hydrogen bond	2.72
1K2J	Ade4	hydrogen bond	2.74
		hydrogen bond	2.17
		hydrogen bond	2.64
		hydrogen bond	3.28
	Gua8	Pi-alkyl	5.35
	Ade10	Acceptor-acceptor	2.81
	Cyt11	hydrogen bond	2.89
	Gua12	hydrogen bond	2.17

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