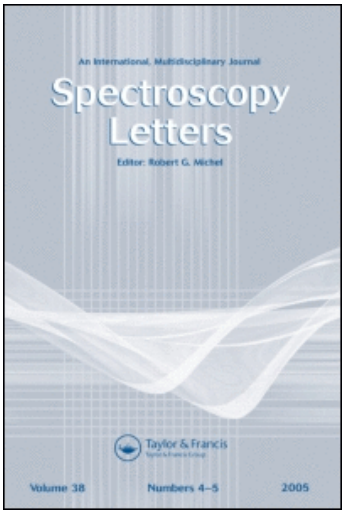

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M. Gopal[a]; S. Veeranna[a]; L. S. Doddamani[b]
[a] Department of Studies in Biochemistry, Kuvempu University, Davangere, Karnataka, India [b] Department of Studies in Zoology, Karnataka University, Dharwad, Karnataka, India

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Intercalation Binding of 4-Butylaminopyrimido[4′,5′: 4,5]selenolo (2,3-*b*)quinoline to DNA: Relationship with In Vitro Cytotoxicity

M. Gopal,[1,*] S. Veeranna,[1] and L. S. Doddamani[2]

[1]Department of Studies in Biochemistry, Kuvempu University, Shivagangotri, Davangere, Karnataka, India
[2]Department of Studies in Zoology, Karnataka University, Dharwad, Karnataka, India

ABSTRACT

The interaction between double-stranded (ds) DNA and the 4-butylamino pyrimido[4′,5′: 4,5]selenolo(2,3-*b*)quinoline (BPSQ) has been studied with spectral luminescence methods. Binding constant values determined by absorption and fluorescence titration indicated a binding constant of 4.24×10^4 and $5.9 \times 10^4\,M^{-1}$, respectively. The interaction was markedly suppressed by increasing the salt concentration. BPSQ exhibited a strong specificity for the guanine–cytosine (GC) sequence in total

*Correspondence: M. Gopal, Department of Studies in Biochemistry, Kuvempu University, Shivagangotri, Davangere, Karnataka 577 002, India; Fax: +91-08192-208420; E-mail: muttagigopal@yahoo.co.in.

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DNA at an ionic strength of 0.01 ($5.54 \times 10^6\,M^{-1}$). BPSQ increased the viscosity of sonicated rod-like DNA fragments, producing a calculated increment in length of 2.4 Å per bound drug molecule. A circular dichroism experiment showed that BPSQ might interact with DNA according to two possible binding modes depending on its structure and concentration. The first mode concerned intercalation of BPSQ with its long axis perpendicular to the long axis of the DNA helix. The same drug was able to bind to external sites, second mode, once the intercalation sites were saturated at high concentration. The results from cytotoxicity assays indicate that BPSQ was found to be toxic to all cell lines tested with IC_{50} values ranging from 2.8 to $\geq 20\,\mu M$. It was concluded that the binding of BPSQ to DNA occurs by a mechanism of intercalation, which probably accounts for its reported antitumor activity.

Key Words: DNA; 4-Butylaminopyrimido[4′,5′: 4,5]selenolo(2,3-*b*) quinoline; Ellipticine; UV spectroscopy; CD spectroscopy; Binding model; Cytotoxicity.

INTRODUCTION

Ellipticine, an alkaloid isolated from *Apocyanaceae* plants and several of its more soluble derivatives (9-hydroxyellipticine, 2*N*-methyl-9-hydroxy ellipticinium, 2*N*-methyl-9-chloroellipticinium, and 2*N*-methyl-9-methoxy ellipticinium) exhibits significant anti-tumor and anti-HIV activities.[1] The main reason for the interest in ellipticine and its derivatives, for clinical purposes, is their high efficiencies against several types of cancer, their rather limited toxic side effects, and their complete lack of hematological toxicity.[2] Ellipticines are anticancer drugs, whose precise mechanisms of action have not yet been explained. It was suggested that the prevalent mechanisms of ellipticine antitumor activities are (i) intercalation into DNA[3,4] and (ii) inhibition of DNA topoisomerase II activity.[2,5–7] Also, ellipticine and 9-hydroxyellipticine cause selective inhibition of p53 protein phosphorylation in several human cancer cell lines,[8] which is correlated with their cytotoxic activity.

The synthesis of compounds containing a novel tetracyclic condensed quinoline system, pyrimido[4′,5′: 4,5]selenolo(2,3-*b*)quinoline, by successive building up of selenophene and pyrimidine rings on quinoline by Dimroth rearrangement has been reported.[9] This ring system is of interest because of its close relationship with anticancer alkaloid ellipticine.

Recently, we have found that 8-methoxy, 4-amino, and 8-methyl-4-(3-diethylamino propylamino)pyrimido[4′,5′: 4,5]thieno(2,3-*b*)quinoline

(MDPTQ) covalently binds to DNA.[10,11] 8-Methoxy and MDPTQ have produced marked cytotoxic effects against HL60, B16 melanoma, neuro2a cells in culture and have displayed good antitumor activity against B16 melanoma and Ehrlich ascites tumors in vivo.[10–12] In addition, we have found that MDPTQ inhibits nucleic acid synthesis strongly in both sarcoma 180 and Ehrlich ascites tumor cells in vitro (manuscript in preparation). 4-Butylaminopyrimido[4′,5′: 4,5]selenolo(2,3-*b*)quinoline (BPSQ) is a new analogue of these substances that was conceived and synthesized to investigate possible structure–activity relationships for antitumor activity.[9]

A number of epidemiological and experimental studies, and clinical intervention trials, have indicated that the micronutrient, selenium has potential anticarcinogenic effects.[13–16] For instance, inverse relationships between serum levels of selenium and cancer incidence have been reported.[13,17] A clinical trial on the effects of dietary supplementation with selenium-enriched yeast on skin cancer incidence has resulted in a significantly reduced incidence in several secondary-site cancers, although skin cancer was not inhibited.[18] In addition to naturally occurring selenium compounds, certain organoselenium compounds such as the synthetic compounds 1,4-phenylenebis(methylene) selenocyanate,[19] triphenylselenonium, and diphenylselenide[20] have also been shown to be inhibitory in different tumour models.

On the basis of these indications, we have tentatively identified pyrimido [4′,5′: 4,5]selenolo(2,3-*b*)quinolines as new member of the class of anticancer drugs whose activity can be attributed to binding to DNA so as to interfere with its function as a template for nucleic acid synthesis in susceptible cells. This paper reports the results of experiments designed to investigate that hypothesis, using physicochemical techniques as well as in vitro cytotoxicity assays on HL60, B16 melanoma, and neuro 2a cells to study the interaction of BPSQ with DNA.

MATERIALS AND METHODS

Calf thymus DNA type I (42% GC) and *Micrococcus lysodeikticus* DNA (72% GC) were purchased from Sigma Corporation. DNA solutions were prepared by dissolving the solid material, normally at 1–2 mg/mL, in *tris*-HCl buffer pH 7.0 (0.01 M). DNA concentrations were determined spectrophotometrically at 260 nm using a molar extinction coefficient of $6600\,M^{-1}\,cm^{-1}$. The compound BPSQ was taken up in the present study (Fig. 1). This drug was a generous gift from Dr. S. Y. Ambekar, Department of Chemistry, Mysore University, Mysore, India. Aqueous stock solution of BPSQ was prepared by wetting the drug crystals with DMSO followed by addition of water. Working stock solutions of the drug were prepared by

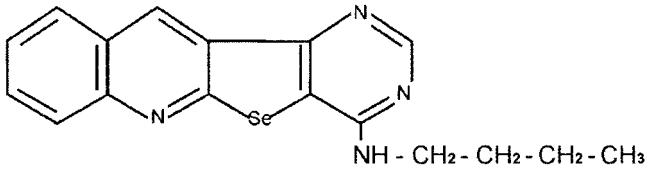

Figure 1. The chemical structure of BPSQ.

dilution of an aqueous stock solution with DNA binding buffer (*tris*-HCl buffer, pH 7.0). The concentrations of BPSQ solutions were determined spectrophotometrically at 352 nm using a molar extinction coefficient of 72,000 $M^{-1}\,cm^{-1}$.

Spectroscopy

Ultraviolet-visible absorption spectra were determined by use of a Perkin–Elmer model 554 UV-VIS recording spectrophotometer using 10 mm light-path. The parameters of interaction between BPSQ and DNA were determined spectrophotometrically using a Beckman 25 double beam spectrophotometer equipped with 10 mm light path quartz cuvettes. Aliquots of a concentrated DNA solution (6.8–123.5 μM) were added to a cuvette filled with a BPSQ solution (28–40 μM) and thoroughly mixed. Extreme care was taken to ensure that optical reference solutions were prepared in an identical manner.

The binding data were expressed in the form of a Scatchard plot.[21] The variables of r (moles of ligand bound/mole of nucleotides) and C (the molar concentration of free drug) were calculated from the absorption measurements according to the method of Peacocke and Skerrett.[22] $K(O)$, the intrinsic association constant for an isolated site, and n, the number of nucleotides occluded by the binding of a single drug molecule, were computed to satisfy Eq. (10) of McGhee and von Hipple.[23]

Fluorescence spectra were determined by use of a Hitachi Model 2000 fluorescence spectrophotometer. Emission spectra of the drug alone, and in the presence of increasing concentrations of DNA, were measured according to methods previously described.[24,25]

Viscometry

Viscosity measurements were made according to published procedures[26,27] using a capillary viscometer with a thermostatted bath D40S

at 20°C ± 1°C. The flow time for water was 71.3 sec. For the viscosity experiments, samples of calf thymus DNA were sonicated into fragments having an estimated molecular weight of approximately 500,000.

Circular Dichroism

The circular dichroism measurements were recorded with a JASCO spectrophotometer model J-20A. Solutions of drug and/or DNA were scanned in 1 cm quartz cuvettes. Measurements were made by progressive addition of DNA to a pure ligand solution to get the desired drug/DNA ratios. Molar circular dichroism ($\Delta\varepsilon$), was used for CD spectra. The drug/oligonucleotide ratios, r' were expressed in mole/base.

Cytotoxicity Test

HL60, B16 melanoma, and neuro 2a cells were cultured in RPMI 1640 medium plus 10% fetal calf serum at 37°C in 5% CO_2. The drug's cytotoxicity was determined with a cell growth inhibition test. Cells (5×10^5 cells/mL) were treated for 8 hr with different drug concentrations (0.1–50 μM). Cells were then centrifuged, washed, seeded at 10^4 cells/mL, and cultured in drug-free medium at 37°C for 2 days. Cells were counted with a Coulter counter, and cell growth was expressed as the fraction of surviving cells in treated samples compared with those in controlled samples.

RESULTS

The effect of progressively increasing concentrations of DNA on the absorption spectra of BPSQ is shown in Fig. 2. The spectral changes involved essentially a progressive red shift and hypochromicity in the complex until saturation was recorded (DNA = 123.5 μM). The absorbance of BPSQ in the peak was decreased by about 24.37% and a maximum red shift of 8–10 nm was observed. No significant change in the absorption pattern was observed and, more particularly, no blue shift resulted even at a very high value of the BPSQ/DNA ratio. An isosbestic point occurred at 380 nm, and no further variations were observed. Binding of BPSQ to calf thymus DNA was measured spectrophotometrically, which resulted in the Scatchard plot[21] shown in Fig. 3. Calculations of values of r and r/C were performed using the absorbances measured at 352 nm. The theoretical curve was drawn according to the excluded-site model developed by Crothers and McGhee and

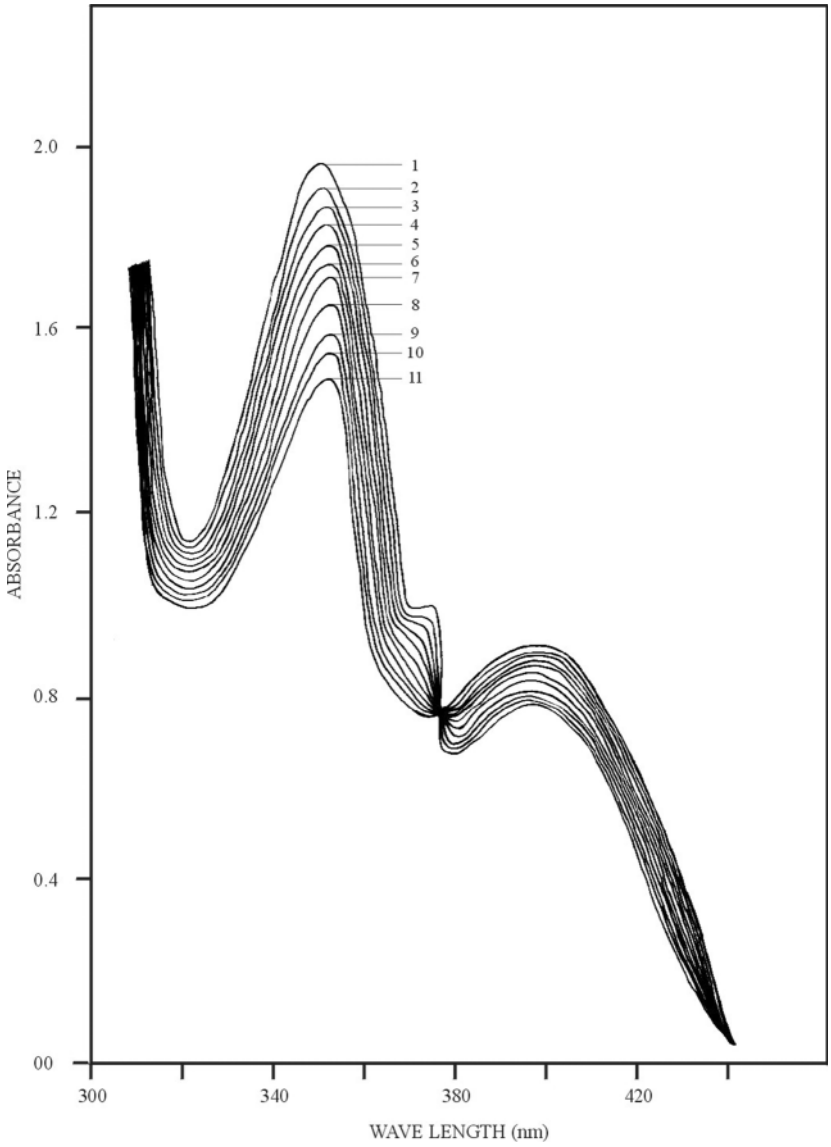

Figure 2. UV-absorption spectra of BPSQ with calf thymus DNA 1, 28 μM drug; 2, 6.8 μM; 3, 13.7 μM; 4, 27.4 μM; 5, 41.1 μM; 6, 54.9 μM; 7, 68.6 μM; 8, 82.3 μM; 9, 96.1 μM; 10, 109.8 μM; and 11, 123.5 μM DNA.

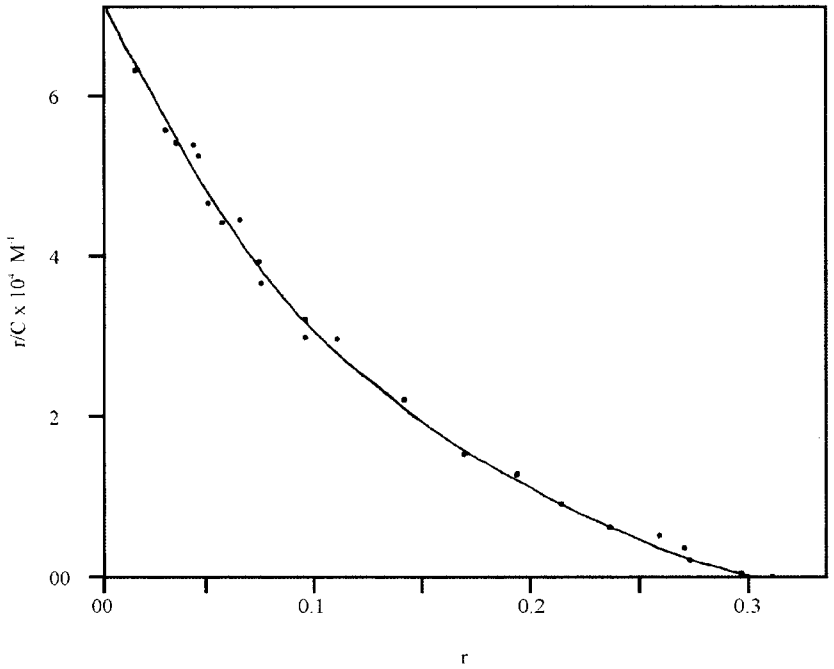

Figure 3. Scatchard plot of the binding of BPSQ to calf thymus DNA in *tris*-HCl buffer. The total drug concentration was 28 μM. The line drawn corresponds to Eq. (10) of McGhee and von-Hipple with $K(O) = 4.2 \times 10^4\,M^{-1}$ and $n = 3.0$ nucleotides.

von Hippel[23] giving a $K(O)$ of $7 \times 10^4\,M^{-1}$ and the site–size parameter $n = 3.0$ nucleotides.

The fluorescence intensity of BPSQ decreased upon addition of DNA [Fig. 4(b), insert], although the shape of the spectrum remained unchanged. The ratio of fluorescence intensity in the presence and in the absence of DNA (I_0/I) can be plotted with respect to the DNA concentration to give a Stern–Volmer type plot [Fig. 4(a)].[28,29] Since the fluorescence intensity of DNA-bound BPSQ is small compared to that of free BPSQ and the resulting Stern–Volmer plot appears to be a straight line, the slope can be directly understood as the equilibrium constant for BPSQ–DNA complex formation. The equilibrium constant of BPSQ was found to be $5.9 \times 10^4\,M^{-1}$. A Benesi–Hilderbrand plot[29,30] can also be constructed [Fig. 4(b)] from the changes in the fluorescence intensity at 433 nm. The association constant for the formation of the BPSQ–DNA complex, which was calculated from the ratio of the slope to the intercept, was $2.6 \times 10^4\,M^{-1}$. Both the results

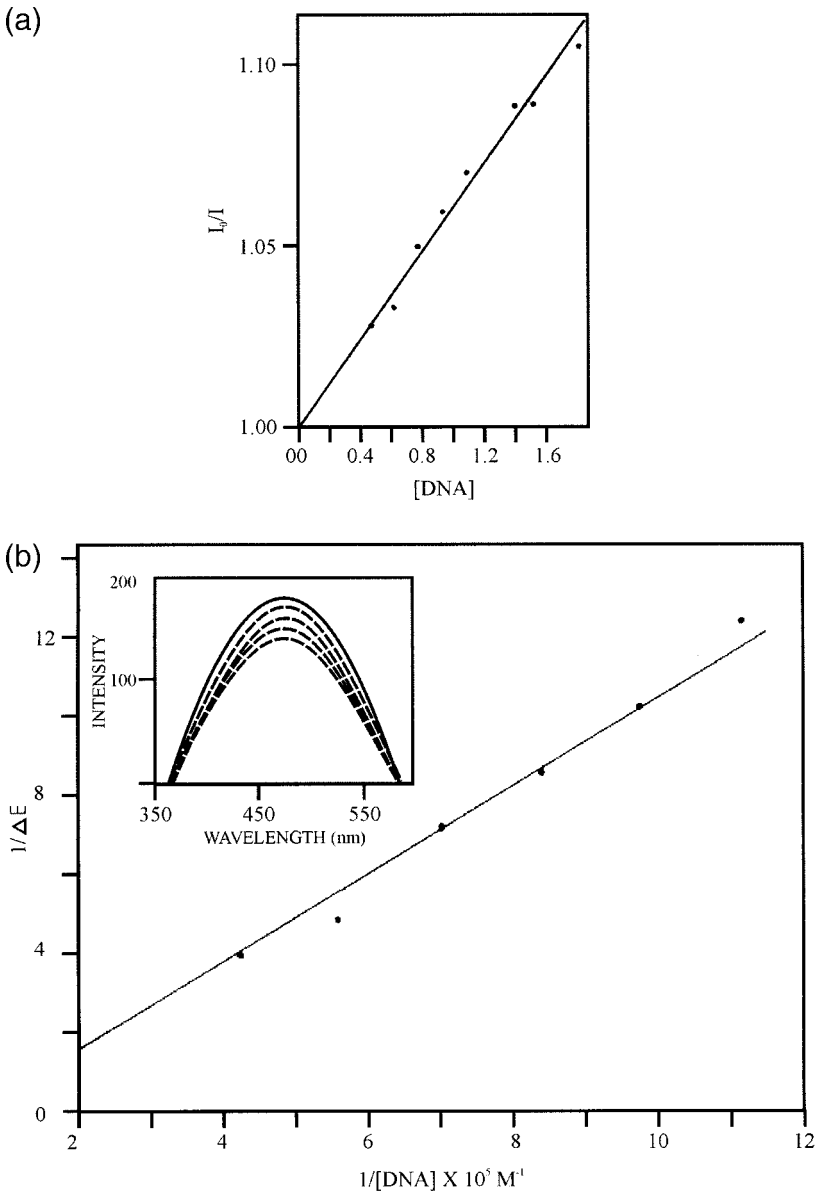

Figure 4. (a) Stern–Volmer plot and (b) Benesi–Hilderbrand plot. Fluorescence emission spectrum of BPSQ (28 μM) in the presence (dotted curve) and absence (solid curve) of DNA (163 μM) is inserted in (b). Excitation was at 352 nm, emission at 433 nm, and slit width at 1.5/3 nm for both (a) and (b).

from the Stern–Volmer and Benesi–Hilderbrand methods indicate that the binding of BPSQ to B-form DNA is far more favorable.[29] From the measured association constant, 12–43% of BPSQ was calculated to be bound when 123.5 μM of DNA and 28 μM of drug were mixed.

Previous studies[10,11] have shown that the ionic strength of the medium often affects the binding of a ligand to DNA. We studied the interaction between BPSQ and calf thymus DNA under the same buffer conditions with the ionic strength increased to 0.1 and 1.0 by addition of NaCl. Binding of BPSQ was strongly dependent on ionic strength, as can be seen in Fig. 5. The binding parameters at an ionic strength of 0.10 revealed that the association constant was reduced 40 fold to $K = 2.8 \times 10^3\,M^{-1}$ and that the frequency of binding sites was changed. When the ionic strength was raised to 100-fold to $I = 1.0$, only a meager reduction in the absorbance of BPSQ

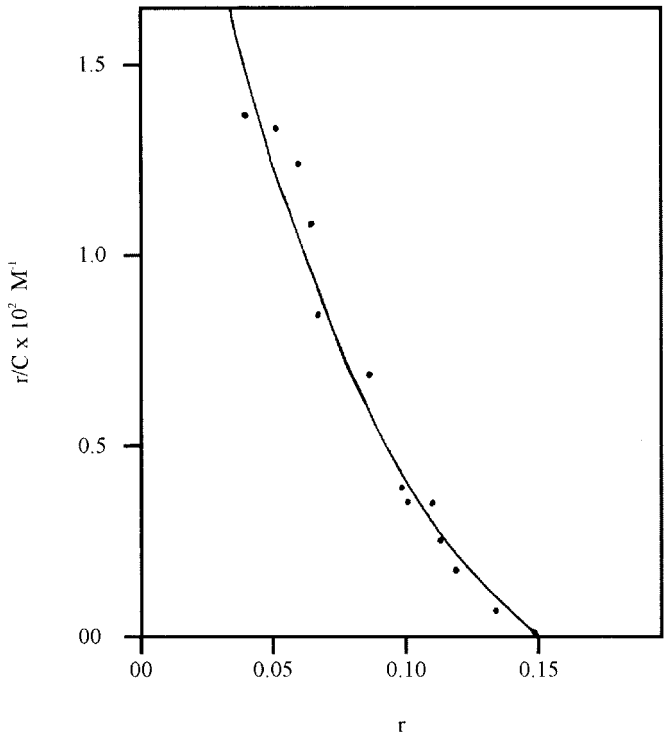

Figure 5. Scatchard plot of the binding of BPSQ to calf thymus DNA at ionic strength $I = 0.10$. The total drug concentration was 28 μM, $K(O) = 2.8 \times 10^3\,M^{-1}$ and $n = 6–7$ nucleotides.

was observed even when the molar ratio of BPSQ : DNA was increased above 0.70, which demonstrated that the ability of the drug to form a complex with DNA was grossly impaired.

Since a number of drugs have been reported to exhibit sequence-specificity in binding to DNA,[31] the interaction of BPSQ with *M. lysodeikticus* DNA was also studied (Fig. 6). The binding isotherm yielded $K(O) = 3.5 \times 10^7\,M^{-1}$ and $n = 2$ nucleotides. It can be seen that both parameters differ from those measured for calf thymus DNA, revealing that this DNA, which is characterized by a higher content of G + C (72%), bound BPSQ more efficiently.

An increase in viscosity of native DNA is regarded as a diagnostic feature of an intercalation process.[31,32] We have measured the viscosity changes in short, rod like DNA fragments. The relative length increase (L/L_0) of the

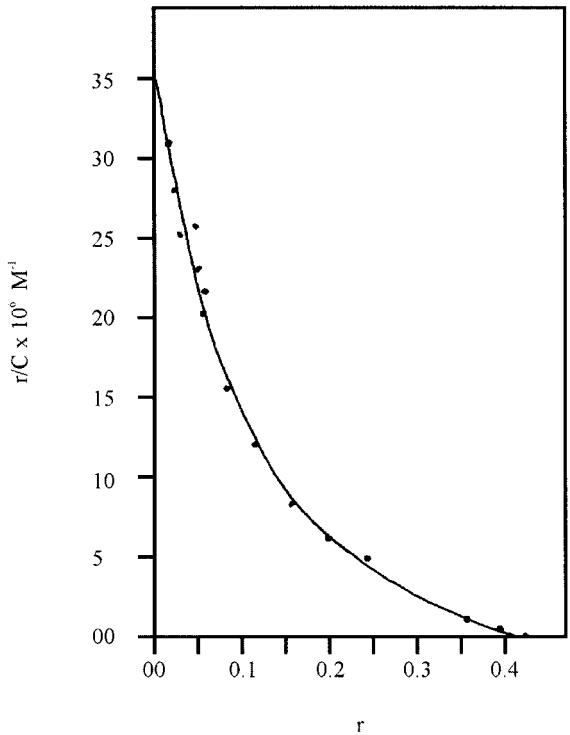

Figure 6. Scatchard plot of the binding of BPSQ to *M. lysodeikticus* DNA in *tris*-HCl buffer. The total drug concentration was 28 μM. The line drawn corresponds to Eq. (10) of McGhee and von-Hipple with $K(O) = 3.5 \times 10^7\,M^{-1}$ and $n = 2.0$ nucleotides.

complex formed between BPSQ and DNA is shown in Fig. 7. It is evident that binding of BPSQ increased the viscosity of DNA, corresponding to an increase in the contour length of the DNA fragments. The measured slope of the plot, 1.16 ± 0.05, falls within 72% of the slope of a theoretical curve for an idealized intercalation process $(1 + 2r)$. On this basis we calculate

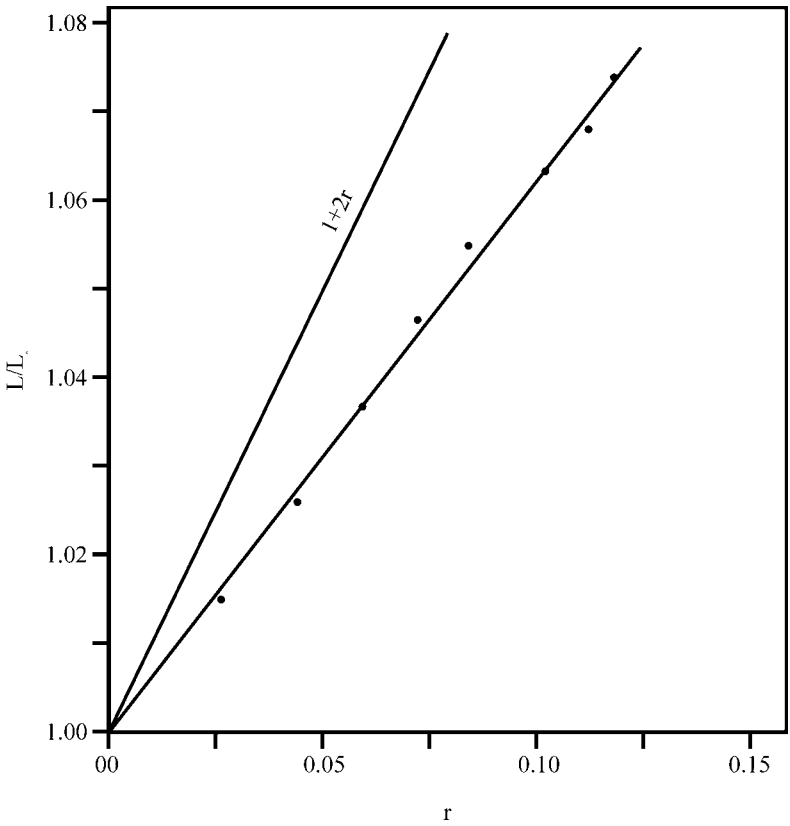

Figure 7. Effect of BPSQ on the relative contour length of sonicated calf thymus DNA fragments. L represents the contour length of fragments with BPSQ bound at the indicated binding ratio r; L_0 is the contour length of control BPSQ free DNA. The line labeled $1 + 2r$ represents the theoretical relation for an idealized intercalation process. The line fitted to the experimental points was computed by the method of least squares and constrained to pass through the point (0,1). Its slope corresponds to the relation $L/L_0 = 1 + (1.16 \pm 0.05)r$. Each point represents the mean of the three experiments.

that intercalation of one drug molecule provoked an increase of 2.4 Å in the contour length of DNA. Since the size of these sonicated fragments was significantly greater than the persistence length, the estimated 2.4 Å lengthening is probably best regarded as a lower limit.

Although the CD spectrum of drug that bound to DNA is not fully understood, it is known to be very sensitive to the environment of the drugs. The appearance of the CD spectrum reflects the binding geometry and binding mode of the drug as well as the arrangement of neighboring DNA bases.[33] The CD spectrum of BPSQ in the presence and absence of DNA is depicted in Fig. 8. In case of BPSQ a signal is induced, for $r' = 0–0.24$, at $\lambda = 352$ nm about the same wavelength as in the corresponding UV spectra. Thus, the CD and UV measurements conclude to an intercalative binding mode for BPSQ at low drug to DNA ratio. The simplicity of the positive CD signal ($\lambda = 352$ nm) suggests that BPSQ intercalates with its chromophore plane parallel to the base pair planes and its long axis oriented perpendicularly to the long axis of the DNA helix as demonstrated for intercalation of trimethine cyanine dye,[34] distamyein–ellipticine,[35] 2-methyl-9-hydroxy ellipticinium acetate.[36] This is consistent with an intercalative binding geometry. Existence of such a geometry is further proved by x-ray analysis of 2-methyl-9-hydroxy ellipticinium acetate with oligonucleotide,[37] daunomycin and adriamycin in their crystallized complexes with an hexanucleotide.[38]

At a higher value ($r' > 0.2$) a negative signal is induced at $\lambda = 332$ nm proving the binding of BPSQ to a new site. The signal position, which appears only weakly, shifted compared to that detected for the free drug in UV-absorption suggests an outside binding mode at high concentration for BPSQ (Fig. 8). These features may suggest equilibrium between large amount of self-stacked drugs bound to the surface of DNA and a small amount of drugs intercalated within base pairs (signal at $\lambda \approx 344$ nm).

Cytotoxic activity was determined on human HL60, B16 melanoma, and neuro 2a cells using a cell growth inhibition assay after 8 hr drug exposures (Table 1). The results from the cytotoxicity assays indicate that BPSQ with an IC_{50} 2.8 μM is highly cytotoxic to HL60, as compared to B16 melanoma 7.2 μM, while neuro 2a ≥ 20 μM in comparison possesses poor cytotoxic activity. Thus, the present results indicate that the intercalating mode illustrated by BPSQ at low drug to DNA ratio is efficient toward the cytotoxic properties.

DISCUSSION

The results presented in this paper provide coherent evidence that association of BPSQ with DNA can be explained by a mechanism of intercalation.

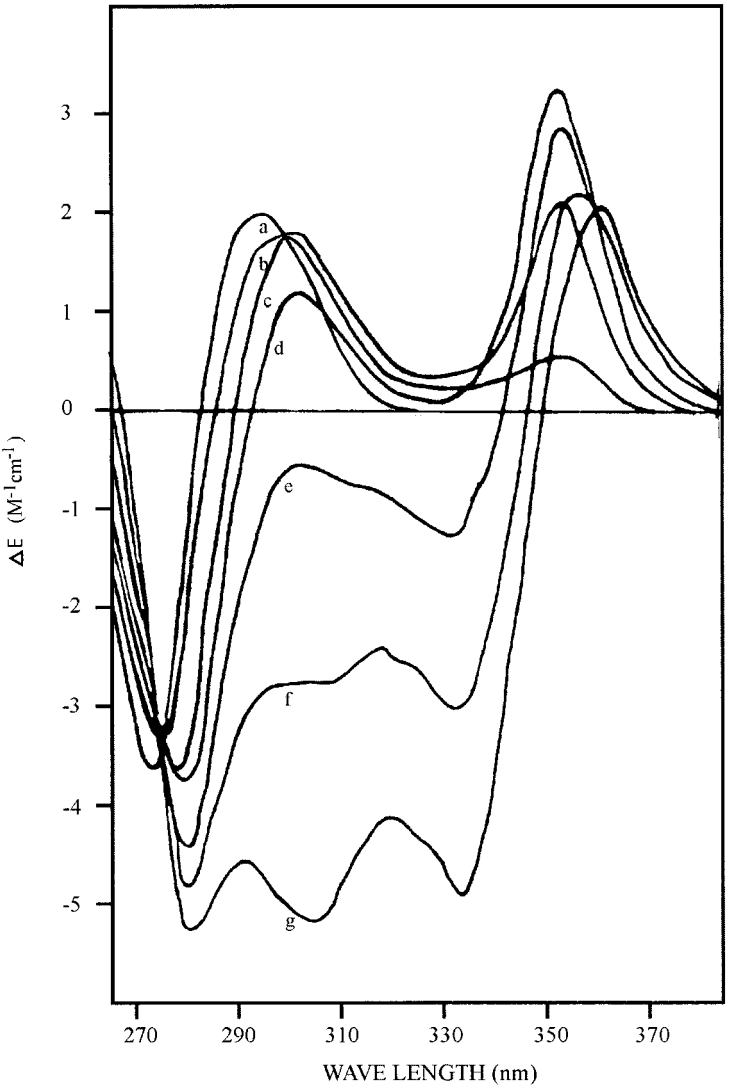

Figure 8. CD spectra of calf thymus DNA titrated with BPSQ; at (a) $r' = 0$; (b) 0.08; (c) 0.16; (d) 0.24; (e) 0.40; and (g) 0.72.

Table 1. In vitro antitumor activity of BPSQ.

	IC_{50} (μM)[a]		
Compounds	HL-60	Neuro-2a	B16 melanoma
BPSQ	2.8 ± 0.09	$\geq 20 \pm 1.02$	7.2 ± 0.10
Ellipticine	1.680 ± 0.01	0.800 ± 0.06	1.430 ± 0.09

Note: Doses that reduce the cell growth by 50%, after 48 hr in vitro as compared to controls.

[a]Results are the means $\pm$ SD of three independent experiments.

In many ways BPSQ behaved as an ideal intercalating drug: on binding to DNA we saw the typical bathochromic and hypochromic shifts in its absorption spectrum; its fluorescence was quenched; the binding constants determined at $I = 0.01$ M were within the range 10^4–$10^5\,M^{-1}$, commonly reported for such drugs; the interaction was suppressed by raising the salt concentration; and hydrodynamic changes consistent with extension and unwinding of the DNA helix were clearly seen.

The importance of electrostatic forces as regards to the formation of the complex is emphasized by the results obtained when the ionic strength of the medium was increased. Adding NaCl upto $I = 0.1$ dramatically lowered the intrinsic association constant and simultaneously reduced the number of nucleotides occluded. Furthermore, when a 100-fold increase in the ionic strength was effected, the binding was so weakened as to verge on the undetectable. These results could be explained by competition of the Na^+ ions for the charged phosphate groups of the DNA, consequently limiting the possibility of charge neutralization with the BPSQ molecules. The dependence of the binding constant on the ionic strength of the environment seems rather more marked than observed with other intercalating drugs, which may indicate a higher dependence of BPSQ binding on electrostatic forces for the formation of the complex with DNA. We considered the possibility that the marked ionic strength-dependence of binding might be artifactual, perhaps arising from aggregation of drug molecules at higher salt concentrations, but found no evidence for such a phenomenon. Moreover, no deviations from Beer's law were noted over the entire range of concentrations employed ($\leq 1.79 \times 10^4$ M).

Binding to DNA showed some dependence on the nucleotide content and/or sequence, as evidenced by the increase in the association constant for *M. lysodeikticus* DNA which has a higher GC content than calf thymus DNA. These findings would be in accordance with the results previously

reported for the 2-*N*-methyl-9-hydroxy ellipticinium,[36] sanguinarine,[26] and anthracycline[39] which ostensibly display a preference for GC basepairs.

The most solid evidence for the intercalation mode of binding comes from the viscometric and circular dichroism experiments. The calculated value for the DNA helix extension of 2.4 Å, although lower than the idealized value expected for the intercalation model of 3.4 Å, lies well within the range of values reported for other intercalating agents, i.e., between 1.8 and 4.5 Å.[40] CD experiments showed that BPSQ interact with DNA according to two possible binding modes: the first mode concerned intercalation of BPSQ with its long axis perpendicular to the long axis of base pairs and second mode, the drug was able to bind to external site once the intercalation sites were saturated at high concentration.

Selenium compounds are purported to interfere with number of possible steps in pathways leading to carcinogenesis, including the inhibition of DNA adduct formation and cell proliferation, and the enhancement of apoptosis.[41,42] Each of these endpoints may be influenced via several possible mechanisms.[41,42] Effects on apoptosis would not be detected in mutagenesis assays.[19] However, the effects of selenium as an antiproliferative agent may result in an antimutagenic effect. As mentioned in Introduction, topoisomerase-II mediated DNA strand breaks are certainly the most important determinants of antitumour activity: comparative experiments have clearly shown that antitumour drugs were able to generate clevable complexes. Moreover, it has recently been possible to correlate cell resistance to elliticine drugs to DNA topoisomerase II activity.[43] Among the possible pharmacological implications of BPSQ sequence specificity, it could be noted that topoisomerase II mediated strand break do not occur randomly, but are highly site-specific as shown in case of NMHE.[44,45] Furthermore, the sites of drug induced DNA cleavage markedly differ with the nature of the drug, leading to characteristic pattern.[46] Such studies, as recently discussed,[47] could open new perspectives for the understanding of the action of intercalating agents, which could be modulated by the accessibility of both drug and topoisomerase II to specific genes.

CONCLUSION

The interaction of BPSQ with ds DNA has been studied by use of absorption, fluorescence, viscosity, and circular dichroism studies. The obtained results—GC specificity of BPSQ as demonstrated by the fluorescence titration experiments, stability of the DNA complexes to the high ionic strength of the solution, and increase in the contour length of the DNA fragments, suggest that BPSQ interacts with ds DNA predominantly by intercalation. Our results

show that CD is useful to discriminate between the different binding modes of BPSQ to DNA. In vitro cytotoxicity assay indicates that the intercalating mode illustrated by BPSQ at a low drug to DNA ratio is efficient toward the cytotoxic properties.

Further experiments to define the range of antitumor efficacy and biochemical basis of action of BPSQ are in progress.

ACKNOWLEDGMENTS

We are grateful to Dr. S. Y. Ambekar for his generous gift of 4-butylamino pyrimido[4′,5′: 4,5]selenolo(2,3-*b*)quinoline (BPSQ). This work was supported by UGC research grant KU/DV-3/26/2001/8587. SV thanks the Kuvempu University for financial assistance through Junior Research Fellowship.

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