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ORIGINAL PAPER

Chemotherapeutic potential of 9-phenyl acridine: biophysical studies on its binding to DNA

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Abstract Acridines and their derivatives are well-known probes for nucleic acids as well as being relevant in the field of drug development to establish new chemotherapeutic agents. We have shown from molecular modelling studies that 9-phenyl acridine and some of its derivatives can act as inhibitors of topoisomerase I and thus have potential to act as anticancer agents. Rational design of new compounds for therapeutics requires knowledge about their structural stability and interactions with various cellular macromolecules. In this regard it is important to know how these molecules would interact with DNA. Here we report the interaction of 9-phenyl acridine (ACPH) with calf thymus DNA (CT-DNA) based on various biophysical and molecular modelling studies. Spectrophotometric studies indicated that ACPH binds to CT-DNA. DNA melting studies revealed that binding of ACPH to CT-DNA resulted in a small increase in melting temperature, which is unlikely in case of classical intercalator; rather, it indicates external binding. Viscosity measurements show that ACPH exhibits groove binding. Competitive binding of ACPH to CT-DNA pre-bound to ethidium bromide (EB) showed slow quenching. Measurement of the binding constant of ACPH by fluorescent intercalator displacement (FID) assay corroborated the notion that there was groove binding. Molecular modelling studies also supported this finding. Results indicate that binding of ACPH is through partial intercalation in the minor groove of DNA.

Keywords 9-Phenyl acridine · DNA · Viscosity · DNA melting · Fluorimetry · Molecular modelling

Abbreviations

ACPH	9-Phenyl acridine
T_m	DNA melting temperature
FID	Fluorescent intercalator displacement
EB	Ethidium bromide

Introduction

Binding of molecules to DNA regulates its function; protein–DNA interactions form the basis of various molecular-biological processes required for functioning of cells (Wang et al. 2003; Bai et al. 2005; Colella et al. 1996). Much effort has also been directed to study DNA–small molecule interactions, such as the interaction of DNA with various types of organic molecules (Arya and Willis 2003; Trieb et al. 2004; Brana et al. 2001; Graves and Velea 2000; Boger et al. 2000), inorganic complexes (Vaidyanathan and Nair 2005; Burkoff and Tullius 1988; Gupta et al. 1992; Tullius and Dombroski 1985; Uma et al. 2005; Vijayalakshmi et al. 2000), fluorescent dyes (Zipper et al. 2004; Biver et al. 2005) and ions (Wu et al. 2005; Burda et al. 1997; Hegde et al. 2004). These studies help to understand the molecular basis of complex diseases and sometimes provide important drug candidates (Bolognesi

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et al. 2008; Dilda and Hogg 2007; Arkin 2005). Notable amongst these are the acridine group of drugs. Acridines are well-known probes for nucleic acids, but a number of acridine-based drugs have been successfully utilized as chemotherapeutic agents, such as pyrizaloacridines (LoRusso et al. 1990), imidazoacridinones (Cholody et al. 1992), anthrapyrazoles (Leopold et al. 1985), acridine carboxamides (Adams et al. 2004) and trizoloacridinones (Cholody et al. 1990). They are used as anticancer, antibacterial or antiprotozoal agents because of their binding ability to DNA. Some acridine derivatives, i.e. the decahydroacridinedione groups of dyes, have been found to mimic nicotinamide adenine dinucleotide (NADH) because of their structural similarity to NADH analogs (Singh et al. 1982).

Small molecules generally bind reversibly to DNA through intercalation between base pairs (Vaidyanathan and Nair 2005; Burkoff and Tullius 1988; Gupta et al. 1992; Tullius and Dombroski 1985; Uma et al. 2005), binding to major (Cate and Doudna 1996) or minor groove (Vijayalakshmi et al. 2000; Rueda et al. 2005) or by binding externally to the DNA helix (Gupta et al. 1992; Zipper et al. 2004). Drugs such as netropsin and distamycin bind to the minor grooves of DNA having AT-rich sequences (Boger et al. 2000). Many eukaryotic transcription factors are known to bind to the major grooves of DNA. Binding of oligonucleotides to homopurine–homopyrimidine sites in the major groove results in formation of triple helices (Thuong and Helene 1993), which are believed to have significance in specific recognition of DNA.

Most acridine derivatives interact with DNA as intercalators. Some bis-acridines bind to DNA through intercalation between consecutive nucleotides (Zee-Cheng and Cheng 1978; Murdock et al. 1979; Cain and Atwell 1974). Binding of acridine molecules interferes with DNA function by blocking the DNA starter required by polymerases to synthesize RNA and DNA, thereby inhibiting protein synthesis (Hurwitz et al. 1962). In many cases the chemotherapeutic action of these acridines is not restricted to DNA binding alone, but they also exert their effects by inhibition of key enzymes such as topoisomerase, telomerase or polymerases (Kohn 1996; Harris 1996; Wesierska-Gadek et al. 2004). These interactions are mainly non-covalent in nature.

In recent times a lot of interest has been generated in the search for acridine-based drugs (Zee-Cheng and Cheng 1978; Murdock et al. 1979; Kohn 1996; Harris 1996). However, there are no reports on the biological activity of 9-phenyl acridine (ACPH), although a number of synthetic derivatives of 9-arylacridine are known (Datta et al. 1990). We have shown from molecular modelling studies that these groups of drugs can act as inhibitors of topoisomerase I and thus have potential to act as anticancer agents (Bhowmik et al. 2008). Rational design of new compounds for therapeutics requires knowledge about their structural stability and interactions with various cellular macromolecules. In this regard, it is important to know how these molecules would interact with DNA. We present herein our findings from experimental as well as computational modelling studies of binding of ACPH with calf thymus DNA. We used biophysical techniques to establish the binding of the derivative with DNA and substantiated the results with the help of molecular modelling studies. The results indicate that ACPH can bind to DNA and may thereby act as a potential therapeutic lead compound for the design of new drugs. Since this is the first report regarding the activity of ACPH as a potential drug candidate, these results may contribute to future rational drug development endeavours.

Materials and methods

ACPH was prepared in the laboratory as described by Datta et al. (1990). Ethidium bromide (EB) was obtained from Sigma, and CT-DNA from Fluka chemicals. A stock solution of DNA was prepared in TE buffer (pH 7.5). The filtered DNA solution gave an ultraviolet (UV) absorbance ratio (A_{260}/A_{280}) of $\sim$1.9, indicating no contamination (Marmur 1961). All experiments were carried out in TE buffer at pH 7.5.

Physico-chemical studies

Absorption spectra of ACPH and the DNA–ACPH complex were recorded on a Shimadzu UV–Vis double-beam spectrophotometer, and λ_{max} of ACPH and also the complex were determined. Absorbance of ACPH alone and that of DNA–ACPH complex at various concentrations of ACPH (0–20 μM) with fixed concentration of DNA (30 μg/ml) was determined at the λ_{max} of ACPH/DNA–ACPH complex. This experiment was repeated thrice, the bars in the graph representing the standard deviation.

DNA melting temperature (T_m) measurements were carried out by monitoring absorbance of CT-DNA (30 μg/ml) in the presence and absence of ACPH (10 μM) at the λ_{max} for DNA at 260 nm, as a function of temperature using the same instrument, which is equipped with a thermoelectric temperature controller (Peltier system, ±0.1°C) and thermostatic cell holder. The resulting absorbance versus temperature curve was differentiated and the weighted maximum was taken as T_m. Uncertainty in T_m was estimated as ±0.5°C by replications. The results shown are mean ± standard deviation from three experiments.

Viscometric experiments were carried out using an Oswald-type viscometer with 3 ml capacity, in a thermostatic oil bath maintained at 25 ± 1°C. The flow rates of the buffer in the presence of ACPH at various concentrations were measured with a manually operated timer at least three times to agree within 0.2 s. The relative specific viscosity was calculated according to the relation

$$(\eta = (t - t_0)/t_0),$$

where t_0 is the flow time for the buffer and t is the observed flow time for DNA in the presence and absence of the dye. A plot of $(\eta/\eta_0)^{1/3}$ versus $1/R$ was constructed from viscometric measurements as described by Cohen and Eisenberg (1969).

$$R = [\text{DNA}]/[\text{ACPH}].$$

The plotted results are mean ± standard deviation from three experiments.

Steady-state fluorescence spectroscopic experiments on the interaction of the dye with CT-DNA-EB complex in TE buffer (pH 7.5) were carried out at room temperature. Emission spectra were recorded on a Perkin Elmer spectrofluorimeter using 1-cm-pathlength cuvette. The excitation and emission slits were both 10 nm, and the scan speed was 200 nm min^{-1}. The complex of CT-DNA (50 μg/ml) and EB (5 μM) in TE buffer was placed in a thermostatic quartz fluorescence cuvette and titrated with 12.5 μM aliquots of 1 mM dye solution with continuous stirring. The total volume of the solution was 2 ml. After each titration, the solution was mixed thoroughly and allowed to equilibrate thermally for 5 min prior to fluorescence measurements. The apparent binding constant (K_{app}) was calculated from the equation

$$K_{EB}[\text{EB}] = K_{app}[\text{ACPH}],$$

where K_{EB} is 1.0×10^7 M^{-1}. [ACPH] corresponds to the concentration of dye at which 50% reduction of emission intensity of pre-bound EB was observed.

Molecular modelling studies

In order to study the structural aspects of DNA–ACPH interactions, a model of ACPH was built using the Biopolymer module of the Discovery Studio software package (Accelrys) (Honig et al. 1993; Nicholls and Honig 1991; Sharp et al. 1991). For model building the different molecular fragments were taken from the built-in library of the Biopolymer module. They were assembled and joined to form the desired ACPH molecule. Bonding parameters such as bond angle, torsion angle and bond length were checked visually and corrected. The molecule was subjected to energy minimization using AMBER force field. The resulting molecule was analyzed further to check for any distortions in the bonding parameters of the molecule. The ACPH molecule so generated was docked into the DNA. For docking purposes, B-DNA systems were chosen, such as the dodecamer duplex of sequences $d(CGCGAATTCGCG)_2$ (Dickerson), 5′CGCGA**AA**TCGCG3′/5′CGCGA**TT**TCGCG3′ (D-AT; purposely modified from the Dickerson sequence to have an AT-rich binding sequence), 5′CGCGA**GG**TCGCG3′/5′CGCGA**CC**TCGCG3′ (D-GC; purposely modified from the Dickerson sequence to have a GC-rich binding sequence), $dA_{12}.T_{12}$ and $dG_{12}.C_{12}$. The DNA coordinates were obtained from the protein data bank (Pdb ID: 1BNA). The B-DNA molecules so obtained were subjected to energy minimization in order to obtain the stable state of the molecule. The resulting energy-minimized coordinates of the DNAs were used for docking purposes. The docking was done using the software HADDOCK and patchdock server (Schneidman-Duhovny et al. 2003). The coordinates of all DNA–ACPH complexes were subjected to energy minimization using AMBER in two steps. In the first step, the DNA of the DNA–ACPH complex was kept fixed and the ACPH was allowed to move. In the next step both the DNA and the ACPH were allowed to move. The conjugate gradient method was used for minimization until the derivative reached 0.001 kcal/mol. The resulting energy-minimized coordinates of the docked complexes were used for thermodynamic analyses using the structural thermodynamic calculator (Lavigne et al. 2000).

Results and discussion

Spectrophotometric studies

The absorption spectrum of ACPH showed absorption maxima at 259 and 355 nm. Figure 1 presents the consolidated results for absorbance at wavelength 259 nm for only ACPH at concentrations ranging from 4 to 20 μM and for DNA (30 μg/ml) with increasing concentration of ACPH. The additive values of absorbance of DNA (30 μg/ml) with different concentrations of ACPH are also plotted. The absorbance of DNA + ACPH at each concentration is less than the additive values of the absorbance of DNA and ACPH separately, indicating binding of ACPH with DNA.

Thermal denaturation study

An important tool to study the interaction of small molecules with nucleic acids is study of DNA melting. It is expected that the structure and mode of binding of the dye molecule may play a role in the double-helical predisposition of CT-DNA. Binding of organic dyes or metallointercalators generally results in considerable stabilization of

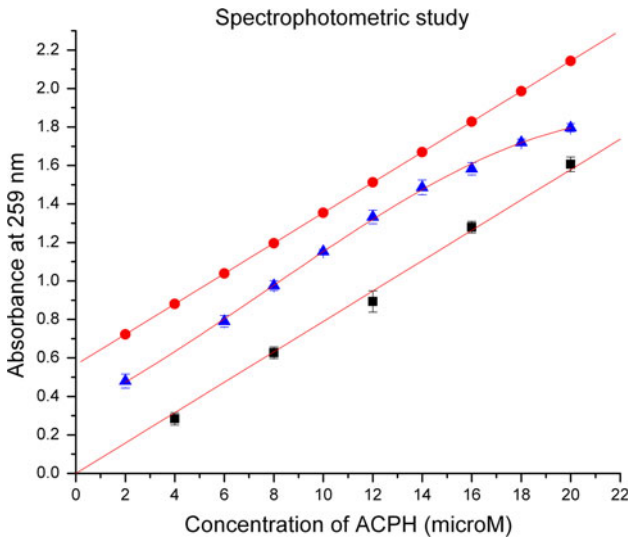

Fig. 1 Absorbance of ACPH at different concentrations (*filled squares*) and after binding with CT-DNA (30 μg/ml) (*filled triangles*). Optical density (OD) values were determined at the absorbance maxima of ACPH at 259 nm. *Filled circles* show the calculated additive value of the absorbance of ACPH and DNA obtained individually at 259 nm. Results are mean ± standard deviation from three experiments

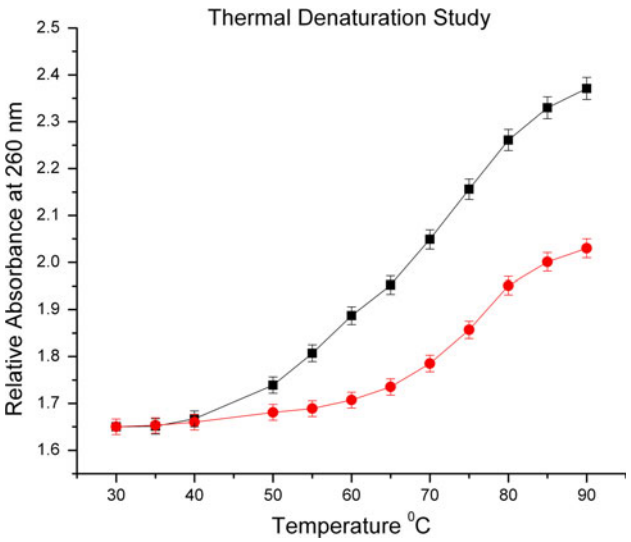

Fig. 2 Thermal denaturation of CT-DNA (30 μg/ml) in absence (*filled squares*) and presence (*filled circles*) of ACPH (10 μM). Results are mean ± standard deviation from three experiments

the DNA duplex, with corresponding increase of melting temperature (T_m) (Neyhart et al. 1993). The amount of increase in T_m is dependent on the mode of binding, but the increasing trend in T_m is independent of the mode of binding. For classical intercalators, the observed increase in T_m was 13–14°C (Neyhart et al. 1993). Thermal denaturation experiment, carried out on CT-DNA in the absence of ACPH, revealed that T_m for the duplex is 69 ± 0.2°C under our experimental conditions (Fig. 2). Addition of ACPH to DNA resulted in a small increase in melting temperature by 4 ± 0.2°C. This change in melting temperature was statistically significant using Student's *t*-test ($P < 0.001$). Small change in melting temperature has also been observed in case of an unusual mode of binding through partial intercalation (Rajendran and Nair 2006).

Viscosity measurements

An unambiguous and critical test for the binding model in solution in the absence of crystallographic structural data is hydrodynamic measurement, which is sensitive to length change of nucleic acid. Of the hydrodynamic measurements such as viscosity, sedimentation, and rotational diffusion as measured by transient electric diffusion, viscosity measurements provide a better tool for investigation of drug–DNA intercalation or groove binding, since optical studies provide necessary but insufficient clues to support binding model. Generally, classical intercalators increase the viscosity of DNA due to the accommodation of the ligand between the base pairs of the helix. In contrast, under the same conditions, drug molecules that bind exclusively in the DNA grooves (e.g. distamycin, netropsin) could bend or kink the DNA helix and reduce the effective length and concomitantly its viscosity. The effect of the addition of different ACPH on the viscosity of CT-DNA (50 μg/ml) is shown in Fig. 3. The results shown are the average from three experiments. The viscosity is found to decrease with increasing concentration of ACPH up to 15 μM, and thus the observation supports the groove-binding nature of the molecule rather than classical intercalation.

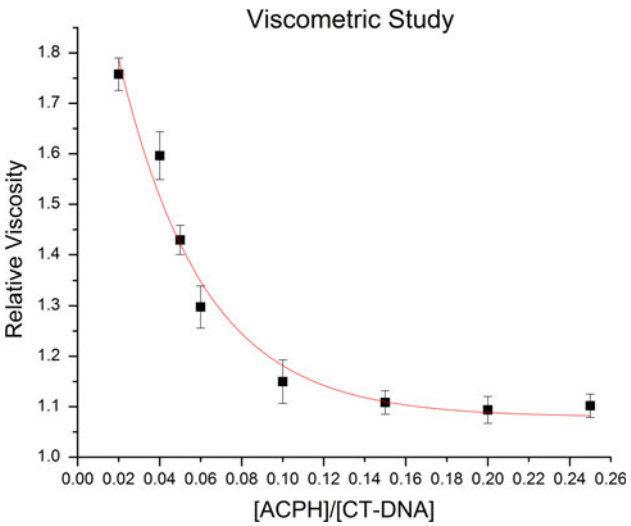

Fig. 3 Effect of concentration of ACPH (0–25 μM) on the relative viscosity of CT-DNA (100 μg/ml). Results are mean ± standard deviation from three experiments

Fluorescent intercalator displacement assay

ACPH shows fluorescence when excited with light of 259 and 355 nm, exhibiting an emission maximum around 480 nm for both excitations. On binding with DNA the fluorescence spectra does not change as much as on binding with ethidium bromide or other intercalative dye. Thus, this dye is not a suitable agent for use in DNA detection.

To assess binding affinity of the dye molecule with DNA, FID assay has been carried out. Ethidium bromide is a classical intercalator with binding affinity on the order of 10^7 (Lee et al. 1993). It displays a dramatic enhancement of its emission intensity when intercalated into DNA due to strong stacking interaction between adjacent base pairs. The enhanced fluorescence of the DNA–EB complex can be quenched by the addition of a second ligand molecule which is either an intercalator (Vaidyanathan and Nair 2003) or a groove binder (Boger et al. 2000; Shim et al. 2004), and such a decrease in the emission intensity of the pre-bound EB is the basis of the FID assay. This experiment was repeated thrice. The result from a typical experiment is shown in Fig. 4, which depicts the variation of emission intensity (at 600 nm) of the pre-bound EB on the addition of dye molecule. We find that with 50 μg/ml CT-DNA, even up to 120 μM ACPH, there is a gradual decrease in the fluorescence intensity without any endpoint. This shows that ACPH is not very efficient at displacing EB, which is a perfect intercalator. The binding constant of the dye with CT-DNA was estimated to be 4.8×10^5 M^{-1}. Ligands that are well-known groove binders show low values of binding constants in comparison with ligands that intercalate into DNA (Lee et al. 1993; Boger et al. 2000). Thus FID assay favours the groove-binding mode.

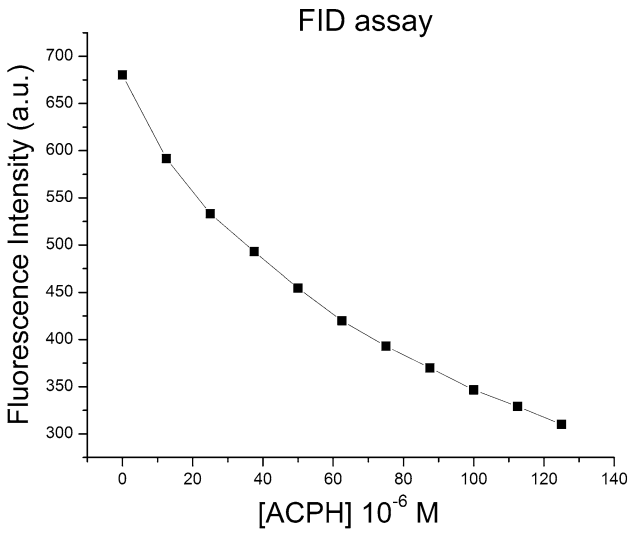

Fig. 4 Variation of emission intensity of CT-DNA (50 μg/ml) with pre-bound EB (5 μM) on addition of varying concentrations of ACPH at 600 nm. The excitation wavelength was 335 nm

Molecular modelling

When ACPH was docked into DNA, the molecule was found to bind to the minor groove of DNA. Interaction of ACPH with B-DNA [dodecamer duplex of sequences $d(CGCGAATTCGCG)_2$ (Dickerson)] obtained from molecular modelling is shown in Fig. 5. The interactions are basically non-covalent interactions, viz. hydrophobic as well as electrostatic interactions between ACPH and DNA. The interactions occur either through the phosphate backbone atoms of the DNA or via the DNA bases. There are some conformational changes in the ACPH molecule after binding. The ACPH binds mainly to the minor groove of the DNA and then bends the DNA in such a way that a part of the molecule comes between the two base pairs of the DNA. There are aromatic pi-stacking interactions between the ring systems of the DNA bases and the ACPH molecules. This implies that ACPH binds to the DNA via partial intercalation also. The binding free energy of the complex was calculated using the structural thermodynamics calculator. The binding free energies are presented in Table 1.

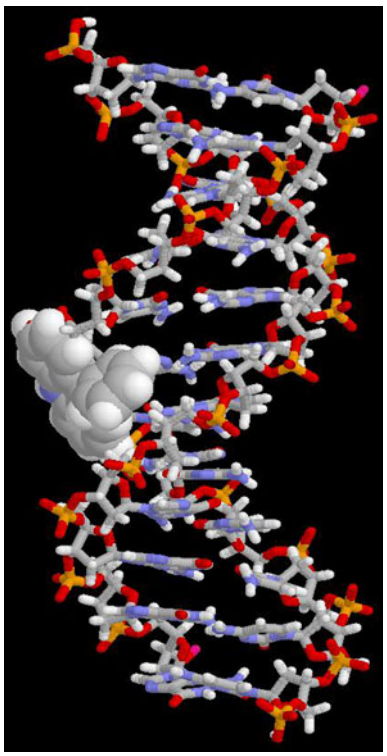

Fig. 5 Interaction of ACPH with B-DNA [dodecamer duplex of sequences $d(CGCGAATTCGCG)_2$ (Dickerson)] obtained from molecular modelling. DNA is depicted by stick model and ACPH is shown by space-filling model; the colour scheme of the atoms is according to the Corey–Pauling–Kultin (CPK) mode

Table 1 Binding free energies of ACPH with different B-DNA sequences

Sequence	Binding free energy (kcal/mol)
$dA_{12}.dT_{12}$	133.77
$dG_{12}.dC_{12}$	110.89
Dickerson	120.67
D-AT	119.66
D-GC	105.54

The molecular modelling analyses revealed that ACPH binds to all the investigated DNA sequences in the minor groove. The interaction energies are more or less comparable, being lower for GC-rich sequences because of the strong interactions between the GC base pairs as compared with AT. The overall modes of interactions in all cases are similar.

Conclusions

In the present study an attempt was made to analyze the DNA binding activity of ACPH. From both biophysical experiments as well as molecular modelling studies it was revealed that ACPH binds to DNA. Increase in DNA melting temperature indicates that binding of ACPH to DNA does not facilitate strand separation. Viscometric studies indicated the groove-binding mode. FID assay and molecular modelling studies corroborated the notion that ACPH binds in the minor groove of DNA and bends the DNA. We have already proved the ability of ACPH to bind to and inhibit topoisomerase I. Therefore, ACPH may be proposed as a potential lead compound for development of new antitumor drugs.

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