Frontiers in Health Informatics
ISSN-Online: 2676-7104
www.healthinformaticsjournal.com
2024; Vol 13: Issue 8
Open Access

Characterization of Quinacrine-Induced Structural and Antigenic Modifications in Calf Thymus DNA Using Biophysical and Immunological Techniques

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Cite this paper as: Dr. Asha K. S, MD, Dr. R. Sripradha, M.Sc, Ph.D , Dr. K. M. Tullanithi, M.Sc, Ph.D (2024). Characterization of Quinacrine-Induced Structural and Antigenic Modifications in Calf Thymus DNA Using Biophysical and Immunological Techniques. *Frontiers in Health Informatics, 13 (8) 1163-1171*

Abstract

Background: Quinacrine, a heterocyclic acridine derivative, has been historically used as an antimalarial drug and is now investigated for its anticancer potential. However, its interaction with DNA raises concerns about potential carcinogenicity due to structural alterations induced in the DNA molecule.

Objective: This study aimed to investigate the structural and antigenic modifications in calf thymus DNA induced by quinacrine in a concentration- and time-dependent manner using biophysical and immunological approaches.

Methods: Calf thymus DNA was treated with quinacrine at various concentrations and incubation times. Structural alterations were characterized using ultraviolet (UV) spectroscopy, circular dichroism (CD), Fourier-transform infrared (FT-IR) spectroscopy, fluorescence emission analysis, dynamic light scattering (DLS), agarose gel electrophoresis, and electron microscopy (TEM and SEM). Antigenic changes were assessed through enzyme-linked immunosorbent assay (ELISA) and inhibition assays using New Zealand White rabbits immunized with native and quinacrine-modified DNA.

Results: UV analysis revealed significant hypochromicity at 260 nm, with 35–52% reduction in absorbance depending on quinacrine concentration and incubation time. CD spectra demonstrated altered ellipticity, suggesting disruptions in the native B-DNA conformation. FT-IR spectroscopy revealed shifts in vibrational bands indicative of perturbations in purine, pyrimidine, and phosphate backbone regions. Fluorescence analysis showed a 60% increase in intensity at 492 nm in quinacrine-modified DNA, confirming binding interactions. ELISA and inhibition assays demonstrated high specificity of induced antibodies for quinacrine-modified DNA, with differential recognition of various inhibitors.

Conclusion: Quinacrine induces significant structural modifications in DNA through intercalation and groove binding, as evidenced by spectroscopic and immunological analyses. These findings underscore the need for caution in the therapeutic use of quinacrine due to potential carcinogenic effects and offer insights into its interaction with DNA for future drug design.

Keywords: Quinacrine, Calf Thymus DNA, Structural Modifications, Antigenicity, Biophysical Techniques, Intercalation, DNA Binding, FT-IR Spectroscopy.

Introduction-

Ehrlich and Benda brought quinacrine, an antimalarial medication, to the world in 1912, more than 80 years ago. Because of its toxicity and resistance, it was outlawed. Quinacrine formulations, which go by a variety of names, were reintroduced during World War II and were later replaced by chloroquine in 1945. Due to its potential to interact with a

number of biological macromolecules, quinacrine is shown to be engaged in numerous pathophysiological processes, just like the majority of other medications. This has been extensively utilized, and quinacrine has now become a polyspecific medication with broad therapeutic potential against prion disorders, asthma, helminthic infections, and other conditions. It is particularly well-known for its adjuvant cancer therapy.[1,2]

Quinacrine is often taken orally with water after meals. It is rapidly absorbed from the digestive tract, with plasma levels peaking within 8–12 hours. The liver, spleen, lungs, and adrenal glands contain the largest amounts of the drug, with the liver having the highest concentration (20 times that of plasma), with 80–90% of the drug bound to plasma proteins. Although 300 mg of quinacrine per day was found to be well tolerated in contemporary clinical settings with no notable toxicity recorded, it has been linked to reversible hepatitis because of its capacity to concentrate in the liver after long-term therapy.[3,4]

Evidence suggests that quinacrine can enter the nucleus and interact with DNA via the acridine backbone, a planer molecule that allows it to stack between base pairs of double-helical DNA to intercalate into it, causing the helix to unwind. In addition, the diaminobutyl side chain stabilizes the DNA double helix by interacting with the minor groove of DNA. The study was created to better investigate and examine the structural changes that DNA (calf thymus DNA) experiences in the presence of quinacrine. Using a variety of biophysical approaches, we give a thorough characterisation of quinacrine-modified commercially available calf thymus DNA. We also analyze the antigenic alterations the DNA molecule experiences in a dosage and time-dependent way when incubated in the presence of quinacrine. [5,6]

Materials and Methods-

Quinacrine is an antimalarial drug which has been evaluated for its anticancer potential. However, all anticancer drugs also possess potential carcinogenic effects. Quinacrine shows well documented interaction with DNA by binding on the major groove of DNA and intercalating between the bases due to its planar structure. These changes in the structure of DNA could be evaluated as one of the important factors for progression and pathophysiology of hepatocellular carcinoma. So, in the present study, commercially available calf thymus DNA was modified with quinacrine in a concentration and time dependent manner and their induced structural alterations were characterised by ultraviolet (UV), circular dichroism (CD), Fourier transform infrared (FT-IR) spectroscopy, thermal denaturation studies, dynamic light scattering (DLS), agarose gel electrophoresis, transmission electron microscope (TEM) and scanning electron microscope (SEM).

Commercial calf thymus DNA was purified to be devoid of single-stranded sections and proteins. First, 0.1 M Salinesodium was used to dissolve DNA (2 mg/ml). In a sealed cylinder or flask, citrate (SSC) buffers (pH 7.3) were extracted using an equivalent amount of chloroform-isoamyl alcohol (24:1) for one hour while being continuously shaken. The DNA found in the separated aqueous layer was extracted again using a chloroform-isoamyl alcohol mixture, and then it was precipitated using two volumes of cold ice.

After being collected on a glass rod, purified DNA was allowed to air dry before being dissolved in acetate buffer (30 mM sodium acetate with 30 mM zinc chloride, pH 5.0) and treated with S1 nuclease (150 units/mg DNA) for 30 minutes at 37 °C to eliminate single-stranded areas. The reaction was then stopped by adding a tenth volume of 200 mM EDTA, pH 8.0. Using chloroform-isoamyl alcohol, S1 nuclease-treated DNA was extracted, and two liters of ice-chilled absolute ethanol were used to precipitate the result. Ultimately, the necessary buffer was used to dissolve the precipitate. The A260/A280 ratio was used to verify that the calf thymus DNA (ct-DNA) was pure and free of proteins, RNA, and single strands. DNA from the calf thymus (50 lg/ml) was treated with different quinacrine concentration as a time function. The reaction mixture was thoroughly dialyzed against PBS for the data, which is for 5 mM Quinacrine at 37 °C for 1 hour in 0.1 M phosphate buffered saline (PBS buffer, pH 7.4).

To evaluate the change in DNA's thermal stability following modification, thermal denaturation analysis was performed. N-DNA's midpoint melting temperature (Tm) and the Shimadzu UV-2400 spectrophotometer, which has a temperature

programmer and controller unit, was used to heat denaturize Q-DNA samples in order to assess them. Every sample was melted at a rate of 1.0 C per minute between 30 and 90 degrees Celsius. As the temperature increased, the change in absorbance at 260 nm was noted.

A Fourier transform infrared spectrometer (FT-IR 8200) (Shimadzu Corp., Kyoto, Japan) was used to measure the infrared (IR) absorption spectra of the N-DNA and Q-DNA Japan. After quinacrine and DNA were incubated for two hours, a spectrum was obtained. For every sample, roughly 128 scans were obtained using 4 cm-1 in the spectral region of 4000–650 cm-1. Background spectra were gathered before every measurement. Every measurement was carried out in a controlled setting with a relative humidity of 45% at room temperature [7, 8]. N-DNA was used as a control in a subtraction of the buffer solution spectra from N-DNA and Q-DNA.

For vaccination, female New Zealand White (NZW) rabbits weighing between 1.0 and 1.5 kg were chosen. Four rabbits received vaccinations, two for each of the N-DNA and Q-DNA antigens. 50 lg of antigen complexed with methylated BSA in a 1:1 (w/w) ratio and emulsified with an equivalent volume of Freund's complete adjuvant were administered intramuscularly at various sites. For seven weeks, booster doses of the same quantity of antigen were administered weekly in Freund's incomplete adjuvant. Seven days after the boost, test bleeds were conducted to measure the antibody titer. After eight weeks, the animals were bled, the serum (immune and preimmune) was extracted from the blood, heated for 30 minutes at 56°C to deactivate complement proteins, and then preserved at -20°C with sodium azide. The study protocol was approved by the Institutional animal ethics committee.

Maxisorp polystyrene immunomodules with a flat bottom were subjected to ELISA. In short, 100 ll of N-DNA or Q-DNA (2.5 lg/ml in TBS, pH) was applied to immunomodules. 7.4) and incubated for two hours at 37°C then for the entire night at 4°C. Duplicates of each sample were coated, and half of the modules which had no antigen acted as a control.

The test immunomodules' wells were cleaned three times with TBST to get rid of any unbound antigen, and any empty spaces were plugged for four to five hours at 37 °C using 150 ll of 1.5% non-fat skimmed dry milk (in TBS, pH 7.4). Competition ELISA was used to determine the antibody specificity. A fixed quantity of IgG was permitted to interact with inhibitors at varying concentrations (0–20 lg/ml). (20 lg/ml) to allow the immunological complex to develop for two hours at 37 °C and overnight at 4 °C. The combination was added to wells coated with antigens, and the bound antibodies were examined in accordance with our lab's established technique. [9].

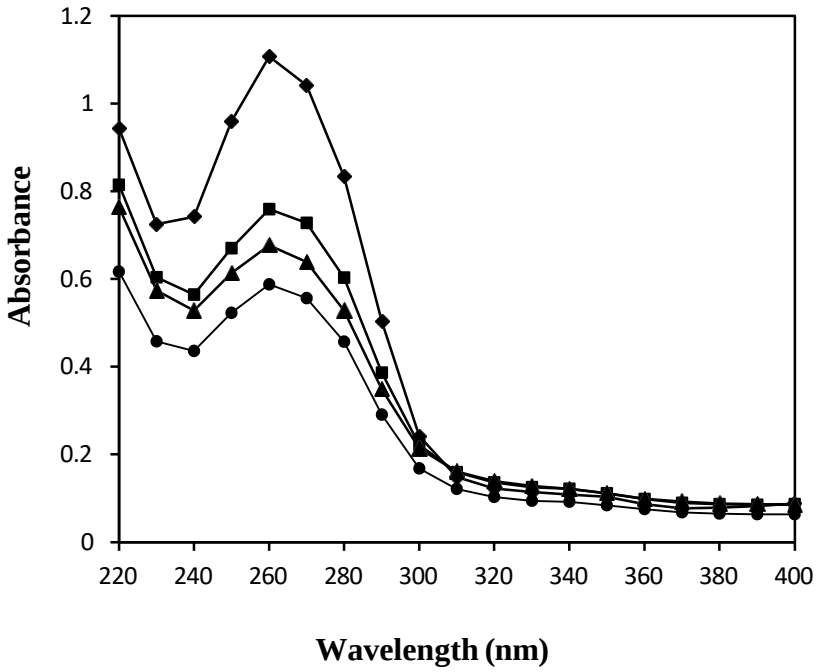
Competitive-inhibition ELISA was used to determine the antigenic specificity against different inhibitors. For two hours at room temperature, 100 of antigen was applied to the ELISA plates then stored at 4 C for the night. A constant quantity of affinity-purified antibody was treated with varying concentrations of inhibitors (0–20 lg/ml) for two hours at room temperature and then overnight at 4 °C. The resulting immunological complex was covered with wells. Using paranitrophenyl phosphate as a substrate, bound antibodies were tested using anti-rabbit alkaline phosphatase. Each well's absorbance was measured using an ELISA microplate reader at 410 nm.

Statistical analysis

Data are expressed as mean ± standard deviation (±SD). Statistical significance of the results was determined by Students's t-test and a p value of <0.05 was considered as significant.

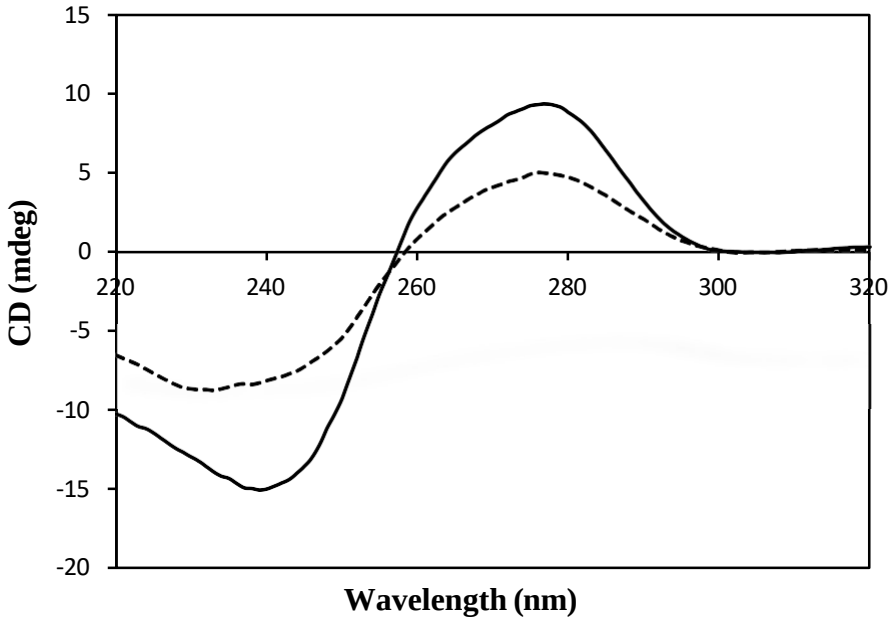
Results-

Figure 1- UV absorbance spectra of native calf thymus DNA (-♦-), modified calf thymus DNA in presence of 5 mM (-■-), 10 mM (-▲-), 15 mM (-●-) increasing concentration of quinacrine after one hr incubation. The above result is an average of 3 independent experiments.



Different concentrations were used to modify calf thymus DNA (50 µg/ml) for different incubation time (1 hr, 2 hr) at 37 °C. Optimum hypochromicity was observed at 260 nm.UV analysis of native and DNA modified by different concentration of quinacrine was performed on UV-1700 spectrophotometer. Native DNA showed maximum absorbance at 260 nm whereas upon modification with different increasing concentrations of quinacrine, DNA showed hypochromicity at 260 nm. The percentage hypochromicity observed for DNA at 5 mM, 10 mM,15 mM at one and two hr were 35%, 39% and 47% and 35%, 43% and 52% respectively compared to native form of DNA.

Figure 2-CD spectra of native (-) and quinacrine modified (---) DNA.



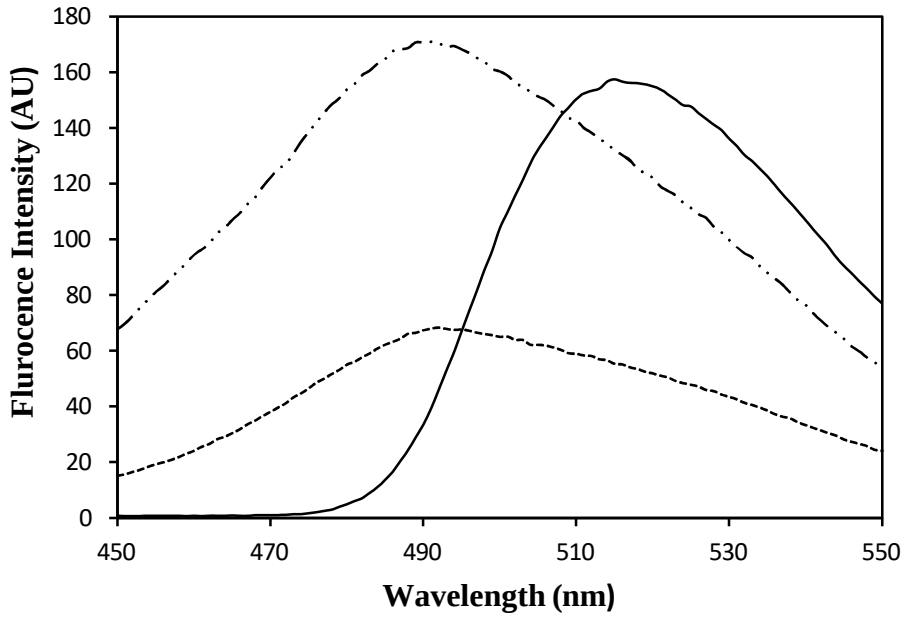
The above result is an average of 3 independent experiments. CD profile of native DNA showed a negative peak at 240 nm and a positive peak 276 nm (Fig 11). However, upon modification at 5 mM of quinacrine, there is decrease in ellipticity from 9 mdeg to 4 mdeg at 276nm and increase in elliptical form -15 mdeg to -8 mdeg at 240 nm.

Table 1- Vibration bands assigned to various components of nucleic acids.

Wave number (cm^{-1})	Assignment
1800–1500	Purine and pyrimidine vibrations
1500–1250	Vibrational coupling between base and sugar
1250–1000	Sugar phosphate chain vibration
1000–700	Sugar and sugar phosphate vibration

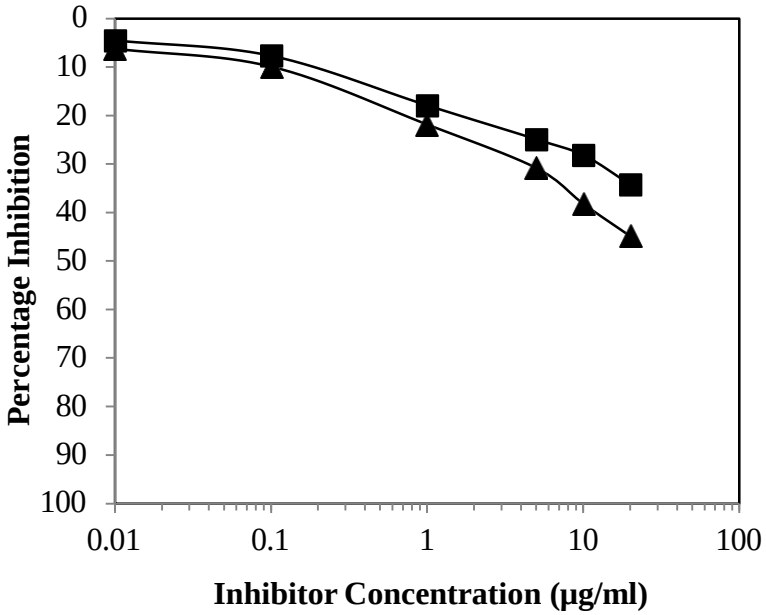
Some important marker bands of nucleic acids are depicted in Table1. Native and quinacrine modified DNA was subjected to FT-IR spectroscopy in the vibration region of 700–1800 cm−1. The band at 1638 cm −1has been assigned to adenine (A) while those at 1384 cm −1 and 1086 cm −1 are attributed to asymmetric and symmetric stretching of phosphate in backbone of DNA upon modification with 5 mM of quinacrine (Fig 14) and three vibration bands of FTIR showed a shift of bands from 1618 cm −1 to 1638 cm −1 , 1083 cm −1 to 1086 cm −1 and 1321 cm-1 to 1384 cm-1(Fig 14).All these shifts observed in FTIR is a clear indication of perturbations in the native conformation of DNA.

Figure 3- Fluorescence emission spectra of quinacrine (-), native DNA (---) and DNA modified with quinacrine 5 mM (-.-.-.) excited at an excitation wavelength of 420 nm. The above result is an average of 3 independent experiments.

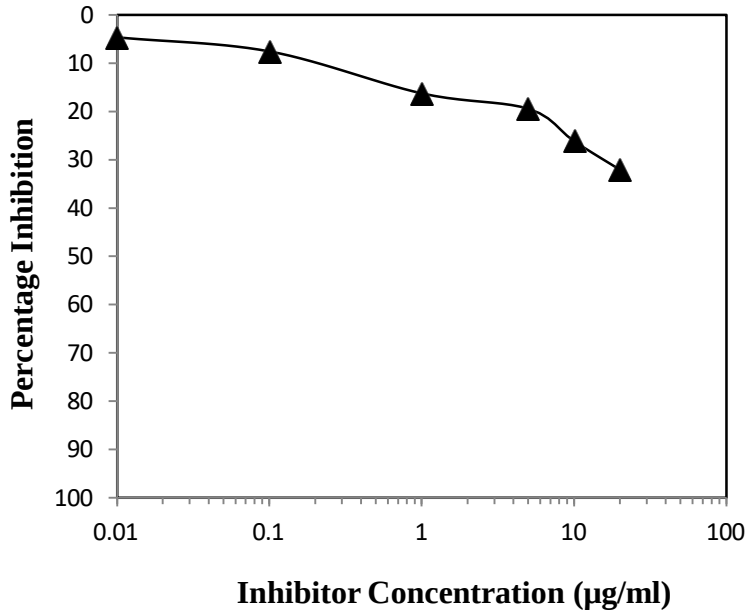


Quinacrine is a compound which has its own intrinsic fluorescence. Quinacrine modified DNA was assessed after excitation of samples at 420 nm. Increase in emission intensity at 492 nm suggests presence of modification in DNA, which accounted to almost 60% increase in fluorescence at 5mM quinacrine modified DNA after 2 hr incubation (Fig. 15) also there was observable shift in the emission wavelength from 490 nm to 492 nm whereas the maximum fluorescence intensity of the quinacrine was found at 515 nm.

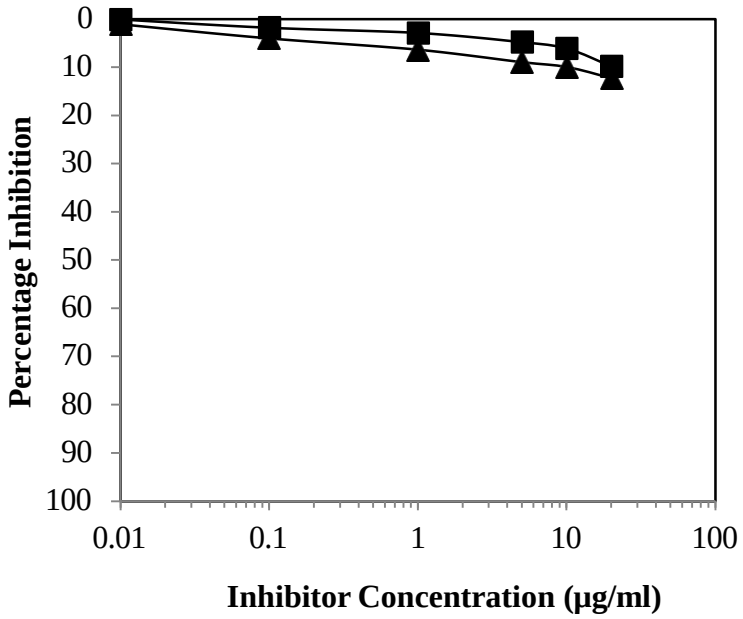
Figure 4- (a,b,c)



a- Inhibition ELISA of anti-quinacrine-DNA IgG by 4-Chloro-o-phenylenediamine modified calf thymus DNA (▲) and carbamylated modified calf thymus DNA (■). Microtitre modules were coated with quinacrine modified DNA (2.5 µg/ml)



b- Inhibition ELISA of anti-quinacrine-DNA IgG by fructose modified calf thymus DNA (▲). Microtitre modules were coated with qunacrine modified DNA (2.5 µg/ml).



c- Inhibition ELISA of anti-quinacrine-DNA IgG by glyoxal modified human LDL (▲) and acetaldehyde modified human IgG (■). Microtitre modules were coated with qunacrine modified DNA (2.5 µg/ml).

Antigenic specificity of anti- quinacrine-DNA IgG with various inhibitors was evaluated by competitive inhibition ELISA to ascertain that the induced antibodies were highly specific for quinacrine modified DNA. The induced antibodies showed heterogeneity in recognizing a variety of inhibitors at maximum inhibitor concentration of 20 µg/ml. 4-Chloro-o- phenylenediamine modified calf thymus DNA, fructose modified calf thymus DNA, carbamylated modified calf thymus DNA, glyoxal modified human LDL and acetaldehyde modified human IgG were used as inhibitors, caused 44.85%, 34.2%, 32%, 12.2% and 9.8% inhibitors respectively in the binding of anti- quinacrine-DNA IgG to quinacrine modified DNA.

Discussion-

Quinacrine is a hetrocyclic three ring compound and an acridine derivative which interacts with double stranded DNA (dsDNA) to form adducts by intercalation, groove binding and covalent binding. It is a FDA approved drug for malaria, giardiasis and tapeworm infection. In this present study, characterization of native and quinacrine modified calf thymus DNA was performed using various physicochemical techniques. The UV absorption spectra of quinacrine modified DNA at increasing concentrations of quinacrine showed hypochromisity at 260 nm after one and two hr of incubation, but significant hypochromisity is observed when DNA was incubated with quinacrine after two hr incubation. This is due to the presence of an acridine “backbone” that confers a planar structure to the molecules, allowing them to intercalate into DNA, stacking and binding with the major groove predominantly and minor groove by diaminobutyl side chain [10].

Circular dichorism (CD) is a technique that is sensitive to the conformation of anisotropic molecules and chiral super assemblies which is used to study the structure of macromolecules including DNA; it also gives information about base pairs stacking. This phenomenon is demonstrated by the absorption bands present in any optically active molecule. As a result, various bio molecules exhibit this phenomenon due to their dextrorotatory and levorotatory components. CD profile of native DNA showed major peak at 240 nm (negative) and 276 (positive) peak whereas quinacrine modified

DNA showed decrease in ellipticity from 9 mdeg to 4 mdeg at 276 nm and increase in elliptical form -15 mdeg to -8 mdeg at 240 nm. These bands are considered as a marker for double helical DNA in B-Conformation [11] where change in the positive peak indicates change in the nitrogenous bases and negative peak represents helixity of DNA.

Further, evaluation of the stability in the structure of DNA was analyzed by studying the melting properties of native and quinacrine modified DNA. Temperature melting profile, showed stability of the quinacrine modified DNA by both increase in onset of unwinding and melting temperature when compared with native calf thymus DNA which were from 70 °C to 76 °C and 84 °C to 87°C respectively [12]. These changes may be attributed due to the formation of more rigid and stable structure as a result of binding of quinacrine to major groove as well as its intercalation between the bases and the diaminobutyl side chain interaction with the minor groove which is considered more important in temperature stabilization of quinacrine modified DNA. A 3 °C rise in the melting temperature was found in case of quinacrine modified DNA when compared to its native counterpart.

The acquired fluorogenic nature of the quinacrine modified DNA was confirmed by increase in the emission intensity as well as shift in peak from 490 nm in case of native DNA to 492 nm in quinacrine modified DNA when excited at 420 nm. Furthermore, the increase in fluorescence intensity of quinacrine modified DNA was calculated to be 60% which is a significant change and this confirms interaction of quinacrine with calf thymus DNA. It is a well-documented that quinacrine itself a highly fluorogenic drug [13] which showed peak at 515 nm.

Antibodies against quinacrine modified DNA were induced in experimental animals by injecting quinacrine modified DNA complexed with methylated bovine serum albumin in presence of Freunds complete and Freunds incomplete adjuvant . The quinacrine-DNA was found to be better imunogen as revealed by the high titre of induced antibodies (>1:6400) in direct binding ELISA and inhibition ELISAs were performed using pre-immune sera and immune sera and it was observed that the antigenic specificity of anti- quinacrine- DNA antibodies showed an inhibition of 89.2% when quinacrine modified DNA is used as an inhibitor at a concentration of 20µg/ml. The concentration required to attain 50% inhibition was calculated to be 3.5 µg/ml whereas only 19.23% inhibition was observed when native DNA was used as an immunogen. These results showed anti-quinacrine-DNA antibodies recognized quinacrine modified DNA more as compared to native DNA therefore in order to assess binding specificities of these antibodies; IgG was isolated from both pre-immune and immune sera. Inhibition ELISAs were performed using pre-immune IgG and immune IgG and it was observed that the antigenic specificity of anti- quinacrine- DNA IgG showed an inhibition of 90.9% when quinacrine modified DNA is used as an inhibitor at a concentration of 20µg/ml . The concentration required to attain 50% inhibition was calculated to be 2.4 µg/ml whereas only 22.8% was observed when native DNA was used as an immunogen and this implies that majority of the antibodies repertoire are directed only against quinacrine modified DNA. These results were significant at $p < 0.05$.These result show the recognition of new epitopes generated by quinacrine modified DNA by the immune IgG. Cross reactivity of anti-quinacrine-DNA IgG with 4-chloro-o-phenylene diamine modified calf thymus DNA, fructosylated calf thymus DNA, carbamylated calf thymus DNA, glyoxal modified human LDL and acetaldehyde modified human IgG showed inhibition about 44.85%, 34.2% 32%, 12.2% and 9.8% respectively in the binding of anti-quinacrine-DNA IgG to quinacrine modified DNA. This also again proves the fact that anti-quinacrine-DNA IgG is more specific to quinacrine modified lesions on DNA.

Conclusion-

Quinacrine an antimalarial drug, a heterocyclic three ring compound and an acridine derivative is known to interact with double stranded DNA (dsDNA) to form adducts by three general modes 1. Intercalation, 2. Groove binding and 3. Covalent binding. Quinacrine modified calf thymus DNA showed 37% hypochromic effect on treatment with 5 mM

quinacrine after 2 hr incubation.Quinacrine modified DNA in Circular dichroism showed change in the positive ellipticity which indicates change in the nitrogenous bases whereas change in negative ellipticity represents changes in the helicity of DNA.

After characterization, quinacrine modified DNA was used as an antigen for immunization in New Zealand White rabbit to raise antibodies against it. High titre antibodies were obtained (>1:6400) which were characterised through direct binding ELISA. Binding specificities were confirmed by competition ELISA and it showed that anti-quinacrine-DNA antibodies recognize quinacrine induced lesions on DNA. Formation of immune complex formation was visualized by band shift assay.

Anti-quinacrine-DNA antibodies were found to be cross reactive with DNA isolated from peripheral lymphocytes of hepatocellular carcinoma patients whereas these antibodies did not show any binding with native form of DNA, so it could be used as an immunological probe to detect early lesion in cases of hepatocellular carcinoma.

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