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Biochimica et Biophysica Acta 1381 (1998) 104–112

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Taxol–DNA interactions: fluorescence and CD studies of DNA groove binding properties of taxol

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Received 17 December 1997; accepted 4 February 1998

Abstract

Taxol is perhaps the most successful drug used for the treatment of various cancers. Comprehensive literature accumulated on therapeutics of the drug has indicated numerous side effects. In this paper, by use of fluorescence spectroscopy, it is shown that taxol binds to DNA with an affinity constant (K_a) of 1.08×10^7 M^{-1}. This binding is accompanied by a large 'red edge excitation shift' (REES) of fluorescence emission maximum in taxol–DNA complex. The results point to an interaction of taxol with its core eight-membered ring in the DNA groove and the three phenyl rings projecting away from the DNA. The drug encompasses about two base pairs of DNA upon binding to it. Systematic studies with taxol analogues confirms such a mode of binding. These interesting findings on hitherto unknown taxol–DNA interactions may have clinical implications in view of its large number of side effects and pharmacokinetics.

Keywords: Taxol; Fluorescence; CAgC; DNA binding; REES; Conformation

1. Introduction

Taxol isolated from the Western yew, *Taxus brevifolia* [1], has emerged as the most promising antineoplastic drug for treatment of colon, ovarian, lung and breast cancer as well as melanoma and lymphoma [2]. Its detailed structure–activity relationship have been worked out [3] and the intense interest in taxol is a consequence of the demonstration by Horwitz et al. [4], that it affects the tubulin–microtubule equilibrium. It decreases the critical concentration and the induction time required for polymerization of tubulin to microtubules. Taxol's inhibitory activity of microtubule depolymerisation arrests cell proliferation at the interjunction of G2 and prophase [5].

Taxol also affects various other cellular processes, e.g., increased synthesis of tumor necrosis factor (TNFα) [6], and inhibition of DNA synthesis initiation by thrombin and epidermal growth factor [7,8]. It is also shown to be associated with disturbances in many other cellular processes that lack direct relation with cell division. It affects transport of several proteins viz., insulin, catecholamine, prothrombinase and collagenase across plasma membrane [8,9] and inhibits phosphatase activity on Bcl-2 protein in fine tuning of the signal transduction pathway and programmed cell death [10]. In mice, taxol is known to accumulate in the plasma and the tissues upon intraperitoneal administration [11]. Therapeutic interest in taxol as a drug has led to exhaustive research that has

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0304-4165/98/$19.00
PII S0304-4165(98)00019-1

pointed out a battery of side effects. The effect of introducing taxol during radiation treatment of cancer has been shown to result in concentration dependent increased death of cells [12].

At a molecular level conformation of taxol is highly solvent sensitive and exhibits the phenomena of 'hydrophobic collapse' in various organic solvents [13]. It is suggested that it binds to tubulin with primary recognition of the taxane ring followed by additional interaction with the side chains [13,14]. A recent study has shown incorporation of taxol into hydrophobic region of model membranes resulting in conformational changes of both the C13 side chain and the main taxane ring [15]. In vitro studies have shown that taxol drastically reduces the effect of externally added DNA, which inhibits the assembly of microtubules from solutions of tubulin isolated from pig brain [16]. In view of the above observations, the direct interaction of taxol with DNA has important relevance especially since the drug is already released for human chemotherapy.

This paper presents results which shows that taxol has favourable molecular interaction with DNA. The binding of taxol with DNA is accompanied by a large 'red edge excitation shift' (REES) of its fluorescence emission maximum in taxol–DNA complex. This together with CD properties suggests that taxol binds to DNA through the taxane ring system, very much similar to its interaction with tubulin [14]. In the bound form taxol inhibits DNA dependent DNA synthesis.

2. Materials and methods

All chemicals used were of the highest purity available and were used without further purification. Unless otherwise mentioned all spectral measurements were performed in 10 mM Tris buffer (pH 7.2), containing 100 mM NaCl and 20 mM $MgCl_2$. Taxol and its analogues were a kind gift from the National Cancer Institute, USA. A stock solution of taxol prepared in 0.1% acetic acid in methanol was diluted with the buffer just before use.

Ethidium bromide displacement assay was performed as reported in literature [17]. Typically, DNA (20 μM) was added incrementally to 1 μM aqueous ethidium bromide solution, until the rise in ethidium bromide fluorescence ($\lambda_{ex} = 475$ nm, $\lambda_{em} = 610$ nm) attained saturation. To this complex, small aliquots (2 μl) of concentrated taxol solutions (820 μM) were added till the drop in intensity reached a constant value. C_{50} value is the concentration of the drug which caused a 50% drop in the fluorescence intensity in EtBr–DNA complex.

Fluorescence spectra were recorded on Perkin Elmer LS 50B luminescence spectrometer. The emission spectra of taxol was obtained in the range 260 to 400 nm upon excitation at 230 nm, using a slit width of 5 nm each and a scan speed of 100 nm/min. The excitation spectra were recorded from 200 to 500 nm, with the excitation and emission slit widths of 10 and 5 nm, respectively. Fluorescence titration experiments were performed by keeping the taxol concentration constant and stoichiometrically varying the DNA concentration. The titration data was fitted into the Stern–Volmer Eq. (1):

$$I_0/I = 1 + K_{SV}[\mathrm{DNA}] \quad (1)$$

where I_0 and I are the fluorescence intensities of the probe in the absence and presence of DNA. K_{SV} is the Stern–Volmer quenching constant which is a measure of the efficiency of fluorescence quenching by DNA.

The binding affinities of taxol to DNA was calculated from the fluorescence titration curves. By using the fluorescence data, concentration of free probe was determined using Eq. (2), where C_T is the total concentration of the added probe, C_F is:

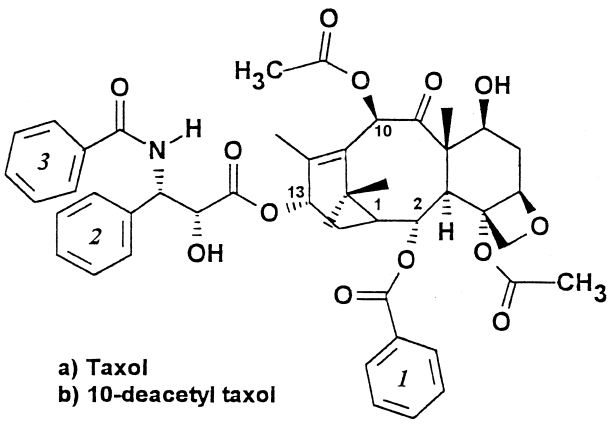
$$C_F = C_T(I/I_0 - P)/(1 - P) \quad (2)$$

the concentration of the free probe and I and I_0 are the fluorescence intensities in the presence and in the absence of DNA, respectively. P is the ratio of observed fluorescence quantum yield of the probe in bound form to that in free form. The value of P was obtained from a plot of I/I_0 vs. $1/[\mathrm{DNA}]$ such that the limiting fluorescence yield is given by the y-intercept. The amount of bound probe (C_B) at any concentration is equal to $C_T - C_F$.

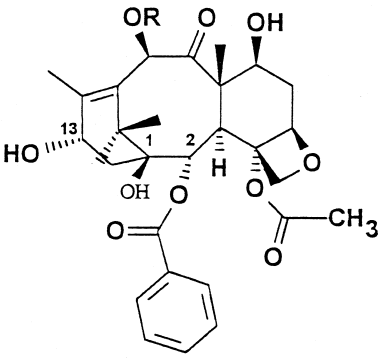
Scatchard analysis was done according to the modified equation of McGhee and von Hippel [18] using a plot of r/C_F vs. r, where r is equal to $C_B/[\mathrm{DNA}]$.

$$r/C_F = K_i(1 - nr)\left[(1 - nr)/[1 - (n-1)r]\right]^{n-1} \quad (3)$$

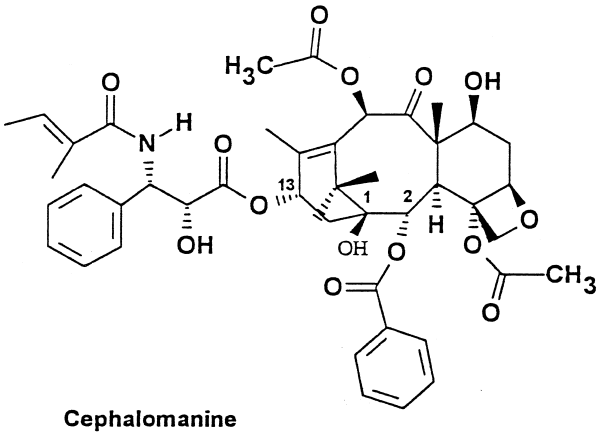
In Eq. (3), K_i is the intrinsic binding constant and n is the binding size in base pairs. These values can be obtained from the linear regression best fit of the data.



a) Taxol
b) 10-deacetyl taxol



c) R = Ac - Baccatin III
d) R = H - 10-deacetyl Baccatin III



Cephalomanine

Scheme 1.

The binding stoichiometry of the polynucleotide–drug complex was estimated from the intersection of the straight lines in a plot of normalized increase in fluorescence against the molar ratio of polynucleotide to ligand. The intersection point gives the number of bound nucleotides per drug molecule.

All CD spectra were recorded on a JASCO J600 machine. Conformation of DNA bound taxol was obtained by subtracting the CD signal of only DNA from that of the complex. This would in effect give the CD signal of taxol bound to DNA. The oligonucleotide d(CGCGAATTCGCG)$_2$ [Dickerson's dodecamer] used for taxol complexation studies was synthesized on a Pharmacia 'Gene Assembler Plus' using standard phosphoramadite chemistry [19] purified by FPLC and the purity rechecked by HPLC. Although all the results presented in this paper use Dickerson's dodecamer, interaction of taxol with λDNA (Boehringer Mannheim) gave similar results.

Primer extension reactions were carried out with pGEM-3Z plasmid as the template and Sp6 primer (both purchased from Promega, Madison, USA). The template (1 μg) and the primer (10 pmol) were annealed in 10 mM Tris (pH 7.2), 20 mM $MgCl_2$ and 50 mM NaCl by heating at 65°C for 5 min followed by slow cooling to room temperature. Primer extension reaction was carried with dNTP mixture containing $\gamma-^{32}P$ dCTP and 1 unit Klenow enzyme (DNA-*Pol*I fragment). In another set of reaction, the annealed sample was incubated with taxol (8 to 64 nM, respectively) for 15 min before primer extension reaction. The reaction was terminated by the addition of (20 mM) EDTA. The samples were then run on a 1% agarose gel. After the run, the gel was dried and exposed to X-ray film, at −70°C (Scheme 1).

3. Results

Fig. 1 shows the fluorescence spectrum of taxol in aqueous solutions, with excitation at 230 nm and emission maximum in the range of 330–340 nm, and the effect of DNA addition on the spectrum. The fluorescence of taxol arises from (i) the C13 side chain containing two phenyl groups, one in the main chain and the other linked by an amide bond, and (ii)

a phenyl ring at C2 position on the eight-membered taxane ring [15]. Other analogues of taxol [viz., 10-deacetyl taxol, Baccatin III, 10-deacetyl baccatin III and cephalomanine (Scheme 1)] used in present study have fluorescence properties very much similar to taxol. This set of analogues have one, two or all the three phenyl systems and the structural basis of binding of taxol and its analogues to DNA can be systematically monitored by fluorescence spectra arising from these segments.

The fluorescent spectra of taxol alone was found to be highly dependent on the solvent polarity, with a change from methanol to acetonitrile resulting in a blue shift of about 30 nm in the emission maximum ($\lambda_{em,max}$) [15]. This behaviour was attributed to a 'hydrophobic collapse' of taxol structure in going from relatively nonpolar acetonitrile to polar methanol. Fig. 2 shows the concentration dependent changes in the fluorescence signature of taxol in aqueous Tris buffer. The emission λ_{max} exhibits a blue shift of 20 nm upon increasing taxol concentration from 0.04 to 102 nM, with a simultaneous rise in the fluorescence intensity that attains saturation beyond ~51 nM concentration. This behaviour suggests an aggregation of taxol with a Critical Aggregation Constant of 6.4 nM (Fig. 2, spectra 4).

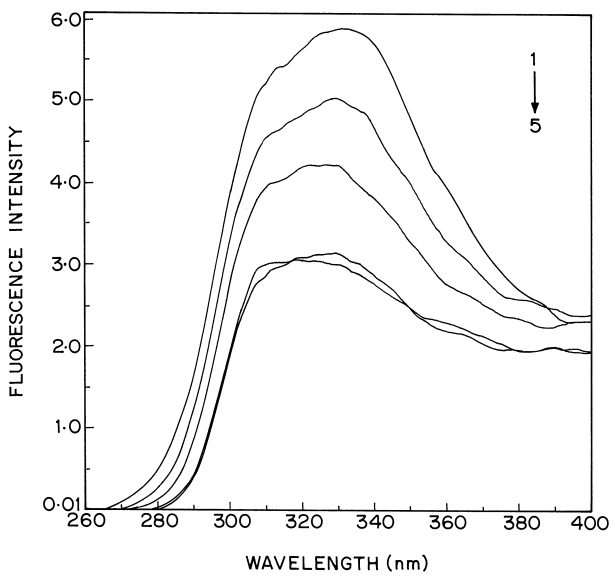


Fig. 1. Effect of DNA on the fluorescence spectra of taxol (3.2 nM). DNA concentrations: (1) none, (2) 1.9 μM, (3) 3.2 μM, (4) 6.3 μM, (5) 6.7 μM. For other conditions, see Section 2.

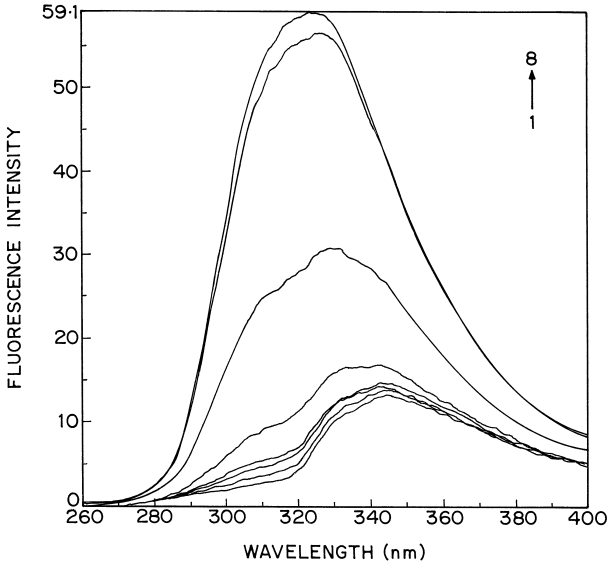


Fig. 2. Fluorescence spectra of taxol in 10 mM Tris buffer, as a function of concentration (1) 0.04 nM, (2) 0.4 nM, (3) 1.6 nM, (4) 3.2 nM, (5) 6.4 nM, (6) 25.6 nM, (7) 51.2 nM, and (8) 102.4 nM.

3.1. Fluorescence studies of taxol–DNA binding

Fig. 1 shows the effect of stoichiometric addition of synthetic oligonucleotide d(CGCGAATTCGCG)$_2$ on fluorescence spectra of taxol, at room temperature. It is seen that increasing the concentration of the oligonucleotide results in a gradual decrease in fluorescence intensity of taxol upto a maximum of ca. 50%, without effecting any perceptible shifts in fluorescence $\lambda_{max,em}$. A similar fluorescence quenching behaviour was observed for 10-deacetyl taxol, 10-deacetyl baccatin, baccatin-III and cephalomanine that have different number of phenyl rings, upon interaction with DNA.

The Stern–Volmer quenching constants (K_q^{-1}) computed from the fluorescence changes of taxol and its analogues upon interaction with DNA was in the range of 0.2 to 0.35 μM^{-1} of DNA, with only a very small variation among the different taxol analogues (Table 1). 10-Deacetyl taxol required highest amount of DNA to achieve a 50% quenching of the initial fluorescence as indicated by the K_q^{-1} values. The quenching of taxol fluorescence by added cesium chloride showed no difference among the free and DNA-bound form. An analysis of the binding affinity of taxol to DNA by Scatchard plot showed a marked

Table 1
Stern–Volmer quenching constants

Compound	K_{SV} μmol^{-1} (Dickerson's 12 mer)	K_q^{-1}
Taxol	0.35	2.8
10-deacetyl–Taxol	0.2	5
Bacattin III	0.27	3.6
10-deacetyl–Bacattin	0.35	2.8
Cephalomanine	0.25	4

deviation from linearity. An affinity constant (K_a) of 1.08×10^7 M^{-1} was obtained for taxol–DNA binding from a linear double reciprocal plot.

A knowledge of the binding stoichiometry of the drug–DNA complex would be helpful in characterizing the geometry of the drug binding to DNA. This was computed from Fig. 3 by the intersection point on the binding isotherms, giving a binding site size of slightly less than two nucleotides per bound drug molecule.

The displacement of DNA intercalated ethidium bromide by groove binding molecules has been used as a standard technique to assay DNA binding agents [20]. The binding constants of drugs to DNA can be estimated by measuring the loss of ethidium fluorescence as a function of added ligand. In such a competitive binding assay, taxol displaced ethidium bromide bound to DNA leading to a drop in fluorescence of ethidium bromide by 50% corresponding to a C_{50} value of 4.6 μM, thereby supporting a strong complexation affinity with DNA as found from Fig. 4.

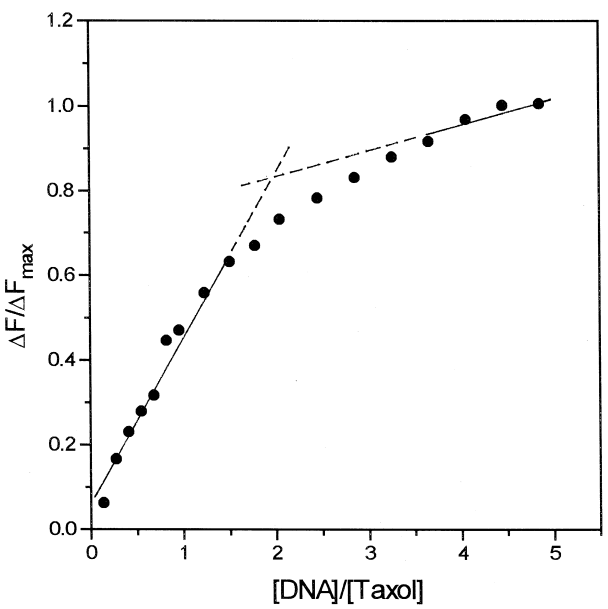


Fig. 3. Fluorescence binding isotherms for the association of taxol with DNA in 10 mM TRIS buffer (pH 7.2), containing 50 mM NaCl and 20 mM $MgCl_2$. The binding stoichiometry in terms of number of nucleotide bases/drug molecule is the value at the intersection of the two straight lines.

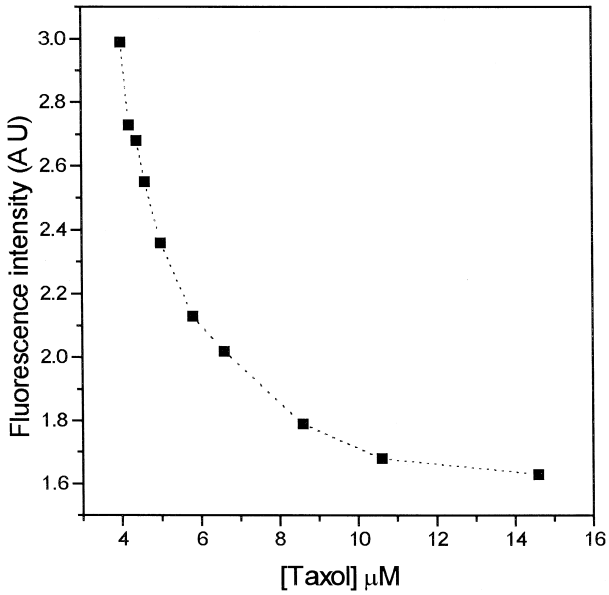


Fig. 4. The displacement of ethidium bound to DNA by addition of taxol as followed by fluorescence of ethidium bromide, Ex. 475 nm, Em. 610 nm. The final drug concentration is indicated on *x*-axis.

3.2. REES

In order to provide further evidence for taxol–DNA binding, a study of the restriction in mobility of taxol upon binding to DNA was attempted. For a fluorophore in bulk nonviscous solvent, the dipolar relaxation of the solvent molecules around the fluorophore in the excited state is much faster than the fluorescence life time. However in highly viscous solvents, where the dipolar relaxation of the solvent molecules in the excited state is much slower, the decay rates and the wavelength of maximum emission show de-

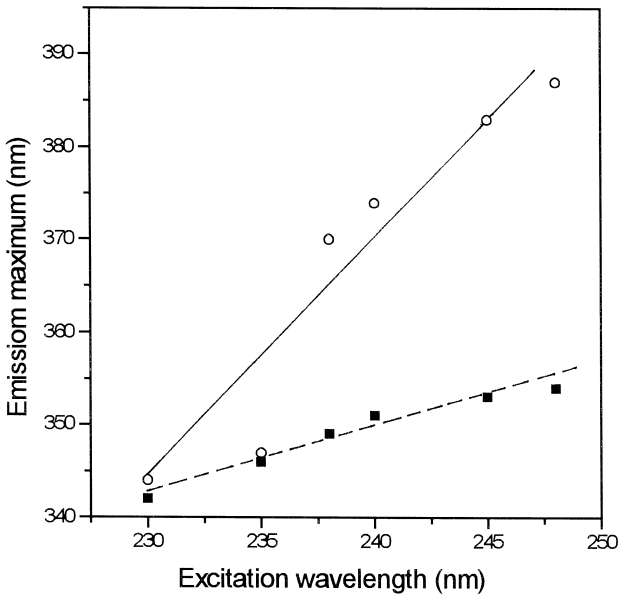


Fig. 5. The effect of excitation wavelength change on the emission wavelength of taxol (3.2 nM) in the absence (- - - -) and presence of DNA (———). Other conditions as in Section 2.

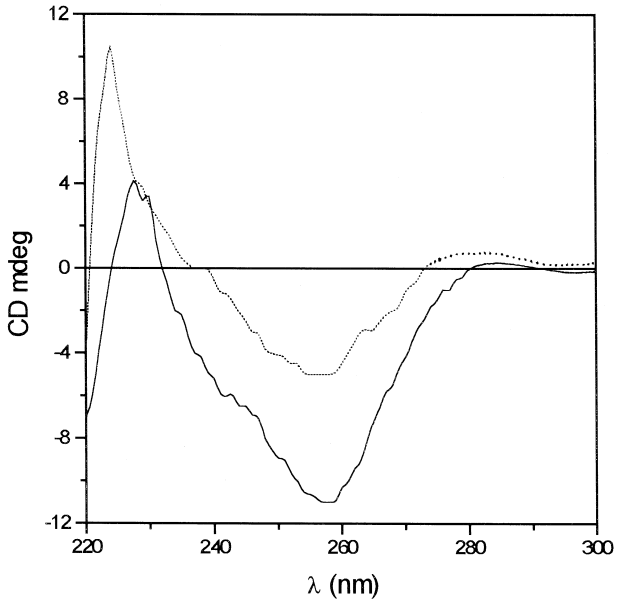


Fig. 6. CD spectra of taxol [3.2 nM] alone (solid line) and when bound to DNA (8 μM). Spectra were recorded in 10 mM TRIS buffer (pH 7.2), containing 50 mM NaCl and 20 mM $MgCl_2$.

pendence on the excitation wavelength [21–23]. Such a shift in the wavelength of maximum emission called REES, is a good indicator of not only the fluorophore environment but also constrained mobility resulting from its binding to another molecule.

Fig. 5 shows the REES exhibited by taxol in both the free form and upon binding to DNA. While free taxol exhibited a REES of about 12 nm in aqueous media, saturation with DNA increased the REES value significantly to 40 nm. This not only confirms the strong binding of taxol to DNA, but also suggests a large restriction in its mobility as a consequence of its binding to DNA.

3.3. CD studies

Taxol exhibits two prominent CD bands: a negative band at 298 nm and a sharp positive band at 232 nm [15]. The interaction of taxol with DNA elicited considerable changes in its CD spectra (Fig. 6). The 232 nm band of taxol upon binding to DNA shifted towards shorter wavelengths (5 nm) with a positive increase in intensity. While no changes occurred in the position of the broad negative band at 285–295 nm, a large increase in the positive intensity was observed upon DNA binding.

3.4. Inhibition of DNA synthesis by taxol

In the present study, we attempted DNA-*Pol*I (Klenow fragment) driven extension of SP6 primer

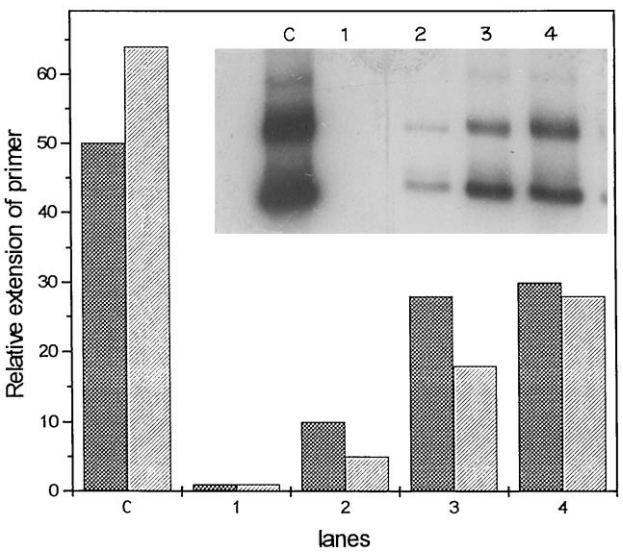


Fig. 7. Histogram showing the extent of primer extended products in the absence (C) and in presence of taxol, (1) 64 nM; (2) 32 nM; (3) 16 nM and (4) 8 nM. Inset: Autoradiogram showing the extended products by Klenow enzyme in absence (C) or presence of taxol (1–4).

using pGEM-3Z plasmid as the template. It was observed that taxol inhibits DNA synthesis in a concentration dependent manner (Fig. 7). A total inhibition of DNA synthesis was observed in presence of 0.064 μM taxol. As low a concentration as 2 nM taxol was sufficient to significantly inhibit DNA synthesis. The inhibition of DNA synthesis due to the binding of taxol to the Klenow fragment of DNA-*Pol*I was ruled out by use of enzyme preincubated with taxol for 30 min and removal of free taxol by passing the mixture through a G-10 column. Such preincubated enzyme had no effect on DNA synthesis activity, suggesting that taxol may inhibit DNA synthesis by binding to the DNA (as a substrate) and not to the enzyme.

Table 2
Emission properties of Taxol

% Dioxane	$\lambda_{ex,\ max}$	$\lambda_{em,\ max}$	Stokes shift
100	216.7	286.3	112,183
90	216.7	286.3	112,183
80	216.1	286.6	116,246
60	215.5	285.7	114,020
50	215.5	285.7	113,774
40	215	285.7	114,019
30	214.9	286.3	116,049
20	213.8	286	118,076
10	212.1	284.4	119,858
5	210.3	284.9	124,511
0	213.8	326.5	161,448
Taxol + DNA	220	345	164,690

4. Discussion

A useful attribute of ethidium binding to DNA is that its fluorescence is enhanced about 50-fold, making it a convenient probe to measure DNA binding of other drugs [20]. The addition of a DNA binding agent induces a progressive decrease in fluorescence of ethidium due to its displacement from the duplex. This also allows distinguishing non-intercalative binding agents from intercalating agents: agents having a large site size (groove binders) require correspondingly smaller concentrations to saturate the sites. As shown in Fig. 4, taxol displaces intercalated ethidium leading to a 50% drop in intensity at a concentration of 4.6 μM, indicating a fairly high binding constant (in the range of 10^6 M^{-1}) with non-intercalation as a probable mode of binding.

The fluorescence of taxol arises from the three aromatic rings. The titration of taxol with DNA causes no significant change in the position of the $\lambda_{em,\ max}$ of taxol (Fig. 1) suggesting that taxol binding to DNA does not change the average local environment of these three phenyl rings (Table 2). An intercalation or a direct groove binding of the phenyl rings attached to C13 and C2 side chains would be expected to elicit strong changes in their fluorescence spectral properties [24]. Thus the binding of taxol with DNA presumably occurs through the core eight-membered taxane ring of taxol rather than by involvement of the phenyl rings. In taxol all three phenyl rings are oriented on the same side of the eight-membered ring system, allowing a facile binding of taxane ring from the opposite side. The relatively different orientation of the ring 3 with respect to the other rings does not affect taxol's interaction with DNA. In this mode of taxane binding, the three phenyl rings are projected away from DNA into bulk water, without significant role for them in DNA recognition. The absence of one or many phenyl rings in taxol analogues, baccatin and cephalomanine, therefore has no effect per se on DNA binding. This type of binding also places the 10-acetyl group towards the DNA and may assist in its recognition since 10-deacetyl taxol showed a weaker DNA binding compared to taxol. Coincidentally, this is similar to the proposed binding of taxol to tubulin, mediated by the taxane ring [14].

Since the aromatic rings, which are the fluorescence reporting groups on taxol, are not involved in DNA binding, it is difficult at this juncture to pin point the groove preference (major/minor) for the drug to bind DNA. The recognition of macrocyclic rings to DNA has precedence in the binding of antitumor antibiotic anthramycin [25]. Its seven-membered core ring is known to fit snugly into the minor groove of the DNA. CsCl mediated fluorescence quenching constants for taxol, either free or in DNA bound form are same, supporting the proposed mode of binding, in which the ring systems would be easily accessible to the quencher in bulk water.

The fluorescence intensity of taxol decreases upon binding to DNA in a manner, qualitatively similar to the effect of dilution (Fig. 2), suggesting that taxol binds to DNA as a monomer rather than in aggre-

gated form. The concentration of taxol used for DNA binding studies was 3.2 nM, much less than its CAgC (6.4 nM) [26]. DNA binding of taxol leads to the break up of even these low number aggregates, consequently leading to a drop in the fluorescence intensity. Earlier studies reported on the effect of taxol on the microtubule assembly have indicated tubulin polymerization with taxol concentration as low as 50 nM [5]. In comparison, the binding of taxol to DNA occurs at a much lower concentration (2 nM) to yield complex in which taxol binds DNA in a monomeric form rather than as an aggregate.

REES data has provided further insight into taxol aggregation behaviour and its DNA binding property. Most fluorophores do not display any REES in free form because the time scale for solvent reorientation in such conditions is of the order of 10^{-12} s [27,28]. Free taxol exhibits a REES (12 nm) which may be a consequence of the 'hydrophobic collapse' assisted formation of 'taxol aggregates'. The relationship between the magnitude of REES and the fluorophore's binding to its substrate has earlier been shown in case of 2-*p*-toulidinyl naphthalene sulfonate (TNS), an extensively used fluorescent probe to study protein structure [28]. In solution, free TNS does not show any REES, whereas when bound to apomyoglobin it exhibits a 11.5 nm REES. In light of these results, a 40 nm REES of taxol emission is a convincing indicator of its strong binding to DNA.

In CD, strong Cotton effects from taxol have been shown to be both solvent and concentration dependent [15]. A correlation of detailed NMR and CD studies in both organic solvents and water have indicated that the positions and the intensity of the CD bands are highly sensitive to the polarity and the aggregation state of taxol. These studies have also provided a molecular basis for the origin of these bands: the 298-nm band arises from the $\pi-\pi*$ transition of the aromatic rings of the side chain, the shoulder at 264 nm due to the C2-*O*-benzoyl group and the 232-nm band corresponds to n-$\pi*$ transition involving the keto C=O group at C9 and the amide carbonyl at C13. A shift in the 232-nm band towards longer wavelength is interpreted to indicate the aggregation of the drug and a shift of the 295-nm negative band an indication of localization of taxol in a nonpolar environment, arising from the C2′–C3′ bond rotation in the C13 side chain.

The binding of taxol to DNA led to a shifting of the positive CD band at 232 nm to shorter wavelengths (5 nm) accompanied by an increase in intensity (Fig. 6). On the other hand, the negative band in the 285–295 nm region showed a change in intensity without any shift. These results strongly suggest that taxol goes from an aggregated form (while in solution) to a non aggregated form due to binding with DNA. In the DNA bound form, taxol would be unable to form self aggregates. Further, the C13 side chain, projecting away from DNA is unaffected by binding as shown by the retention of the 285-nm band position. The slight positive increase in intensity of this band is however, similar to that observed in dilute, unaggregated solutions of taxol. This observation is consistent with the results from fluorescence quenching and REES experiments.

Under similar conditions of ionic strength and pH, intercalators like ethidium bromide and 9-aminoacridine show association constants in the range of 10^5 M^{-1} [29] and the minor groove binding drugs such as netropsin, distamycin and HOECHST 33258 have higher association constants, in the range of 10^7 M^{-1} [30]. The computed K_a of taxol (1.08×10^7 M^{-1}) points it to be a groove binder.

It was recently demonstrated that some DNA binding drugs inhibit DNA amplification by PCR and this concept was employed to assess the sequence selectivity of DNA binding drugs [31]. Earlier studies have elucidated the effect of mithramycin on transcription [32] and netropsin on enhancement of *Pol* II termination [33]. It would therefore be interesting to ponder on the biological significance of present observations that taxol binding to DNA inhibits in vitro, template directed DNA primer extension. In view of the fact that DNA synthesis in vitro could be inhibited by taxol at concentration (2 nM) much lower than that affecting tubulin polymerisation (50 nM), it is tempting to suggest that, low amounts taxol permeating into cells may inhibit DNA replication and possibly translation, leading to cell death. Further experiments are needed to explore such ideas.

5. Conclusions

In summary, this paper demonstrates the hitherto unknown interaction of taxol and some of its ana-

logues directly with DNA. The association occurs in the DNA groove through the eight-membered taxane core ring, with the three phenyl rings pointing away and is similar to that proposed for taxol–tubulin interaction. In light of such evidence for DNA–taxol interactions it remains to be seen whether DNA may be an additional site of action for taxol. Very low concentrations of the drug reaching normal cells may cause damage by interacting with DNA. Unlike the earlier spectroscopic studies most of the present observations are in aqueous medium under physiological conditions. The observed significant binding of taxol to DNA (ca. 10^7 M^{-1}) has possible clinical implications even at low dose treatment, thus assuming biological importance in the context of the side effects and pharmacokinetics of the drug.

Acknowledgements

AGK and DVK thank CSIR and UGC, India, respectively for financial assistance. KNG acknowledges Jawaharlal Nehru Center for Advanced Scientific Research of which he is a Senior Honorary Fellow. We are thankful to Dr. S. Mitra, TIFR, Bombay for allowing us to use the CD machine.

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