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DNA minor groove binder Netropsin alters chromatin structure

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Introduction

Netropsin is an oligopeptide with antibiotic and antiviral activity. (Hahn et al, 1975). Netropsin was first isolated from the actinobacterium *Streptomyces netropsis*. It belongs to the class of pyrrole-amidine antibiotics. This drug is considered to be a classic minor groove binder (Wartell et al, 1974). Netropsin displaces the spine of hydration and fits within minor groove in A-A-T-T center. It widens the groove slightly and bends the helix axis back by 8 degrees, but neither unwinds nor elongates the double helix. The drug molecule is held in place by amide NH hydrogen bonds that bridge adenine N-3 and O-2 atoms, exactly as with spine of hydration (Kopka et al, 1985).

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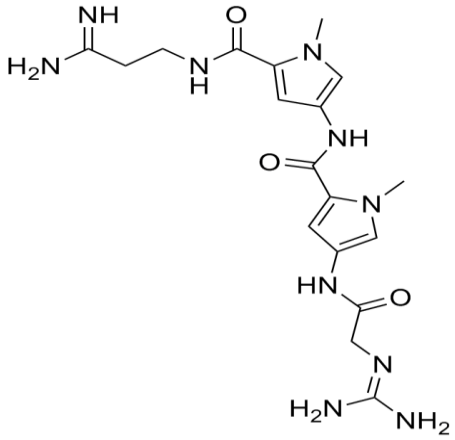

Netropsin

Over the past few decades, the interactions of small molecules, such as putative antitumor agents to chromatin and DNA have been extensively studied using various biophysical techniques. Most of the molecules interfere with gene regulation, and therefore some of them act as chemotherapeutic agents. Netropsin has preference for A/T rich sites; it is an effective probe to characterize the behaviour of different DNA backbone structures towards DNA binding ligands (Rentzeperis et al, 1995). DNA-ligand interactions in cell have higher level of complexity due to the presence of protein that is intimately associated with the template DNA. These proteins scaffold the DNA to form a higher order packaged structure called chromatin (Luger et al). The proteins give it structure and at the same time, regulate its accessibility towards various ligands. The basic repeat element of chromatin is the nucleosome, interconnected by sections of linker DNA. The nucleosome core particle, together with histone H1, is known as a chromatosome.

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Many biophysical studies have helped to elucidate the mode of binding to the chromosomal DNA, the stoichiometry, and the kinetics of the association of the drugs to DNA. However most of the studies have focused on natural DNA and chromatin. It is also important to examine how the presence of histone structure proteins might affect the affinity and stoichiometry of the ligand binding.

In the present study mainly two biophysical approaches, spectrofluorimetry and isothermal titration calorimetry have been employed. First we have done fluorimetric titration of netropsin with chromatin and chromosomal DNA to evaluate the dissociation constants. We also employed isothermal titration calorimetry to evaluate the binding parameters and thermodynamic features for the association of netropsin with soluble chromatin and chromosomal DNA. It helps us to know about the binding preference of the molecule. Dynamic light scattering was done to elucidate the effect of classical minor groove binder netropsin upon hydrodynamic size of soluble chromatin.

Materials and methods

Materials

Netropsin, chicken liver, Tris, $MgCl_2$ (1M) ,$CaCl_2$ (0.1M), PMSF (phenyl-methyl-sulfonylfluoride), EDTA (0.5M) , Triton X 100 (10%) , $NaHSO_3$ (0.5M), sucrose, sodium acetate, sodium chloride, micrococcal nuclease, 100 base pair

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DNA ladder, 6x loading dye, agarose, boric acid, isopropanol, phenol:chloroform:isoamyl alcohol.

Methods

Preparation of netropsin

Netropsin was dissolved in 10 mM Tris-HCl pH 7.5 and 15 mM NaCl and left overnight at -20^{0}C. Concentration was determined using molar extinction coefficient of 21500$M^{-1}cm^{-1}$ at 295nm with CECIL 7500 spectrophotometer. Stock solutions were stored at 4^{0}C.

Isolation of chromatin from chicken liver

Chicken livers were stored at -80^0 C for long term. Prior to isolation of chromatin, the livers were kept at -20^{0}C overnight and transferred to 4^{0}C on the day of isolation.

Chromatin was isolated from the nuclei by micrococcal nuclease digestion (Kornberg, 1974, Blobel and Potter et al, 1966). Nuclei were suspended in appropriate amount of digestion buffer. For micrococcal nuclease digestion of the nuclear suspension, calcium chloride was added to a final concentration of 2mM and incubated at 37°C for 2 minutes. In general, 20µl of micrococcal nuclease was added to 400µl nuclear suspension. Enzymatic digestion was carried out for 30 seconds. The reaction was stopped by addition of 20mM

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EDTA, followed by chilling on ice. The suspension was centrifuged at 8,000 rpm for 8 minutes, and the supernatant was discarded.

Chromatin was extracted by lysis of the micrococcal nuclease digested nuclei.

This was achieved by suspension of the pellet in appropriate amount of lysis buffer and incubation on ice for 30minutes, followed by centrifugation at 10,000 rpm for 10 minutes. Chromatin was obtained in the supernatant. The soluble chromatin was dialysed against 10 mM Tris pH 7.5 and 15 mM NaCl buffer at 4°C over night.

Agarose gel electrophoresis of chromatin

Chromatin was mixed with DNA loading dye in 5:1 proportion and loaded onto a 1.5% agarose gel in 0.5X TBE and containing 10 µl ethidium bromide. Electrophoresis was carried out in 0.5X TBE at 80V for 1 hour and 10 minutes. Chromatin was visualized under U.V. trans illuminator and photograph was taken.

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Figure 1

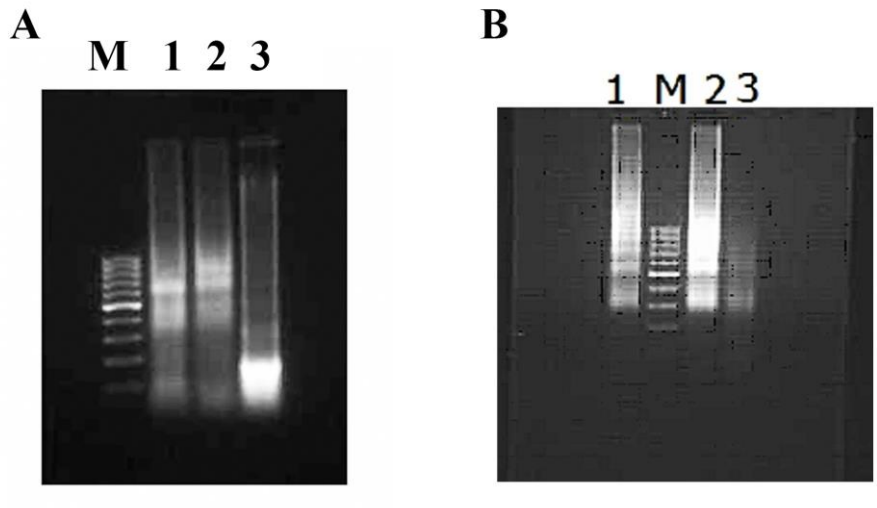

Figure 1: **A:** M indicates 100 bp DNA ladder. Lane 1 indicates undialyzed chromatin. Lane 2 indicates dialyzed chromatin. Lane 3 indicates chromosomal DNA. **B:** M indicates 100 bp DNA ladder. Lane 1 and 2 indicate dialyzed chromatin.

Preparation of chromatosome

Liver nuclei, isolated as described, were suspended in digestion buffer. For digestion, calcium chloride was added to the nuclear suspension. The final concentration of calcium chloride was adjusted to 2 mM. Prior to addition of micrococcal nuclease, the reaction mix was incubated at 37°C for 2 minutes. 20µl of micrococcal nuclease was added to 400µl of nuclear suspension and incubated at 37°C for 5 minutes. The reaction was stopped by addition of 20 mM EDTA, followed by chilling on ice. It was centrifuged for 8 minutes at 8,000 rpm at 4°C.

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The supernatant was rejected. The pellet was resuspended in appropriate amount of lysis buffer, incubated on ice for 30 minutes, and recentrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant contained chromatosomes, along with some longer chromatin fragments. The volume of the supernatant was reduced to a certain volume in a concentrator. Sucrose gradient was prepared with different concentrations of sucrose (5%, 10%.15%, 20%, 25%, 30%). These sucrose gradient contained Tris (final concentration 10 mM) and EDTA (final concentration 2 mM). The supernatant was layered on the top of the gradient. Centrifugation was carried out for 17 hours at 35,000 rpm in AH650 rotor of OTD65B Sorvall Ultracentrifuge. Fractions were collected by tube puncture method and analyzed on 1.5% agarose gel containing ethidium bromide.

Concentration of soluble chromatin and chromatosome were determined using molar extinction coefficient of 6600 $M^{-1}cm^{-1}$ at 260 nm with CECIL7500 spectrophotometer.

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Figure 2

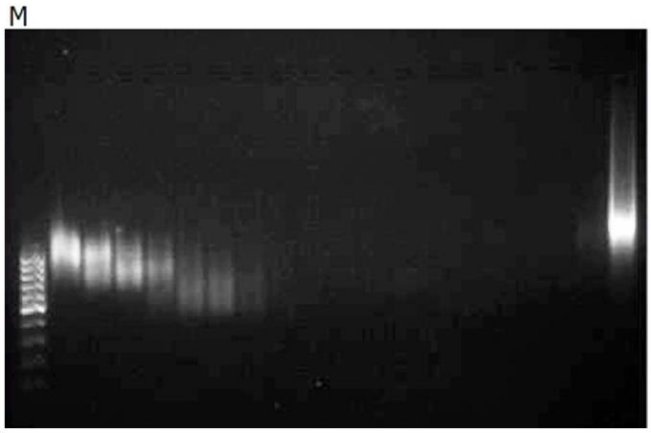

Figure 2: Size fractionation of chromatin. M indicates a 100 bp ladder. Lanes indicated from left to right contain samples fractions collected from the bottom of the tube. So, they correspond to gradually decreasing concentration of sucrose.

Preparation of chromosomal DNA from chromatin

Chromosomal DNA was isolated from unfractionated chromatin. Chromatin sample was mixed with 0.1% SDS solution and vortexed well for 1 hour. This step ensured the denaturation of proteins present in chromatin, and their subsequent separation from chromosomal DNA. Equal volume of phenol: chloroform: isoamyl alcohol (25:24:1 v/v) was added, and vortexed vigorously. The emulsion was then centrifuged at 13,000rpm for 10 minutes at 4^0C. The aqueous layer was carefully removed into a clean tube and re-extracted in a similar manner. After 2 extractions with phenol:chloroform:isoamyl alcohol, the

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aqueous layer was extracted with an equal volume of chloroform by centrifuging at13000r.p.m for 10 minutes at 4^0C. For precipitation of DNA, the pH of the solution was adjusted to 5.2 by addition of 1/10th volume of 3M sodium acetate (pH 5.2). Equal volume of isopropanol was added, and the mixture was chilled at -20°C overnight or at -80°C for 1 hour. The DNA was collected by centrifugation at 13000 r.p.m for 15 minutes at 4°C. The DNA pellet was washed with 70% ethanol at 13000 r.p.m for 25 minutes and the pellet was dried in air. It was dissolved in appropriate amount of 10 mM Tris pH 7.5 and 15 mM NaCl buffer and checked for purity and concentration at 260 nm with molar extinction coefficient 6600$M^{-1}Cm^{-1}$ spectrophotometrically, as well as by 1.5% agarose gel electrophoresis.

Figure 3

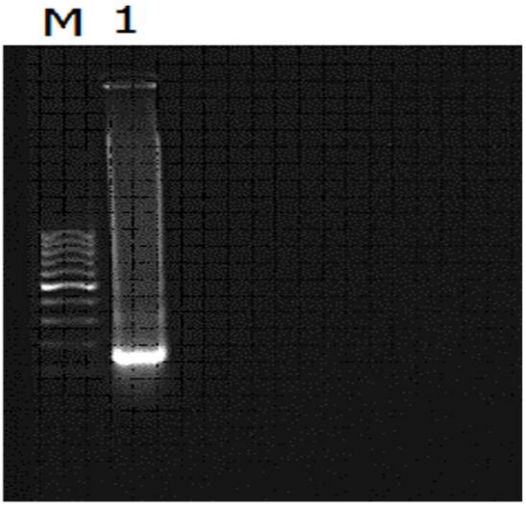

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Figure 3: M indicates 100 bp DNA ladder. Lane 1 indicates chromosomal DNA isolated from chromatin

Fluorescence spectroscopy

Fluorescence titrations of netropsin and chromatin and chromosomal DNA were performed on Perkin Elmer LS55 Luminescence Spectrometer using 1cm path length cuvettes. 10 μM netropsin was titrated against increasing concentrations of chromatin. Samples were excited at 330 nm and the emission spectra were recorded from 400nm to 600nm. The excitation and emission slit widths were adjusted to 10nm each and the spectra obtained were averaged for 4 accumulations, each at 200nm/min scan speed. Fluorescence intensity values at 440nm were utilized to obtain the titration curve at 25°C. Results from fluorimetric titrations were analyzed to evaluate binding parameters in the following manner.

The binding of ligand to polynucleotide (i.e. DNA or chromatin) was assumed to follow the equilibrium (Chakrabarti et al., 2000, Mir et al., 2003):

L + P= L-P

Where L, P and L-P denote the ligand, polynucleotide, and ligand polynucleotide complexes respectively.

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$\mathbf{K_d = [L][P]/ [L-P]}$

$\mathbf{L = [L]^0 – [L-P]}$ and

$\mathbf{P = [P]^0 – [L-P]}$

(Superscript zero denotes the input concentration for each of them.)

$\mathbf{[L]^0}$ = initial concentration of the drug

$\mathbf{[P]^0}$ = initial concentration of the polymer

[L-P] = concentration of Ligand- polymer complex

K_d = dissociation constant

$\mathbf{K_d = [L][P] / [L-P] = \{[L]^0 – [L-P]\}.\{[P]^0 – [L-P]\} / [L-P]}$

As discussed, difference in fluorescence intensity from initial fluorescence of drug in absence of DNA is a function of amount of drug-DNA complex formed, i.e.

$\mathbf{\Delta F = f [L-P]}$

Fraction abound = $\mathbf{\Delta F / \Delta F_{max}}$ where $\mathbf{\Delta F_{max}}$ is the intensity change at maximum binding.

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Hence $\Delta F / \Delta F_{max} = [L-P]/ [L]^0$

$K_d = \{[L]^0 – (\Delta F / \Delta F_{max}).[L]^0\}.\{[P]^0 – (\Delta F / \Delta F_{max}).[L]^0] / \{(\Delta F / \Delta F_{max}).[L]^0\}$

Considering C_p = concentration of polymer and C_0 = initial concentration of drug we can write

$K_d = [C_0- (\Delta F / \Delta F_{max}). C_0] [C_p – (\Delta F / \Delta F_{max}). C_0] / [(\Delta F / \Delta F_{max}). C_0]$

$C_0 (\Delta F / \Delta F_{max})^2 – (C_0 + C_p + K_d) (\Delta F / \Delta F_{max}) + C_p = 0$

This is of a quadratic form: $ax^2 + bx + c = 0$. Fitting the titration data to this equation using Origin software evaluated the K_d value which is a part of the

root evaluated by the quadratic root determination formula. Note that K_d is the apparent dissociation constant as binding was considered 1:1 in the equation.

A double reciprocal plot of 1/ΔFmax against 1/ (C_p-C_0) was used for the determination of ΔF_{max} using

$1 / \Delta F = 1 / \Delta F_{max} + K_d/ [\Delta F_{max} (C_p- C_0)]$

ΔFmax was calculated from the slope of the best fit line corresponding to the above plot. ΔF/ ΔFmax denotes the fraction of ligand bound to DNA. This equation is valid under the condition $C_p >> C_0$ is maintained during the collection of data for the construction of the plot.

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Isothermal titration calorimetry (ITC)

Soluble chromatin, chromosomal DNA were titrated against netropsin solution in 10mM Tris HCl (pH 7.5), 15mM NaCl. For titrations with chromatin, and chromosomal DNA, typically 2 ml of macromolecule (cell concentration 0.2mM) were loaded in the calorimetric cell was titrated against 1mM of the netropsin solution (29 injections of 10 µl each with an initial injection of 1µl) using a 289 µl syringe, rotating at 307 r.p.m. ITC measurements of netropsin dilution in buffer served as control. Calorimetric titrations were performed at 25^0C for chromatin and chromosomal DNA in a MicroCal VP-ITC microcalorimeter. Reference power was12 µcal/sec.Spacing between 2 injections is 300 seconds. Duration of injections is 20 seconds. The resulting thermograms were analyzed using 'single set of binding sites' model of Levenberg – Marquardt non-linear least squares curve fitting algorithm, inbuilt in the MicroCal LLC software. The apparent association constant *Ka*, site size *n* and molar heat of binding ΔHb were obtained using the following relation:

$K_a = \theta/(1-\theta)[X]\theta/(1-\theta)[X]$

Where θ = fraction of sites occupied by ligand X, and $[X]$ = concentration of free ligand. Therefore, the total concentration of ligand (free and bound), X_t is given by

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$X_t=[X] + n\theta M_t$

Where M_t is the bulk concentration of macromolecule in the active cell volume *Vcell*. The total heat content *Q* of the solution in the active cell volume is

$$\mathbf{Q = n_{\theta} M \Delta H V_{cell}}$$

Binding free energy and entropy were obtained using the relation $\Delta G = -RTlnK_a = \Delta H - T\Delta S$, where R signifies the universal gas constant

Dynamic light scattering (DLS)

Dynamic light scattering was performed on a Zetasizer Nano S particle analyzer from Malvern Instruments, UK. The light source was a He-Ne laser (632.8 nm) that utilizes 4 mW power at the same wavelength.

Scattered light from the samples was collected at an angle of 173° and the intensity autocorrelation function was utilized to generate a correlation curve.

Translational diffusion coefficients (D) were obtained from the homodyne autocorrelation function defined by:

$$G(\tau) = A_{[}1+B\exp_{(-}{}^{2\Gamma}{}_{\tau})]$$

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Where $G(\tau)$ is the correlation coefficient, A is the amplitude of the correlation function, and B is the baseline.

$$\Gamma = Dq2$$

Where D is the Stokes-Einstein diffusion coefficient and q is the scattering vector.

Cumulants analysis of the correlation curve was used to obtain the intensity weighted mean hydrodynamic diameter or Z_{av} diameter of the ensemble of particles in the measurement window.

In order to study the effect of netropsin on the hydrodynamic size of soluble chromatin , the sample (400µM) was treated with netropsin in drug to DNA base ratio of 0, 0.05, 0.1 and 0.2,0.3 at 25°C and the hydrodynamic measurements were monitored by DLS. The mean of the Z_{av} diameters were obtained from 10 measurements for each sample.

Chromatosome stability assay

A solution of netropsin was prepared in 10 mM NaCl containing 15mM Tris HCl (pH 7.5), and the concentration was determined using molar extinction coefficient of 21500 $M^{-1}cm^{-1}$ at 295 nm and diluted to 1mM stock concentration. Chromatosome isolated from chicken liver, was incubated with netropsin in drug

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to DNA base ratio of 0, 0.05, 0.1 and 0.2, 0.3 for 90 minutes at 30^0C water bath. The samples were then analyzed by electrophoresis on 1.5% agarose gel in 0.5X TBE, followed by staining with ethidium bromide.

Results

Steady state fluorescence measurements

Figure 4

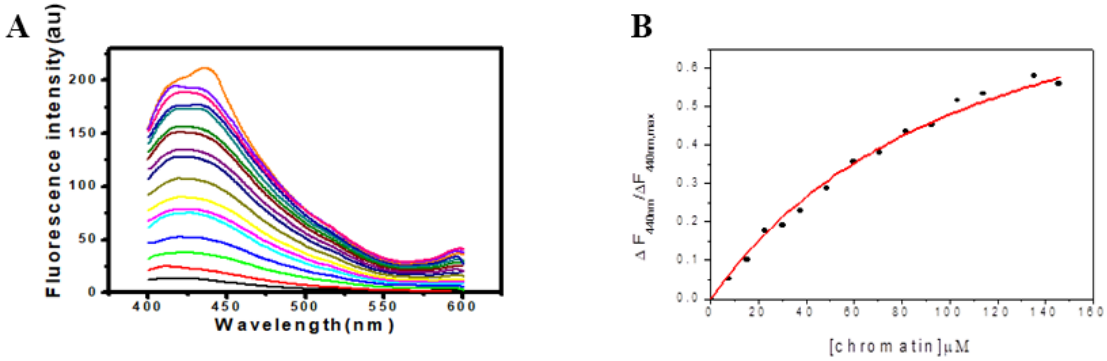

Figure 4: (A) - Analysis of binding netropsin with soluble chromatin by fluorescence spectroscopy. Fluorescence emission spectra (400-600 nm, λ_{ex}=330nm) of netropsin in prescence of increasing concentration of chromatin. (B)- Curve fitting analysis to evaluate dissociation constant for the interaction of netropsin with chromatin

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Figure 5

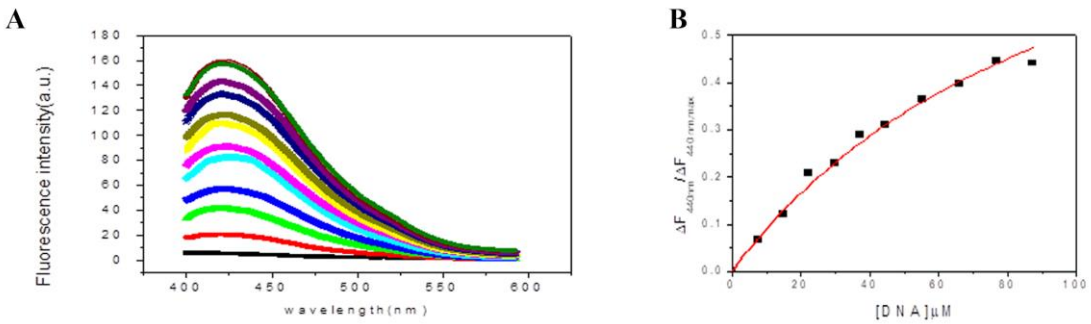

Figure 5: (A) - Analysis of binding netropsin with chromosomal DNA by fluorescence spectroscopy. Fluorescence emission spectra (400 – 600 nm, λex=330nm) of netropsin in presence of increasing concentration of chromosomal DNA. (B) - Curve fitting analysis to evaluate dissociation constant for the interaction of netropsin with chromosomal DNA.

Table 1:

K_d (soluble chromatin)	K_d (chromosomal DNA)
103.47μM	92.18μM

Free netropsin has very low intrinsic fluorescence. The fluorescence intensity is found to increase steadily upon complexation with DNA. The results of fluorescence titrations of netropsin with chromatin, and chromosomal DNA are shown in Figure 4 and 5 (A-B). Increase in the fluorescence quantum yield of the

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ligand upon binding to the polynucleotide originates either from a change in the local environment of the ligand or from an overall change in the conformation of the ligand upon binding to the polynucleotide. In all cases, the nature of the curves show a non-cooperative type of binding, characteristic of a single type of complex formation. Dissociation constant (K_d) values were estimated by means of non-linear curve fit analysis as described earlier. However, the method gives an average value of the dissociation constant. At 25°C, Netropsin binds to chromatin and chromosomal DNA with almost equal affinity. Comparatively, the affinity towards chromosomal DNA is higher than soluble chromatin.

Dynamic light scattering (DLS)

Dynamic light scattering (DLS) was used to study the effect of netropsin on the structure of soluble chromatin in solution. Netropsin causes compaction of soluble chromatin.

Table 2: Hydrodynamic diameter for interaction of soluble chromatin with netropsin

Sample	Netropsin (µM)	Z_{av} diameter (nm)
Chromatin(400µM)	0 20	102.27 97.40

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	40 80 120	93.23 84.89 82.45

The Z_{av} diameter obtained for each corresponding netropsin to DNA base ratios are shown in Table 2. The Z_{av} diameters are observed to decrease from 102.27nm to 82.45 nm (Table 2) in presence of increasing netropsin concentration.

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Figure 6

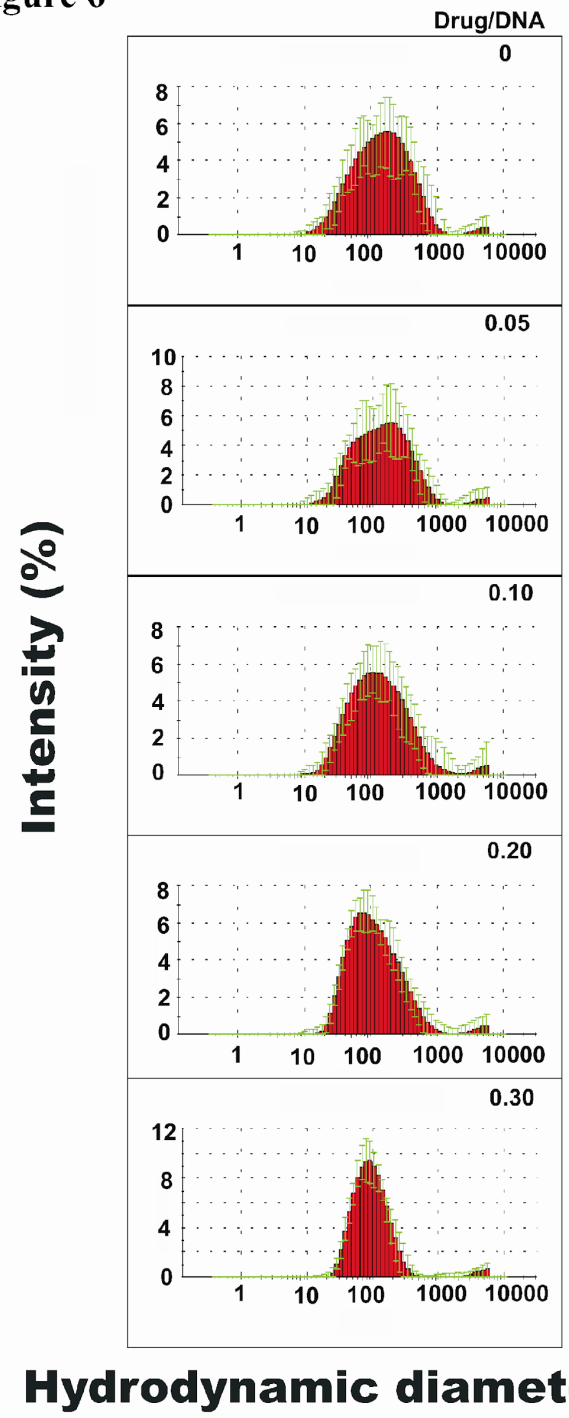

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Figure 6: Dynamic Light Scattering (DLS) to study the effect of netropsin on the hydrodynamic diameters of soluble chromatin. The intensity statistics of 10 measurements each are plotted for soluble chromatin (400μM) in presence of increasing concentrations of netropsin. Error bars indicate standard deviation.

Isothermal titration calorimetry (ITC)

Binding of netropsin to chromatin, and its chromosomal DNA was quantitated by means of isothermal titration calorimetry (ITC). Representative thermograms for the titration are shown in Figure 7. Binding parameters obtained by ITC are summarized in Table 3. At 25°C, the apparent association constants (K_a), for chromatin and chromosomal DNA are comparable. The binding constant of netropsin varies with increased histone content from $3.36x10^4\ M^{-1}$ to $1.47x10^4\ M^{-1}$. On the other hand, drug/bases ratio is 0.0383 for chromosomal DNA and 0.318 for chromatin.

At 25^0C the binding constant varies as follows,

K_a (chromosomal DNA)>K_a (chromatin)

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Figure 7

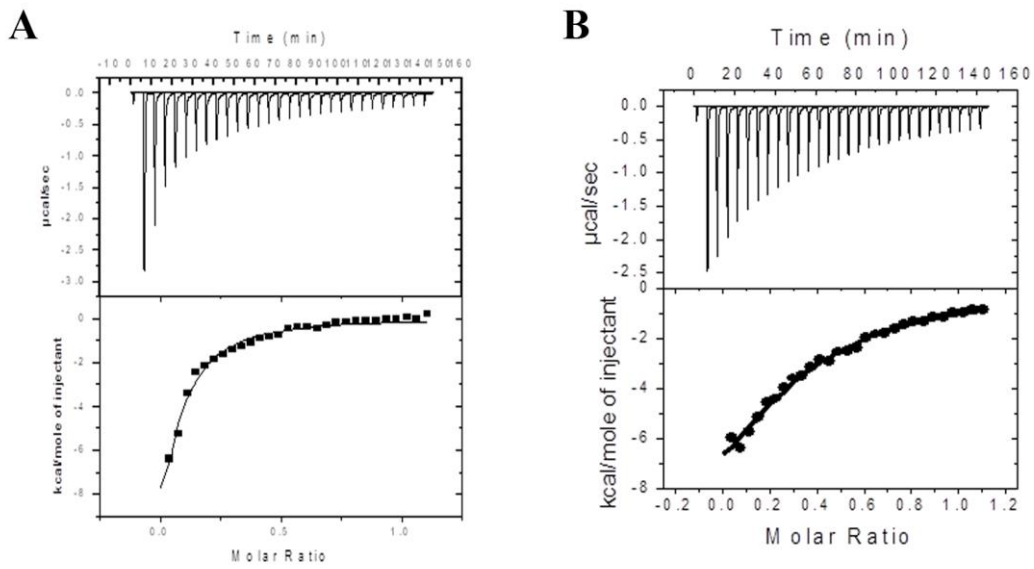

Figure 7: Isothermal titration calorimetry (ITC) profiles for the binding of netropsin to chromatin components. Titration profiles for the interaction of netropsin with (A) chromosomal DNA, (B) soluble chromatin. The experiments were performed in 10mM Tris HCl (pH 7.5), 15mM NaCl at 25ºC.

Table 3: Thermodynamic Data

Sample	Temp (^{0}C)	N (drug/ bases)	ΔH (kcal/ mol)	ΔS (kcal/ K/mol)	K_a (M^{-1})	ΔG (kcal/ mol)

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Chromatin	25^0C	0.318	-13.66	-26.7	$1.47x10^4$	-5.70
Chromosomal DNA	25^0C	0.0383	-38.17	-107	$3.36x10^4$	-6.28

Netropsin affects chromatosome stability

To study the effect of netropsin on chromatosome, we have compared the electrophoretic mobility of netropsin treated and untreated chromatosome on agarose gel. Chromatosomes were incubated with netropsin for 90 minutes at room temperature and it showed a distinctly different pattern of mobility, at and above drug to DNA base ratio of 0.05. DNA was released from chromatosomes in the netropsin treated samples, which was absent in case of netropsin untreated samples under similar conditions. Netropsin causes DNA release from chromatosomes at and above drug/bases ratio of 0.05.

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Figure 8

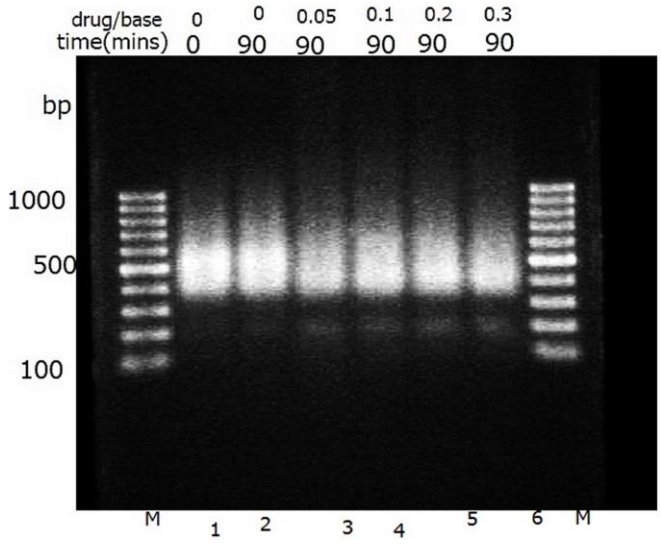

Figure 8: DNA release experiment of chromatosome by netropsin. Agarose gel electrophoresis to study the effect of netropsin on chromatosome. Chromatosome samples (255 μM) were incubated with netropsin at room temperature for 90 minutes at the drug/DNA base ratio indicated and analyzed on 1.5% agarose gel. M indicates 100bp DNA ladder. Chromatosomes incubated with buffer (lanes 1 and 2) served as negative controls. Lanes 3,4,5,6 are the netropsin treated samples at drug/DNA bases ratios of 0.05, 0.1, 0.2, and 0.3.

Discussion

Netropsin is a minor groove binder. Its interaction with soluble chromatin, chromosomal DNA and chromatosome has been studied extensively by various methods like fluorescence titration, isothermal titration calorimetry, dynamic

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light scattering and agarose gel electrophoresis. Chromatin and chromosomal DNA templates are expected to account for the binding of netropsin to histone wrapped DNA and protein free DNA respectively. The DNA component in all the systems is similar. This ensures that the difference in thermodynamic parameters obtained, does not arise from difference in DNA sequences (Breslauer et al., 1987). A comparative study of the systems would therefore help to identify the preferred ligand binding site in chromatin. Results from fluorescence and ITC are comparable. ITC results reveal comparable values of the binding constant K_a for chromatin and chromosomal DNA. Dynamic light scattering was used to investigate netropsin induced structural changes of soluble chromatin. DLS indicates that the hydrodynamic diameter of bulk soluble chromatin decreases upon netropsin treatment in a concentration dependent manner. The concentration dependence implies that the process occurs as a result of the association of chromatin with netropsin. Compaction of chromatin is hypothesized to occur by either of two mechanisms – linker DNA bending and internucleosomal angle contraction. DNA release experiment reveals that netropsin causes DNA release from chromatosomes at and above drug/bases ratio of 0.05.

The thermodynamic data indicates that the presence of histones modifies groove insertion process. The average stoichiometry and binding constants of netropsin are affected in case of chromatin as compared to naked DNA. These parameters gradually change from histone free DNA to chromatin. This supports that the ligand association is affected by incorporation of DNA into nucleosomal

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structures as well as by reduced DNA accessibility resulting from condensation in histone containing chromatin. These experiments (DLS and DNA release experiment) also help us to know that how the structure of chromatin and chromatosome changes in presence of netropsin.

Conclusion

From fluorimetric analysis and ITC results, it was observed that soluble chromatin and chromosomal DNA bind with netropsin with comparable affinities. Netropsin also causes compaction of soluble chromatin. It also releases DNA from chromatosome isolated from chicken liver.

Acknowledgment

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