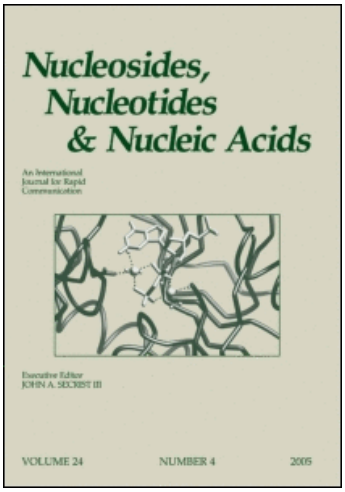

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

A Spectroscopic and Thermodynamic Study of Taxol Nucleic Acid Complexes

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To cite this Article Bischoff, G. , Gromann, U. , Lindau, S. , Meister, W. V. and Hoffmann, S.(1999) 'A Spectroscopic and Thermodynamic Study of Taxol Nucleic Acid Complexes', Nucleosides, Nucleotides and Nucleic Acids, 18: 10, 2201 — 2217
To link to this Article: DOI: 10.1080/07328319908044876
URL: http://dx.doi.org/10.1080/07328319908044876

NUCLEOSIDES & NUCLEOTIDES, 18(10), 2201–2217 (1999)

A SPECTROSCOPIC AND THERMODYNAMIC STUDY OF TAXOL NUCLEIC ACID COMPLEXES

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ABSTRACT: The interactions of natural and synthetic polynucleotide double strands with the antitumor agent paclitaxel and the oncological product "Taxol® for Injection Concentrate" (abbreviated as taxol) were examined in diluted aqueous solutions by thermal denaturation profiles (T_m), CD spectra and UV-absorption measurements. Furthermore, DNA-paclitaxel and -taxol complexes in condensed nucleic acid solutions were studied by differential scanning calorimetry. As polynucleotides alternating and homologous poly[d(AT)] and poly[d(GC)] and calf thymus DNA were used. The results point to stabilizing interactions of paclitaxel to AT nucleotides, whereas in the presence of GC base pairings no interaction took place. Thereby the interaction to homologous (dA)·(dT)-tracts seems to be preferred.

1 INTRODUCTION

Drugs with antiviral or antitumor potential have often been examined by their binding to DNA, since this interaction is expected to play a determining role in therapeutic activities [14-16]. Examining the binding abilities to DNA by varying the nucleic acid sequences and observing the complexes with different methods (CD, T_m, DSC, NMR, and others), leads to useful informations about the nature of the binding and offers possibilities of definite, decided drug modifications [15, 17].

Investigations of the pharmacological product "Taxol® for Injection Concentrate" (abbreviated as taxol) containing paclitaxel as effective drug, (Fig. 1) received much

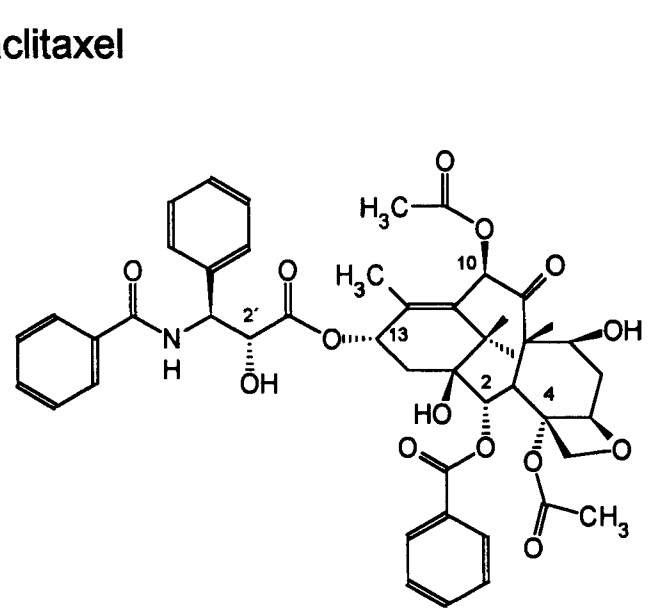

FIG. 1: Chemical structure of paclitaxel

publicity in the past years, since paclitaxel (isolated by M. C. Wani et al. from the extract of the inner bark of the "Pacific yew tree" [1]) seems to be an anticancerous agent [1-3]. Taxol would be regarded as a promising agent for the treatment of several types of cancer like ovarian, breast, lung, and melanoma [4, 5]. Experiments on different cancer cells by treatment with paclitaxel (or taxol) show a stabilization of the microtubules and increased tubulin assembly kinetics [6, 7]. In the last years a great number of medical (and clinical) studies were done by changing the chemical structure of paclitaxel and examining its cytotoxity and tubulin assembly facilities [18-21], while only little is known about the binding modes of DNA-paclitaxel complexes. In the study of M.G. Solis Recendez et al. (1996) a twofold higher concentration of paclitaxel in the nucleus than in the cytosol of a human lung tumor cell line was found [8]. Supplementary to the medical studies, we observed here the interaction of DNA-paclitaxel complexes of selected polynucleotides with common methods.

Paclitaxel (Fig. 1) in alcoholic solution and in the oncologically used drug combination of "Taxol® for Injection Concentrate" with ethanol and polyoxyethylated castor oil was applied to synthetic polynucleotides with alternating sequences, for instance

poly[d(A-T)]·poly[d(A-T)] and poly[d(G-C)]·poly[d(G-C)], and polynucleotides with homologous sequences, for instance poly(dA)·poly(dT), and poly(dG)·poly(dC), as well as calf thymus DNA (48 mol% AT bases) in aqueous solutions. UV-absorption thermal denaturation, circular dichroism (CD) and differential scanning calorimetry (DSC) studies were done at different ratios of paclitaxel to base pairs DNA, increasing amount of ethanol and increasing amount of castor oil.

2 EXPERIMENTAL

2.1 Materials

Poly[d(A-T)]·poly[d(A-T)], poly[d(G-C)]·poly[d(G-C)], poly(dA)·poly(dT) and poly(dG)·poly(dC) were received from Boehringer GmbH, hybridized in 1 mM NaCl (pH ≈ 7) and used without further purifications. A stock solution of calf thymus DNA (Reanal Budapest) was prepared in neutral saline (0.5 to 10 mM NaCl, pH ≈ 7). Concentrations in base pairs were determined by ultraviolet spectroscopy (ε_{270} = 1.32 x 10^4 $M^{-1}cm^{-1}$ poly[d(A-T)]·poly[d(A-T)], ε_{260} = 1.42 x 10^4 $M^{-1}cm^{-1}$ poly[d(G-C)]·poly[d(G-C)], ε_{260} = 1.20 x 10^4 $M^{-1}cm^{-1}$ poly(dA)·poly(dT), ε_{253} = 1.48 x 10^4 $M^{-1}cm^{-1}$ poly(dG)·poly(dC) and ε_{260} = 1.32 x 10^4 $M^{-1}cm^{-1}$ calf thymus-DNA (48 mole-% AT bases)) [9, 10].

Paclitaxel is poorly soluble in water. So in medicine a mixture of dehydrated alcohol, polyoxyethylated castor oil and water is used (taxol). In this study we treated dissolved nucleic acid duplexes with injections of few portions of paclitaxel or taxol (diluted solutions: paclitaxel or taxol concentration 1 nmol/ml, 1 mM NaCl; condensed phases: paclitaxel or taxol concentration 1 nmol/ml, 1-100 mM NaCl).

5 mg (5.9 μmol) paclitaxel powder (Fluka) were dissolved in 200 μl ethanol (paclitaxel concentration 29.5 μmol/ml) and used without further purification. Taxol ("Taxol® for Injection Concentrate) was purchased from MeadJohnson, Bristol-Myers Squibb Co. (Princeton, USA), containing 6 mg/ml paclitaxel (7.0 μmol/ml), 527 mg/ml Cremophor® EL (polyoxyethylated castor oil) and 49.7 % ethanol (8.5 mmol/ml).

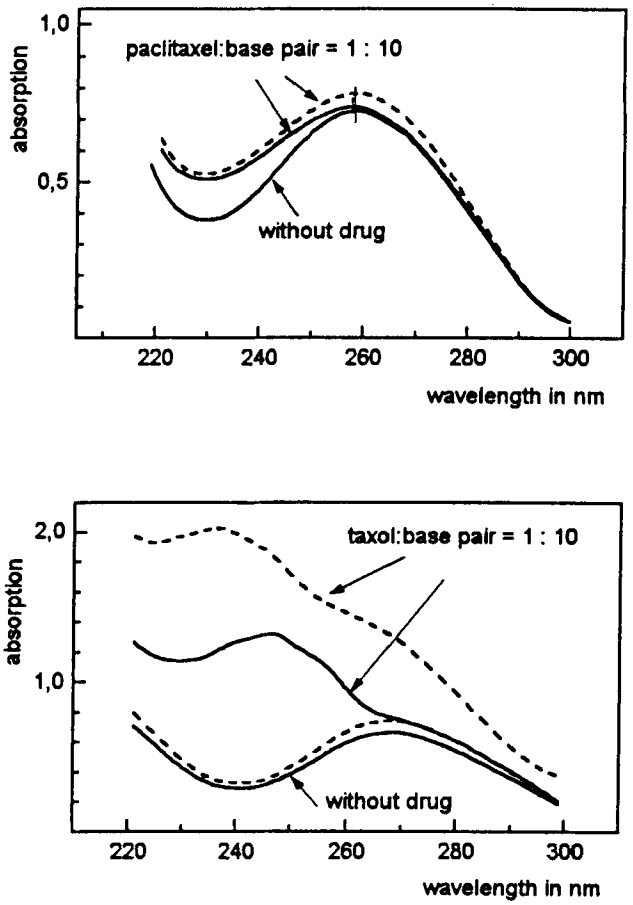

FIG. 2: UV spectra of calf thymus DNA-paclitaxel (top) and -taxol (bottom) complexes at different temperatures; solid lines (20 °C), dashed lines (85 °C)

All DNA and drug solutions were kept at -20 °C and were allowed to warm to room temperature before sample preparation. Portions of the alcoholic paclitaxel and taxol solutions were applied to aqueous DNA-solutions (1 mM NaCl, pH = 6.8). Ethanol ratios of 0.07 Vol% per pmol of paclitaxel (Fluka paclitaxel) and 0.01 Vol% per pmol of paclitaxel (taxol) were used. The paclitaxel Fluka solution contains no castor oil, the diluted taxol-solution contains 0.07 mg/ml polyoxyethylated castor oil per pmol paclitaxel.

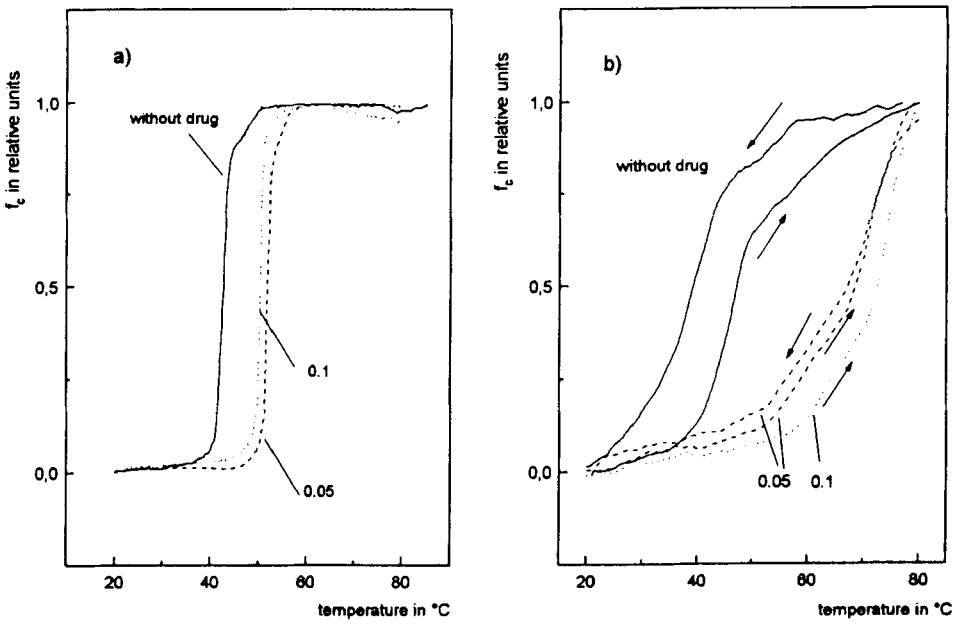

FIG. 3 Thermal denaturation curves of a) poly(d[A-T])·poly(d[A-T]) and b) poly(dA)·poly(dT), pure and complexed with paclitaxel; at f_c =0 no strand, at f_c=1 all strands are separated; solid lines (without drug), dashed lines (ratio paclitaxel:base pairs =0.05), dotted lines (ratio paclitaxel:base pairs = 0.1), temperature increase and decrease marked by arrows

2.2 Methods

UV-absorption spectra in the range of 200 - 500 nm (e.g., Fig. 2) and thermal denaturation profiles at 260 nm (Fig. 3) were obtained with a Perkin Elmer UV/VIS Spectrometer Lambda 2 (equipped with a Peltier temperature control device, PTP-1 Perkin Elmer) by increasing temperatures (0.5 °C/min) at different DNA-paclitaxel, -taxol, -ethanol and -castor oil proportions. The cell holder was thermostated and the temperature was measured inside the cuvette via thermoresistance. Reading of absorbance at 260 nm was measured by increasing and decreasing temperatures in cycles of 20 °C to 85 °C and 85 °C to 20 °C.

CD-spectra (Fig. 4 and 5) were obtained at T = 15 °C on a spectropolarimeter JASCO J-720. Spectra of the nucleic acids, the drug themselves and the nucleic acid-drug complexes were recorded between 200 and 400 nm.

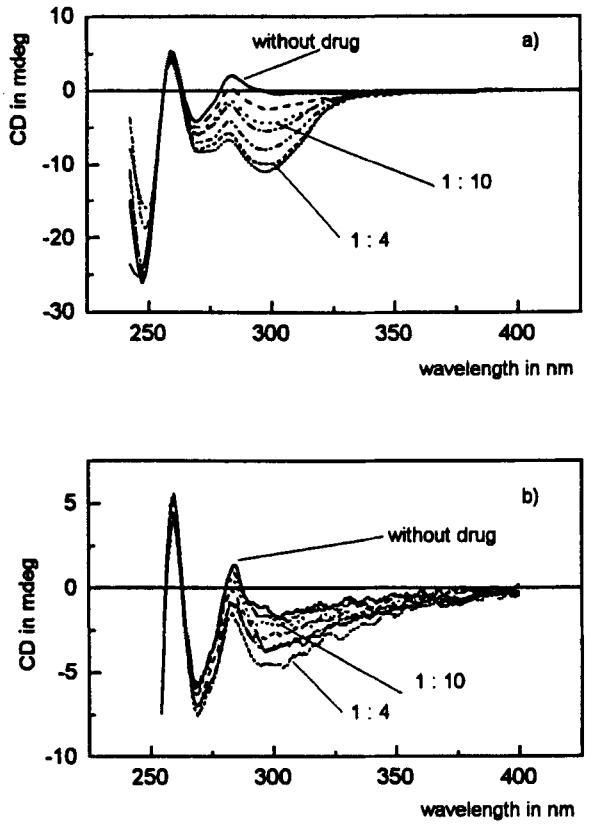

FIG. 4 CD-spectra of poly(dA)·poly(dT) with increasing amount of taxol; (a) measuring, (b) taxol reference subtracted

DSC experiments of pure calf thymus DNA and calf thymus DNA-drug complexes were carried out using a Perkin-Elmer DSC-7 (heating and cooling rates of 5 °C/min). Normally, calf thymus DNA concentrations between 50 and 100 mg/ml (0.1 to 0.2 mmol/ml) in aqueous salt solutions (0 to 300 mM NaCl) were used.

3 Results

3.1 UV Absorption Spectra

The maxima light absorptions of the pure reagents were determined at 230 nm

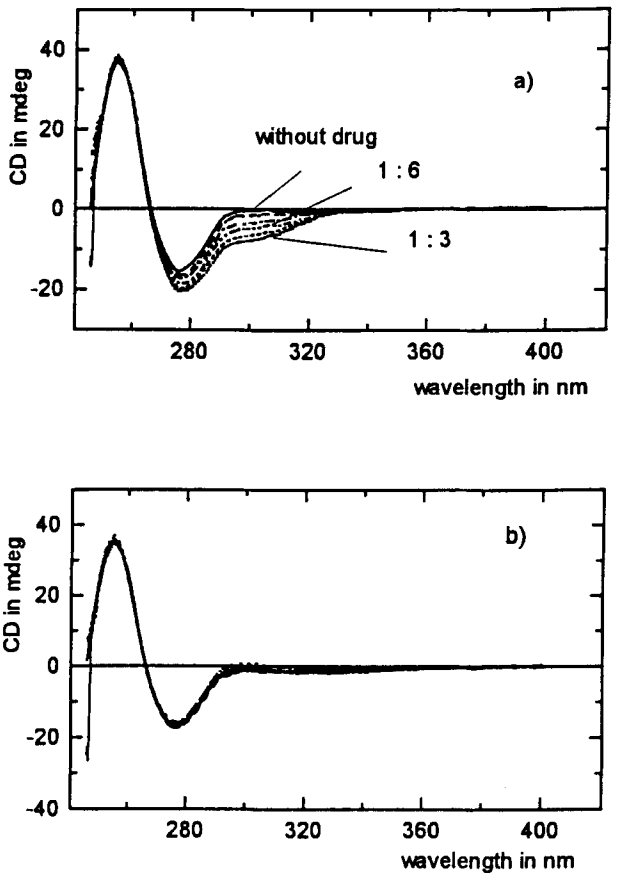

FIG. 5 CD-spectra of poly(dG)·poly(dC) with increasing amount of taxol; (a) measuring, (b) taxol reference subtracted

(paclitaxel, $\varepsilon_{230} = 8.54 \times 10^4\ M^{-1}cm^{-1}$), at 234 nm (taxol, $\varepsilon_{234} = 1.32 \times 10^4\ M^{-1}cm^{-1}$). UV-absorption spectra of the DNA solutions after treatment with paclitaxel or taxol show an increase at around 230 nm proportional to the paclitaxel extinction (Fig. 2). Above 320 nm no light absorption could be found (tested until 500 nm). UV-data are given in Table 1.

The absorption maxima of the here investigated DNA-paclitaxel complexes (around 260 nm) are generally shifted to lower wavelength at 20 °C dependent on the amount of drug (Fig. 2, Table 1). Separating the DNA strands by heating up the DNA-paclitaxel complex leads in most cases to UV absorption maxima similar to those observed at

Table 1: Maxima of UV-absorption (λ_{max}) of DNA and DNA-paclitaxel complexes at 20 and 80 °C in aqueous solution, 1 mM NaCl, λ_{max} (paclitaxel) = 230 nm (ε_{230} = 8.54 x 10^4 M^{-1} cm^{-1})

nucleic acids	without paclitaxel	ratio paclitaxel : base pair 1 : 20	ratio paclitaxel : base pair 1 : 10	ratio paclitaxel : base pair 1 : 5
poly[d(A-T)] · poly[d(A-T)]	262 nm (20 °C) 261 nm (80 °C)	262 nm (20 °C) 261 nm (80 °C)	262 nm (20 °C) 261nm (80 °C)	261 nm (20 °C) 260 nm (80 °C)
poly(dA)·poly(dT)	260 nm (20 °C) 261 nm (80 °C)	260 nm (20 °C) 261 nm (80 °C)	259 nm (20 °C) 260 nm (80 °C)	258 nm (20 °C) 260 nm (80 °C)
poly[d(G-C)] · poly[d(G-C)]	254 nm (20 °C) 248 nm (80 °C)	254 nm (20 °C) 247 nm (80 °C)	252 nm (20 °C) 248 nm (80 °C)	250 nm (20 °C) 248 nm (80 °C)
poly(dG)·poly(dC)	252 nm (20 °C) 246 nm (80 °C)	251 nm (20 °C) 246 nm (80 °C)	250 nm (20 °C) 246 nm (80 °C)	247 nm (20 °C) 246 nm (80 °C)
calf thymus DNA	258 nm (20 °C) 258 nm (80 °C)	258 nm (20 °C) 258 nm (80 °C)	258 nm (20 °C) 258 nm (80 °C)	255 nm (20 °C) 258 nm (80 °C)

80 °C of nucleic acid solutions without drug. This indicates that at 85 °C no drug is bound to the separated strands. An interpretation of the UV-absorption of the DNA-taxol complexes is quite more difficult, because the UV absorptions of paclitaxel, castor oil and DNA are in the same wavelength range.

Absorption measurements of aqueous paclitaxel solutions without nucleic acids by heating up result in no changes of UV absorption at 260 nm. Surprisingly, by heating up aqueous taxol solutions to 85 °C the UV absorption increases strongly above T = 68 °C. Aqueous castor oil solutions (without and containing paclitaxel) on the other hand show a similar behavior with increasing UV-absorption at higher temperatures (above 68 °C), comparable to the absorption changes of aqueous taxol solutions. So this phenomenon could be explained by formation of a colloidal structure of the castor oil molecules in water, which distribute under heating, and do not depend on the presence of paclitaxel.

UV-absorption measurements of ethanolic solutions (tested between 0.02 to 0.8 Vol% ethanol) did not show changes in the absorption by heating up the solution. So the less solubility of castor oil in water may change under increasing temperature by dispersing the castor oil colloids resulting in the UV absorption changes above 68 °C. For that reason, the thermal denaturation curves and UV absorption spectra of the DNA-taxol

Table 2: T_m-values of pure nucleic acids in 1 mM NaCl-solution at increasing ratio of paclitaxel or taxol; the concentrations of DNA were between 40 and 80 pmol/µl

drug	ratio of drug:base pairs	poly[d(A-T)]·poly[d(A-T)]	poly(dA)·poly(dT)	poly[d(G-C)]·poly[d(G-C)]	poly(dG)·poly(dC)	calf thymus DNA
without	0	44.3°C	45.8 °C	>85 °C	>85 °C	43.3 °C
paclitaxel	0.05	51.9 °C	58.8 and 70.7 °C	>85 °C	>85 °C	47.4 and 62 °C
	0.1	50.5 °C	62.5 and 74.3 °C	>85 °C	>85 °C	45.7 and 68 °C
	0.2	53.8 °C	61.9 and 74.8 °C	>85 °C	>85 °C	46.3 and 72 °C
taxol	0.05	41.5 °C	46.1 °C	>85 °C	>85 °C	42.0 and 68 °C
	0.1	45.2 °C	52.6 °C	>85 °C	>85 °C	41.3 and 72 °C
	0.2	47.4 °C	66.5 °C	>85 °C	>85 °C	41.3 and 75 °C

complexes could only be interpreted in the temperature range of 20 to 68 °C by consideration of the reference spectra. But UV melting studies of DNA-paclitaxel complexes could be analyzed in the full measured temperature range (20 - 85°C).

3.2. Thermal Denaturation Curves

UV absorption thermal denaturation curves (Fig. 3) of poly[d(A-T)]·poly[d(A-T)], poly(dA)·poly(dT) and calf thymus DNA, show increasing T_m-values strongly shifted up to higher values by increasing amount of paclitaxel or taxol (under consideration of the reference curves). The T_m-values examined at different ratios of paclitaxel:base pair and taxol:base pair (0 to 0.2) are given in Table 2.

In the temperature range of 20 to 85 °C no separation of poly[d(G-C)]·poly[d(G-C)] or poly(dG)·poly(dC) strands was observed neither in the presence of taxol or paclitaxel

nor without the drugs. It is well known, that polynucleotides with a major part of GC bases do not denature before 90 °C (at given salt concentration). Therefore a definite statement to the stabilizing or destabilizing interaction between GC bases and paclitaxel or taxol could not be done with this method.

The absorption of poly(dA)·poly(dT) strands (Fig. 3b) complexed with paclitaxel (or taxol) increased over a broad temperature range (60 to 70 °C) by separating the strands. Here the highest T_m-shift (paclitaxel: ΔT_m = +29 °C, taxol: ΔT_m = +20 °C) was found. Heating up solutions of calf thymus DNA-paclitaxel (taxol) complexes results in two increases of absorption around T_m = 46 °C (ΔT_m = +3 °C) (taxol: 41 °C, ΔT_m = -2 °C) and T_m = 72 °C (ΔT_m = +19 °C) (taxol: 75 °C, ΔT_m = +22 °C) instead of one transitions (T_m) observed of pure calf thymus DNA (approximately 43 °C).

Studies of nucleic acids with comparable or higher amount of ethanol and/or castor oil in combination with nucleic acids were performed to analyze the T_m-shift of the DNA-drug complexes. The amounts of ethanol were varied in the range between 0.08 and 0.8 Vol%, yielding a T_m-shift of ΔT_m = -0.7 °C at highest amount of ethanol (0.8 Vol% ≈ 0.14 mmol/ml). Portions of 9 to 35 ng/ml castor oil, dissolved in ethanol added to the nucleic acid solutions, yield a decreasing shift in the T_m-values of around ΔT_m = -1 °C. Therefore, the thermal denaturation of DNA-paclitaxel- and DNA-taxol-complexes have to be interpreted preferably as interactions between paclitaxel-molecules and the nucleotides.

3.3 CD-Spectra

Paclitaxel as an optical active molecule absorbs light in the UV-range and shows CD values in the range of 200 to 320 nm. Because of the strong light absorption of paclitaxel at wavelengths between 200 and 250 nm, no interpretation of the CD-spectra at the paclitaxel concentrations employed could be performed. The CD-spectra of aqueous paclitaxel and taxol solutions show at λ>270 nm a strong negative signal, which enlarges at increasing amount of taxol or paclitaxel in the absence of DNA. The largest negative intensity is around 297 nm.

Interaction of paclitaxel or taxol with poly(dA)·poly(dT) or poly[d(A-T)]·poly[d(A-T)] induces negative CD-signals at wavelengths higher than 270 nm, depending on the

amount of drug. After subtraction of the reference spectra (taxol or paclitaxel without nucleotides) an induced negative CD-signal at 269 nm and reduced positive CD-signals at 259 and 283 nm were found, revealing interactions with homologous poly(dA)·poly(dT) and paclitaxel (e.g., Fig. 4). By investigating alternating poly[d(A-T)]·poly[d(A-T)]-paclitaxel- and -taxol-complexes, a decrease of the intensities of the positive CD-signal at 262 nm and the negative CD-signal at 248 nm was observed. The reduced intensities of the nucleic acid assigned CD-signals are indicative of a distortion of the base arrangements in the strands.

Different from this, by investigating the CD-spectra of alternating poly[d(G-C)]·poly[d(G-C)] or homologous poly(dG)·poly(dC), after subtraction of the reference data (paclitaxel or taxol without nucleotides) the characteristic CD-spectra of the pure nucleic acids could be observed (Fig. 5). The results give no indication on an interaction between paclitaxel (or taxol) and GC nucleotides.

Supplementary informations could be obtained by analyzing the CD-signal at 297 nm. Taking the nucleic acid spectra as reference into consideration, the spectra of the pure drug solutions occur. The negative paclitaxel CD-signal does not shift to higher or lower wavelengths.

3.4 Differential Scanning Calorimetry (DSC)

Polydisperse nucleic acid double, triple and quadruple strands in condensed solutions form liquid crystalline phases depending on the ionic solvation [11-13]. As viscous solutions, aqueous calf thymus DNA was used in DNA concentrations between 50 and 100 mg/ml. In all cases typical liquid crystalline textures could be observed by polarizing microscopy at room temperature. DSC measurement of the calf thymus DNA, the calf thymus DNA-paclitaxel complexes and the calf thymus DNA-taxol complexes were performed (NaCl concentrations, 0-300 mM). The value of the transition enthalpy ΔH°_{DSC} of the DNA (helix to coil transition by losing liquid crystalline arrangements) is a helpful tool to examine stabilizing effects of the drug. Unfortunately, paclitaxel is only soluble in water to a less extent, so DSC measurements could only be performed with a ratio of drug to base pairs below 1 : 30 (1 nmol/ml). At higher drug ratio isotropic phases are performed at room temperature, locally separated from the polydisperse

Table 3: Enthalpy observed by T_m (poly(dA)·poly(dT), 10-40 µmol/l) and DSC (calf thymus DNA, 50 mg/ml) measurements

ratio paclitaxel : base pair.	ΔH°_{Tm} (paclitaxel) [kJ/mol base pair]	ΔH°_{DSC} (paclitaxel) [kJ/mol base pair]	ΔH°_{DSC} (taxol) [kJ/mol base pair]
0	-22	8-13	7-13
1 : 100		12-13	14-15
1 : 50	-23	16-18	14-16
1 : 30			26-29
1 : 20	-27		
1 : 10	-30		

double strands. DSC study of calf thymus DNA results in a transition enthalpy ΔH°_{DSC} around 13 kJ/mol (50 mg/ml DNA). This enthalpy increased at increasing amount of drug (Table 3).

4 DISCUSSION

In this study strong interactions of nucleic acids containing AT nucleotides with paclitaxel, as well as with the oncological preparation taxol (containing paclitaxel), were found. The sharp transition point (T_m) of the homologous and alternated nucleic acids shifts to higher temperatures, depending on the amount of drug (Fig. 3). Van't Hoff analysis of the thermal denaturation profiles by using formula 1 was performed, calculating the thermodynamic parameters (ΔH°_{Tm}) of poly(dA)·poly(dT)- and poly[d(A-T)]·poly[d(A-T)]-drug complexes.

$$1/T_m = R/\Delta H^\circ_{Tm} \ln C_T + \Delta S^\circ/\Delta H^\circ_{Tm} \qquad (1)$$

C_T is the concentration of the single strands obtained at 85 °C from UV absorption values, ΔH°_{Tm} the enthalpy and ΔS° the entropy of the complex. R was employed with

8.314 J/mol. Linear plots of $1/T_m$ versus ln C_T by using the above equation gives ΔH°_{Tm}-values with an average error around 10 %. The increase of the absolute value of ΔH°_{Tm} is indicative of the stabilization of the complexes. In general it was found, that at higher amount of paclitaxel the absolute value increased.

A second method to determine thermodynamic parameters involves DSC measurements (using formula 2),

$$\Delta H^\circ_{DSC} = \int(\Delta C_p)\, dT \qquad (2),$$

where ΔC_p is the caloric capacity of the helix coil transition. The received enthalpy (ΔH°_{DSC}) increases with increasing stabilization of the double strands. The stabilizing effect determined by T_m-shift is consistent with the thermodynamical data of calf thymus DNA-drug complexes obtained by DSC. ΔH° data received by both methods are given in Table 3.

In addition to this, CD-spectra could give informations about the arrangements of the bases and the helices. CD-results of mixtures of poly[d(G-C)]·poly[d(G-C)] or poly(dG)·poly(dC) with paclitaxel or taxol indicate, that no interaction between paclitaxel and GC nucleotides takes place. By investigating the CD-spectra of the poly[d(A-T)]·poly[d(A-T)]- or poly(dA)·poly(dT)-drug complexes, reduced intensities of the nucleic acid signals were observed, explained by a certain distortion of the bases under influence of paclitaxel. By trying to disextangle, these apparently contradictory results of T_m, DSC and CD-studies should be explained on closer inspection of the chemical structures.

Watson-Crick arrangement assumed, AT base pairings (alternated and non-alternated) are different from GC base pairings by 2 in contrast to 3 hydrogen bonds. Moreover, the major and the minor groove of the helix are closer resulting in a more twisted helix [22-24]. Homologous AT nucleotides are different from alternating AT nucleotides by an additional interaction between the bases. Their homologous bases (e.g., AAAA) have possibilities to interact with the neighbors of the faced base on the other strand (e.g., TTTT) leading to the broad melting profile. Nelson et al. (1987) [22] found in crystal

structure analysis of homologous (dA)·(dT) strands a narrow minor groove of 9 Å, while in double strands with statistic base sequences the minor groove distance is approximately 12 Å. The major groove was determined with 15.5 Å less smaller than determined for other sequences. These differences may explain why homologous AT nucleotides interact with low molecular weight drugs different from other sequences.

As described above, the greatest stabilization between paclitaxel and nucleic acids was found surprisingly by observing the homologous poly(dA)·poly(dT)-paclitaxel complexes (Table 2). Taking the crystal analysis as foundation to the here described DNA-paclitaxel interaction, paclitaxel molecules with their geometrical possibilities may be arranged in a preferred state by interacting in the narrower major groove of homologous AT nucleotides. The chemical structure of paclitaxel shows 11 chiral atoms of the molecule (Fig. 1). However, the paclitaxel assigned CD-signal does not change by treatment with DNA. So groups of the paclitaxel molecule which have no optical activity may perform the interaction with the DNA. Hydrophilic side groups (e.g. acetyl) and hydrophobic parts (e.g. benzoyl) are combined in the paclitaxel molecule. This special formation seems to have the possibility to interact with the hydrophilic phosphate groups of the DNA backbone (explanation for the strong T_m-shift) and the hydrophobic bases (explanation for the CD-signals). Our proposed structure consists of the speculation that a strong stabilization occurs by clipping the backbones together under paclitaxel influence. Unfortunately, a specific assignment to the binding could not be done at this moment. But this study may be the first step to further investigations.

5 CONCLUSION

Along with the potential, that taxol has been shown as an anticancer drug, clinical problems with toxicity and development of drug resistance are sufficiently severe. It seems, that the antitumor activity mechanism of action not only can occur by microtubules stabilization reactions. The customary studies of paclitaxel (or taxol), focused on microtubules interaction facilities, are extended with the present spectroscopic and calorimetric study by an unexpected specific binding of the drug to

DNA AT sequences, resulting in an astonishing stabilizing effect, especially in homologous (dA)·(dT)-tracts. Perhaps in future, DNA or RNA sequences (gene parts) are important in the molecular medicine to cure human diseases, or to control cytostatic drug resistances.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the excellent facilities generously provided by Prof. G. Pelzl, Department of Physical Chemistry of the Martin Luther University Halle-Wittenberg. Prof. C. Zimmer, Dr. G. Burkhardt and Dr. I. Förtsch, Friedrich Schiller University Jena, are thanked for the possibility to use the CD-spectropolarimeter.

The studies were supported by the Ministry for Research and Technology (FRG), contracts 03119844 and 03119845, the Ministry for Science and Research (Saxony Anhalt), contract 226 A 0731, the Funds of Chemical Industries (FRG) and Employment Office Halle/Saale (FRG).

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Received 11/6/98
Accepted 7/6/99