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SUBCELLULAR DISTRIBUTION OF A NITROXIDE SPIN-LABELED NETROPSIN IN LIVING KB CELLS

ELECTRON PARAMAGNETIC RESONANCE AND SEQUENCE SPECIFICITY STUDIES

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Abstract—A nitroxide spin-labeled netropsin was studied by EPR spectroscopy with respect to its uptake and localization in living KB cells. Whereas the drug was taken up readily, there was relatively little drug in the cytoplasm, but a significant concentration of the drug in the cell nucleus. The EPR signal in the latter site corresponded to a relatively freely rotating radical. The drug exhibited good intracellular stability up to 25 hr. While a ΔT_m of 24° between the spin-labeled netropsin and calf thymus DNA confirmed strong binding, the absence of any DNA elongation by viscometry was consistent with non-intercalative exterior binding which was confirmed to be minor groove specific by binding of the agent to T4 DNA with a ΔT_m of 17.5°. The sequence specificity of the DNA binding of the spin-labeled drug was confirmed by methidiumpropyl-EDTA (MPE) footprinting on a fragment of pBR322 DNA to be very similar to that of the parent netropsin, i.e. selective for AT-rich sites, with minor differences of protection afforded by introduction of the nitroxide label.

There is considerable interest currently in the development of sequence specific DNA binding agents for application as anti-sense cell regulatory agents in diagnosis and therapy [1–5]. Promising results have been obtained with anti-sense probes based on β-oligodeoxyribonucleotides [1, 2, 4] or modifications thereof [6] and, more recently, with the unnatural α-oligodeoxyribonucleotides [7]. While these oligonucleotide-based agents meet some of the criteria for viable anti-sense probes, i.e. high binding specificity, and resistance to intracellular nucleases in the case of β-oligomer methylphosphonates [5] and α-oligomers [7], they suffer the serious disadvantage of difficulty of penetration of the cellular membrane [4]. The methyl phosphonate β-oligomers, prepared in response to this problem, present additional difficulties in that a sequence containing n phosphate residues represents 2^n diastereomers [8]. In an alternative approach we are developing lexitropsins, or information-reading oligopeptides, for the sequence specific delivery of DNA effectors [9–12]. We report an examination of the uptake of a nitroxide spin-labeled netropsin (Fig. 1) into living KB cells and their preferential concentration in the cell nucleus by EPR spectroscopy. Additional experiments with purified DNAs provided detailed information on the sequence specificity and mode of binding of the spin-labeled agent.

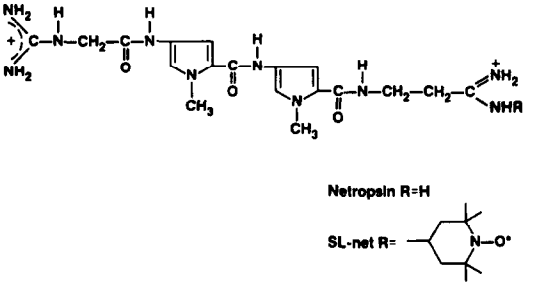

Fig. 1. Structural formulae of netropsin and spin-labeled netropsin.

MATERIALS AND METHODS

Chemicals

IR spectra were recorded on a Nicolet 7199 FT spectrophotometer and only the principal sharply defined peaks are given. FAB mass spectra were determined on an Associated Electrical Industries (AEI) MS-9 mass spectrometer. Melting points were determined on a Fisher–Johns apparatus and are uncorrected.

1 - *Methyl* - 4 - (4 - *guanidineacetylamino* - 1 - *methylpyrrole* - 2 - *carboxamido)pyrrole* - 2 - *carboxamido*-N - *(2,2,6,6-tetramethylpiperidinyl* - N - *oxide)propionamidine hydrochloride (SL-net*).*

1 - Methyl - 4 - (4 - guanidineacetylamino - 1 - methylpyrrole - 2 - carboxamido)pyrrole - 2 - carbo-

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* Abbreviations: SL-net, spin-labeled netropsin; net, netropsin; MPE, methidiumpropyl-EDTA; bp, base pairs; and ct DNA, calf thymus DNA.

xamidopropionitrile [13] (140 mg, 0.3 mmol) was dissolved in 5 ml of dry ethanol and the solution was saturated with dry HCl gas with cooling. After 1.5 hr the solvent was removed *in vacuo* and the residue was washed with dry ether. The residual solid was redissolved in dry ethanol and the solution concentrated to dryness and washed with dry ether. The residual solid was dissolved in 5 ml of dry ethanol, and 4-amino-2,2,6,6-tetramethylpiperidinyl-*N*-oxide (104 mg, 0.6 mmol) was added. After 2 hr the mixture was concentrated to dryness *in vacuo* and the excess of the free radical reagent was extracted with acetone. The residue was dissolved in a small volume of methanol and precipitated by addition of acetone. This operation was repeated twice to afford 137 mg (69% yield) of SL-net of no distinct m.p. (softens at 195° with dec.); IR ν_{max} (Nujol) 1375, 1405, 1460, 1530, 1580, 1645, 3120, 3260 cm^{-1}; MS-FAB, *m/z* 585(M-HCl-Cl)$^+$; Anal. Calcd. for $C_{27}H_{44}Cl_2N_{11}O_4$: C, 49.3; H, 6.7; Cl, 10.8; N, 23.4. Found: C, 49.6; H, 7.0; Cl, 11.0; N, 23.8.

Biochemicals

T4 viral DNA was from Miles Laboratories, Inc. (Elkhart, IN). Restriction enzymes Hind III and Eco RI; pBR322 DNA and sonicated calf thymus DNA (ct DNA) were from Pharmacia Inc. Dithiothreitol (DTT) was from Calbiochem. Netropsin, acrylamide, bromophenol blue, and xylene cyanol were from Serva. Ultra pure urea was from Bethesda Research Laboratories. MPE was a gift from Professor P. B. Dervan. Ferrous ammonium sulfate was from BDH Chemicals, Ltd. [γ^{32}P]ATP was from New England Nuclear.

Methods for cell studies

Cell cultures. KB cells were grown as suspension cultures in Joklik modified Eagle's medium (Seromed, Munich, F.R.G.) supplemented with 5% heat-inactivated Colt serum at 4×10^5 cells/ml.

Spin-labeling. Nitroxide-labeled netropsin (SL-net) was added to cell cultures at 3.5×10^{-5} M (initial concentration) for various incubation periods.

Cell fractionation: Nuclear and cytoplasmic fractions. Spin-labeled cells (i.e. 40×10^6 cells) were collected by low-speed centrifugation, washed once in saline, and allowed to swell for 10 min in ice in 1 ml of hypotonic buffer (0.01 M Tris–HCl, pH 8.1, 0.05 M NaCl 0.001 M EDTA) [14, 15]. The cells were then disrupted by ten strokes of a tight-fitting Dounce homogenizer. The nuclei were pelleted at 600 *g* for 10 min and stored at −80° in 400 μl of hypotonic buffer; the resulting supernatant, referred to as the cytoplasmic fraction, was kept at −80°.

EPR spectra. Prior to EPR examination the cellular fractions were defrosted and sonicated (two 5-sec bursts with the microtip probe of a Bransen sonicator (Danbury, CT, maximum power). Each sample was treated with H_2O_2 and sodium phosphotungstate [16] to reoxidize all the reduced forms of the nitroxide prior to EPR examination. EPR measurements were recorded on a Varian E 109 X-band spectrometer equipped with an E 238 cavity operating in the TM_{110} mode. A 100 kHz high frequency modulation was used with a 20 mW microwave power. The sample solutions were examined in a flat quartz cell.

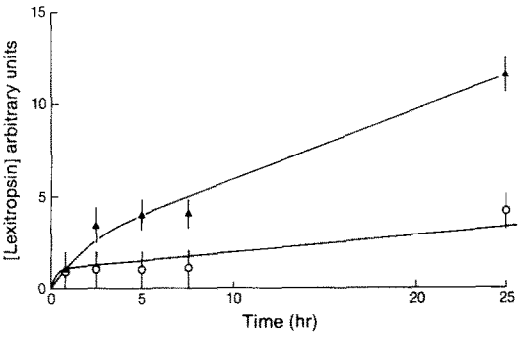

Fig. 2. Time course showing selective uptake of the nitroxide spin-labeled netropsin preferentially into the nucleus of living KB human tumor nasopharengal cells. Key: (-▲-▲-) nuclear fraction; and (-O-O-) cytoplasmic fraction.

Methods for studies with purified DNAs

Footprinting. Hind III digested pBR322 DNA was 5′-^{32}P-labeled and then digested with Eco RI. The resulting fragments (31 and 4332 bp) were not separated prior to footprinting. Solutions for footprinting experiments were prepared by mixing calf thymus DNA, radiolabeled DNA (approximately 10,000 dpm/sample) and ligand (omitted in the control) in Tris buffer, pH 7.4. The stock ligand concentration was determined by weight; ligand quantities are reported as r_t (ratio of ligand to DNA base pairs). After equilibration of the ligand–DNA mixtures for 30 min at 37°, MPE-Fe(II) [from a freshly prepared mixture of $Fe(NH_4)_2(SO_4)_2$ plus MPE] was added to each, followed by DTT. The final reaction mixtures contained 100 μM DNA base pairs, 7.5 mM Tris-HCl, 15 mM NaCl, 2 μM EDTA, 10 μM MPE-Fe(II), and 2.5 mM DTT. Reactions were run for 5 min at room temperature and were then stopped by freezing at −78°. The mixtures were lyophilized, resuspended in formamide loading buffer [17], heated at 90° for 1 min, chilled on ice, and loaded onto a polyacrylamide sequencing gel (0.4 mm thick, 55 cm long, 6% acrylamide, 7 M urea) which was run at 2500 V, 55°, on an LKB Macraphor Electrophoresis unit. After the gel was dried (Bio-Rad model 483 slab dryer) onto filter paper, autoradiography was conducted using Kodak X-Omat AR film at −70° without an intensifying screen. The resulting autoradiographs were scanned using an LKB Ultroscan XL laser densitometer.

EPR studies of DNA binding of spin-labeled netropsin. For the EPR measurements, sonicated ct DNA was dissolved in 0.1 × SSC buffer (15 mM NaCl, 1.5 mM sodium citrate), pH 7.0, to a final concentration of 553 μM in base pairs. The actual DNA base pair concentrations was estimated measuring absorbance at 260 nm of the relevant DNA solution and using the relationship: OD(260) = 1 corresponds to a base pair concentration of 150 μM. The effect of a salt concentration on the drug binding was evaluated from measurements in the presence of 150 mM, 15 mM and 6 mM NaCl in the buffer solutions.

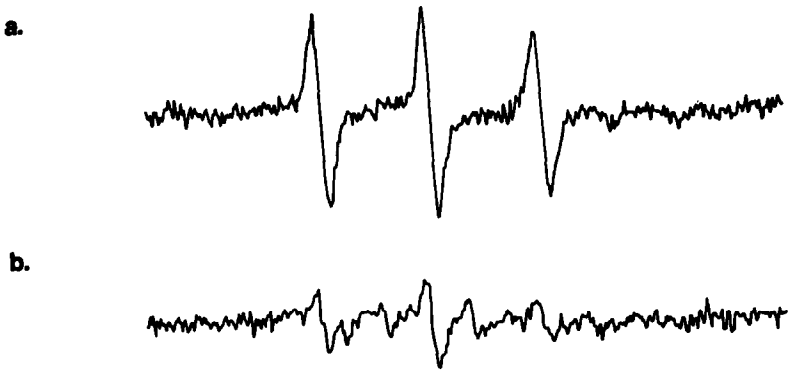

Fig. 3. Typical EPR spectra after 25 hr of incubation of nitroxide-labeled netropsin with KB cells. Key: (a) nuclear fraction; and (b) cytoplasmic fraction.

Measurements of binding of SL-net to ct DNA were performed using a Bruker ER-400 EPR spectrometer operating at 9.5 GHz with 100 kHz field modulation. Samples of a total volume 0.5 to 0.6 ml were introduced into the quartz flat EPR cuvette and experiments were performed at room temperature. Spectra were recorded at the following instrumental settings: microwave power, 20 mW; modulation amplitude, 1.0 G; time constant, 0.5 sec; scan rate, 1000 sec; and the appropriate gain level. To determine the fraction of free (*F*) and DNA-bound (*B*) drug (SL-net) and to construct the Scatchard plot, the procedure described earlier by Bernier *et al.* [18] was adopted.

DNA thermal denaturation determinations. "Melting" curves were measured by using a Uvikon Kontron 810/820 spectrophotometer coupled to a Uvikon Recorder 21 and a Uvikon Thermoprinter 48. Samples were placed in a thermostatically controlled cell-holder (10 mm path length). The cuvette was heated by circulating water from a Haake unit set. The temperature inside the cuvette was monitored by using a thermocouple in contact with the solution. The absorbance at 260 nm was measured over the range 20–95° with a heating rate of 1°/min. The "melting" temperature (T_m) was taken to be the midpoint of the hyperchromic transition.

DNA viscometric determinations. Helical lengthening measurements were made by using an Ubbelohde semimicro dilution viscometer. Temperature was maintained at 20 ± 0.01° in a thermostatically controlled water bath. Flow times were electronically measured to an accuracy of 0.1 sec (Schott ABS/G type detector). Calf thymus DNA was sonicated as described by Wakelin and Waring [19]. Solutions were filtered through 0.45 μm Millipore filters before measurements. The viscometer contained 2.0 ml of a 150 μM solution of DNA. Drugs were added in increments of 5–10 μl from a stock solution (concn = 150 μM). Flow times were measured with an accuracy of 0.1 sec.

RESULTS

Penetration of spin-labeled netropsin into living KB cells

KB cells were incubated with the nitroxide-labeled lexitropsin (Fig. 2) at a concentration of 3.5×10^{-5} M for various times (45 min, 5 hr, 7.5 hr, 25 hr). Penetration of the drug into the cells proved to be smooth and progressive since a significant EPR signal was observed in the nuclear fraction after 45 min. Subsequently, EPR signals were detected in both the nuclear and cytoplasmic fractions up to 25 hr (Fig. 2). The EPR technique has proven to be an extremely sensitive and direct tool to study such small molecule–macromolecule interactions [14, 15, 18, 20].

Localization and environment

At low concentration of the drug (3.5×10^{-5} M) and after 45 min of incubation no detectable EPR signal resulted in either the cytoplasmic fraction or the cell membrane, whereas a significant signal was observed in the nuclear fraction. This indicated a fairly rapid passage through the cell membrane and cytoplasm and relatively high affinity of the drug for the nucleus. Subsequently, some binding was observed in the cytoplasm but the preferential binding in the nucleus expressed by the ratio $[\text{SL-net}]_{nucl} : [\text{SL-net}]_{cyt} \approx 3.0$ was maintained up to 25 hr. A somewhat broadened triplet EPR signal characteristic of a slightly restricted but essentially freely rotating nitroxide label [14, 15] was observed in both nuclear and cytoplasmic fractions (Fig. 3).

To determine if any reduction of the nitroxide-labeled portion of the drug had occurred in the cytoplasm, treatment with H_2O_2 and sodium phosphotungstate was performed. No additional EPR signals resulted. The rotational correlation time of the nucleus bound label, calculated as described by Ortner *et al.* [21], was determined to be $\tau = 0.15$ to 0.17 nsec. Under comparable conditions a control experiment with a spin-label alone, i.e. the ATEMPO probe, was found in equal concentrations either in the cytoplasm or in the nucleus, exhibiting a sharp triplet [14]. The results suggest a passive diffusion of the spin-label in the whole cell but, in contrast, ready uptake of the spin-labeled netropsin with preferential binding in the nuclear receptor. In addition, the survival of the nuclear-bound drug for 25 hr suggests substantial resistance to intracellular degradation.

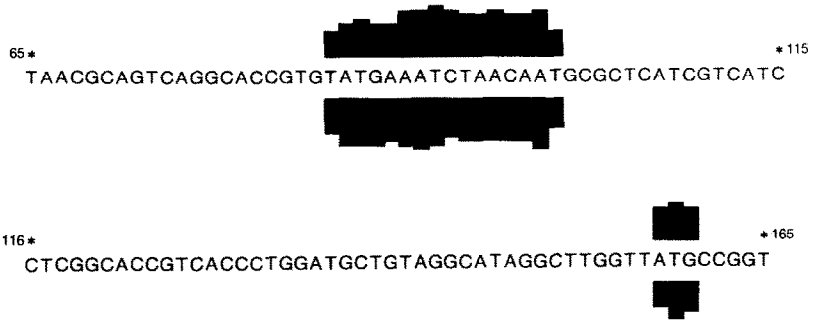

Fig. 4. Summary histogram of the results of MPE footprinting experiments showing inhibition of DNA cleavage. Key: upper trace, SL-netropsin; and lower trace, netropsin.

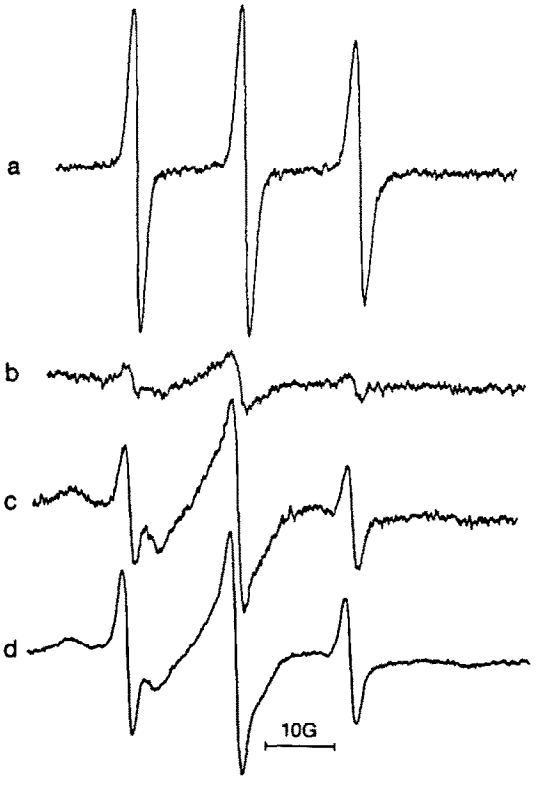

Fig. 5. EPR spectra from samples in 0.1 × SSC buffer, pH 7, containing (a) SL-net (9.8 μM); (b) SL-net (9.8 μM) and ct DNA, DNA/drug = 119; (c) SL-net (37 μM) and ct DNA, DNA/drug = 32; (d) SL-net (65 μM) and ct DNA, DNA/drug = 18. Instrumental gain conditions: (a), (b) and (c), 3.2×10^5; (d) 1.6×10^5.

Interaction of spin-labeled netropsin with purified DNAs

MPE footprinting. The protection afforded the DNA by SL-net compared with the parent netropsin was determined by MPE footprinting. The results are summarized in Fig. 4. Analysis of bases 65–165 revealed that both the degree and the location of cleavage protection by the SL-net were nearly identical to those of netropsin (Fig. 4). Both compounds yielded strong footprints (r_t 0.2) at positions 85–100 and 158–160. These bases are within the four highest affinity netropsin binding locations present in the 100 bp region studied [22].

Thermal denaturation. Thermal denaturation experiments of the spin-labeled agent with calf thymus DNA showed a $\Delta T_m = 16.5°$, indicative of a strong interaction with double helical DNA. Netropsin under comparable conditions showed a $\Delta T_m = 20.0°$. However, viscometric studies revealed no significant elongation of calf thymus DNA with the drug. This result rules out intercalative binding. Thermal denaturation of T4 DNA (in which the major groove is occluded with glucosyl moieties [23]) in the presence of the drug showed a $\Delta T_m = 17.5°$. This result confirms that the minor groove specificity of the parent netropsin (which under comparable conditions gives $\Delta T_m = 20.5°$) is maintained in the spin-labeled derivative.

Determination of binding constants of spin-labeled netropsin. EPR measurements with ct DNA were performed for DNA bp/drug ratios ranging from 5.6 to 122. Recorded EPR spectra consisted of a sharp triplet, $a_N \approx 16$ G, originating from free SL-net in solution, superimposed on a broad spectrum from DNA-bound drug molecules. Figure 5 shows examples of such spectra recorded at some selected DNA bp/drug ratios (a–0; b–119; c–32; d–18). The spectra are similar to those obtained from systems containing DNA and spin-labeled acridines [18, 24, 25]. Fractions of DNA-bound (B) and free (F) drug were estimated from EPR spectra recorded at various DNA base pair/drug ratios. Binding constant K, and number of base pairs per binding site n, were obtained from the non-linear least square best fit of the experimental data to Equation 1 (McGhee and von Hippel [26]);

$$\frac{r}{F} = K[1 - nr]\left[\frac{1 - nr}{1 - (n-1)r}\right]^{n-1} \qquad (1)$$

where r is the number of moles of compound bound per mole of DNA base pairs. The relevant Scatchard plot is shown in Fig. 6. The best fit gave $K = 3.6 \times 10^4\ M^{-1}$ and $n = 5$. These values may be compared with those reported earlier for netropsin [27] $K = 2.9 \times 10^5\ M^{-1}$ and $n = 3$.

The effects of salt concentration on the SL-net binding to ct DNA were also studied. Table 1 shows

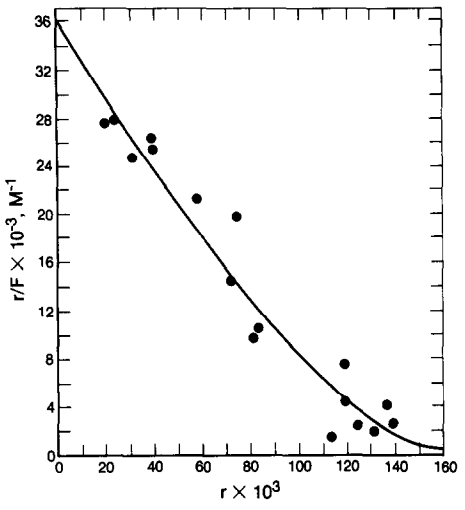

Fig. 6. Scatchard plot for binding of SL-net to sonicated ct DNA in 0.1 × SSC buffer, pH 7. Points shown are from titrations and the curve is the non-linear best fit line according to Equation 1. r is the number of moles of compound bound per mole of DNA base pair; F is the molarity of free compound.

Table 1. Effect of salt concentrations on the binding of nitroxide-labeled netropsin to calf thymus DNA

[NaCl], mM	$[B]/[F]$
6	34.6
15	23.9
150	13.1

B = bound drug; F = free drug. [SL-net] = 19 μM; DNA bp/drug = 23.

the results obtained from the EPR measurements at the DNA bp/drug ratio of 23 ([SL-net] = 19.0 μM) and at three concentrations of NaCl. It is apparent that the fraction of the DNA-bound SL-net increases as the salt concentration decreases. A similar influence of changes in ionic strength on the binding of netropsin to various DNAs was reported earlier by Luck *et al.* [27] and Wartell *et al.* [28].

DISCUSSION

The results of this study with a nitroxide spin-labeled netropsin suggest that the drug has relatively free access into living KB cells. Concentration occurs in the cell nucleus, relative to the cytoplasm. In contrast, a control nitroxide, which does not bear a DNA interactive group, diffuses to all parts of the cell equally [14]. It is reasonable to expect a higher initial concentration of SL-net in the cytoplasm than in the nucleus at some point in the uptake process. However, this was not proven because of the difficulty in obtaining early time points. The results suggest a fairly rapid passage through the cell membrane and cytoplasm. There is no evidence that the drug binds to other cellular macromolecules, e.g. a carrier protein. The results also show that the drug, whether in the cell nucleus or the cytoplasm, is relatively long-lived (i.e. up to 25 hr) and, therefore, apparently not subject to rapid intracellular degradation.

The thermal denaturation and viscosity experiments with the drug and purified calf thymus DNA confirmed strong binding, which is non-intercalative in nature, to double helical DNA. The complementary thermal denaturation experiments with T4 DNA and the labeled lexitropsin confirmed minor groove specificity as in the case of the parent antibiotic netropsin. The influence of selective groove binding to the cellular DNA was corroborated by the EPR linewidths, which were characteristic of a relatively freely-rotating, i.e. non-intercalative, interaction. It may be noted that the broad spectral components seen when the SL-net binds to purified DNA were not evident when the drug accumulated in the nucleus. This may be due to binding of the drug to other components besides DNA in the nucleus or, because of the presence of DNA proteins of chromatin, the minor groove of DNA is less accessible so that no anisotropic effect can be observed. The results of the footprinting studies confirmed that introduction of the bulky tetramethylpiperidinyloxy moiety does not alter significantly the DNA sequence specificity of SL-net compared with the parent antibiotic. This is in keeping with the view that the AAAT sequence specificity is determined principally by a combination of electrostatic interactions, bifurcated amide hydrogen bonds to the DNA bases, and van der Waals contacts with the floor of the minor groove, none of which was apparently affected by introduction of the spin-label. Similarly, the independent binding studies of SL-net to DNA, together with the salt effects, were in accord with non-intercalative binding with a significant electrostatic component. Binding of SL-net to DNA was analyzed in terms of the site exclusion method of McGhee and von Hippel [26]. The EPR study did not provide any information as to the existence of another class of binding sites, with very high binding constant ($K \sim 10^8\ M^{-1}$) as suggested earlier [27, 29]. It should be noted, however, that the strong, specific binding of netropsin to DNA occurs only at very low total drug/nucleotide ratios and could not be measured using the EPR technique employed in this study.

These results represent the first direct evidence, of which we are aware, of preferential minor groove binding of such oligopeptide antitumor antibiotics to the nucleus of living tumor cells. The results also demonstrate that lexitropsins meet many of the criteria for viable anti-sense cell regulatory probes, i.e. relative ease of access into the cell, preferential binding in the cell nucleus and acceptable intracellular stability in contrast to oligonucleotide-based probes. The implications of these results for the development of sequence specific gene probes will be reported in due course.

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