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Interaction of anthracyclines with nucleotides and related compounds studied by spectroscopy

Francisca Barcelo [a], Isabel Barcelo [a], Francisco Gavilanes [b], Jose A. Ferragut [c], Saul Yanovich [d] and Jose M. Gonzalez-Ros [a]

[a] *Department of Biology, School of Sciences, University of Palma de Mallorca, 07071 Palma de Mallorca,* [b] *Department of Biochemistry (Faculty of Sciences), Complutensis University of Madrid, 28040 Madrid,* [c] *Department of Biochemistry (School of Medicine), University of Alicante, Alicante (Spain),* and [d] *Department of Internal Medicine and Pharmacology, Medical College of Virginia (VCU), Richmond, VA 23298 (U.S.A.)*

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Interaction of the antitumour anthracyclines with mononucleotides and related compounds can be assessed through the perturbation of the spectral properties of the drugs. Purine-derived compounds induce spectral changes more efficiently than pyrimidine derivatives. No marked differences are observed when mono-, di- or triphosphate derivatives, deoxy forms, nucleosides or free nitrogen bases are used for the experiments. Visible absorbance data indicate the existence of a drug/purine nucleotide complex in solution. Assuming a simple equilibrium, this complex would be of low affinity ($K_{eq} \approx 100\ M^{-1}$). Circular dichroism spectra of daunomycin in the presence of ATP suggest that the resulting daunomycin/ATP complexes are not comparable to those formed by intercalation of the anthracycline into DNA. ^{31}P-NMR of ATP in the presence of daunomycin does not support the notion that anthracycline/nucleotide complex formation involves interaction through the phosphate group(s) of the nucleotide. Analysis of the quenching of the drug's intrinsic fluorescence in the presence of nucleotides indicates a predominantly collisional, dynamic quenching mechanism. Values in the 2–6 mM and 85–100 mM range, respectively, are estimated for the reciprocal of the Stern-Volmer quenching constant for a variety of purine and pyrimidine derivatives. This indicates that purine derivatives are highly efficient quenchers of the fluorescence of anthracyclines, while pyrimidine derivatives are not. The fluorescence lifetime of daunomycin in the absence of quencher and the Stern-Volmer quenching constants obtained for different nucleotides are used to calculate the apparent bimolecular rate constants for collisions between fluorophore and quencher to occur. Values of $(2–3) \cdot 10^{11}$ and $1 \cdot 10^{10}\ M^{-1} \cdot s^{-1}$ are obtained, respectively, for purine and pyrimidine derivatives. This suggests a combination of static and dynamic quenching processes for purine compounds, which is consistent with the drug/purine nucleotide complex formation detected by visible absorbance. Because of the high intracellular concentration of certain nucleotides, particularly ATP, the above processes are predicted to be highly significant 'in vivo'.

Introduction

Anthracycline antibiotics, such as daunomycin or adriamycin, are potent cytotoxic drugs currently used as antitumour agents. Daunomycin and adriamycin have long been known to associ-

Correspondence address: Dr. J.M. Gonzalez-Ros, Department of Biology, School of Sciences, University of Palma de Mallorca, Carretera de Valldemossa Km 7, 07071 Palma de Mallorca, Spain.

ate with nucleic acid, and this is generally regarded as one of the major cytotoxic effects of these drugs (for reviews, see Refs. 1, 2). Moreover, the supposition that binding to DNA is the key to drug cytotoxicity has provided the chemical basis for the design of several hundred anthracycline derivatives [1–3] as potentially improved chemotherapy agents.

'In vitro' studies on the interaction between anthracyclines and isolated DNA have shown that these drugs behave as intercalating agents, and spectroscopic methods have been widely used to characterize the interactions involved [4–10]. From these studies, a mechanism of binding to DNA has been postulated which involves the rapid formation of an 'outside' DNA-drug complex prior to drug intercalation. This is followed by either conformational adjustments or redistribution of bound drug to preferred sites within the DNA molecule [11,12]. Also, several studies have suggested that the drug–nucleic acid interaction exhibits a base, sequence and conformation dependence [5,9,13–15].

In this report, we have examined 'in vitro' interactions between anthracyclines and mononucleotides and related compounds. For this, we have applied most of the spectroscopic techniques used in the characterization of drug-nucleic acid interactions. Our primary interests are (i) to develop a simple model system for the study of the interaction between anthracyclines and nucleotides that could presumably account for some of the interactions observed with nucleic acids, such as those involved in the initial formation of a drug/DNA 'outside' complex, (ii) to identify low-molecular-weight cytosolic compounds which associate with the drugs and might therefore contribute to overall anthracycline cellular uptake, and (iii) to contribute to the understanding of the base selectivity observed in the formation of anthracycline/DNA complexes.

Materials and Methods

Materials

Daunomycin and adriamycin hydrochlorides were purchased from Sigma Chemical Co. and used without further purification. Stock solutions of the anthracyclines were prepared in water (about 1 mg/ml) and stored in the dark at −10°C. The integrity of the drugs in such solutions was periodically checked by high-performance liquid chromatography, which demonstrated the absence of breakdown products or impurities over periods of storage exceeding 1 month. Dilutions of drug stocks into the appropriate buffers were freshly prepared immediately before use. Anthracycline concentration was determined spectrophotometrically [5,7–9].

Sodium salts of adenosine 5′-phosphate (AMP); adenosine 5′-diphosphate (ADP); adenosine 5′-triphosphate (ATP); 2′-deoxyadenosine 5′-phosphate, (dAMP); guanosine 5′-phosphate, (GMP), uridine 5′-phosphate, (UMP) and citidine 5′-phosphate, (CMP), adenine (free base), adenosine (free base) and calf thymus DNA were obtained from P-L Biochemicals, Sigma Chemical Co. or Boehringer-Mannheim. These reagents were dissolved in distilled water and aliquots of a 1 M NaOH or 1 M HCl solution added to give a final pH value of 7.0, unless noted otherwise. The primary stock solutions were stored frozen until just prior to use. Concentration of nucleotides and related compounds in solution was determined spectrophotometrically from the absorbance at 260 nm (N.R.C. reference constants in P-L Biochemicals catalog). Calf thymus DNA concentration was determined by assuming that a 50 μg/ml solution of double stranded DNA has an absorbance at 260 nm of 1 unit.

Most commonly used buffers are designated as follows: buffer A, 6 mM Na_2HPO_4/2 mM NaH_2PO_4/1 mM EDTA (pH 7.0); buffer B, 6 mM Na_2HPO_4/2 mM NaH_2PO_4/1 mM EDTA/185 mM NaCl (pH 7.0); buffer C, 10 mM Tris/1 mM EDTA (pH 7.0); buffer D, Dulbecco's phosphate-buffered saline.

Absorbance measurements

Absorbance spectra were recorded in a Zeiss DMR 11 spectrophotometer, using 1 or 5 cm path quartz cuvettes. Temperature was maintained by a Heto Denmark circulating water-bath.

Steady-state and dynamic fluorescence measurements

Fluorescence excitation and emission spectra were recorded in a FICA 55 MK spectrofluorome-

ter equipped with a thermostatically controlled cell holder. Excitation and emission wavelength maxima used for daunomycin and adriamycin were 473 and 557 nm, respectively.

Quenching of fluorescence intensity was determined upon addition of microliter volumes of a 0.1 M stock solution of the quencher, by monitoring emission at 557 nm while exciting at 473 nm. Quenching data were cast into a Stern-Volmer plot, according to the equation:

$$F_0/F = 1 + K_Q[Q]$$

where [Q] is the molar concentration of the quencher, F_0 and F are the fluorescence intensity in the absence and in the presence of a quencher concentration [Q], respectively, and K_Q is the Stern-Volmer quenching constant.

Fluorescence lifetimes were measured by the cross-correlation phase method described by Spencer and Weber [16], using an SLM 4800 series subnanosecond fluorimeter with the excitation light (473 nm) modulated at 18 MHz [17]. The average of phase lifetime values relative to a glycogen scattering solution are presented. Corning filters 3-68 were used in the emission pathway. Quenching titrations of the lifetime of the fluorescence samples were performed by adding increasing aliquots of a stock solution of the quencher into the sample cuvette.

Circular dichroism (CD)

CD spectra were measured in a Jovin Yvon Mark III dichrograph at 0.2 nm/s scanning speed, using 5 cm path quartz cuvettes. CD results are the mean values of at least three determinations and are expressed as molar ellipticities (θ) in units of degrees · cm^2 · $dmol^{-1}$.

NMR studies

^{31}P-NMR spectra were obtained on an IBM AF-270 spectrometer operating at a frequency of 109.3 MHz for phosphorus detection. A 10 mm probe tuned for phosphorus was employed. Samples (about 3–4 ml) were prepared in 10% ^2H_2O, which served as a field-frequency lock. All spectra were obtained at a sweep-width of 6024 Hz, employing a flip angle of 90° and an acquisition time of 2.7 s. Broadband proton decoupling was not employed. Routinely, 2000–2500 scans were taken per spectrum. Chemical shifts are reported relative to 85% phosphoric acid.

Results

Visible absorbance

The absorbance of daunomycin solutions at the 480 nm maximum is linearly proportional with concentration up to 10–12 μM. Above this concentration range, self-association of the drug causes a decrease in the apparent extinction coefficient [18]. Addition of either purine or pyrimidine nucleotides to daunomycin solutions at concentrations below the upper concentration limit indicated above produces spectral changes which can be quantitated when using 5 cm path (or longer) cuvettes. Addition of pyrimidine derivatives at concentrations up to 200 mM produces negligible spectral changes which are within the limits of reproducibility by the spectrophotometer. Neither isosbestic points nor shifts of the daunomycin absorbance maxima are observed. Conversely, addition of ATP (Fig. 1), or in general, purine-derived compounds at concentrations ranging from 1 to 200 mM (about 200–40 000-fold molar excess over drug concentration), produces discrete spectral changes whose characteristics (Table I) suggested the occurrence of a single equilibrium be-

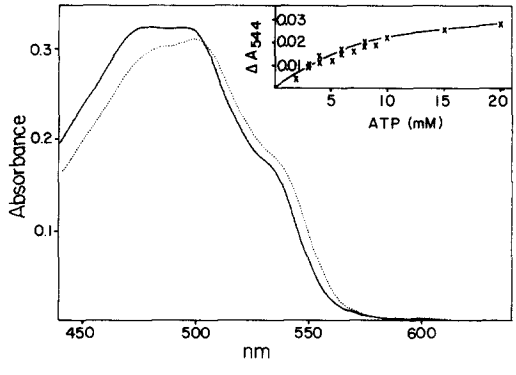

Fig. 1. Visible absorbance spectra of daunomycin (5.5 μM) in 50 mM Hepes (pH 7.0)/200 mM NaCl/5 mM EDTA (solid line), and the same solution containing 50 mM ATP (dotted line). Insert shows the initial part of a titration curve when monitoring the sample's absorbance at 544 nm in the presence of increasing concentrations of ATP. Spectra were taken at 25°C, using 5 cm path quartz cuvettes.

TABLE I

OPTICAL PROPERTIES OF DAUNOMYCIN IN THE PRESENCE AND ABSENCE OF EXCESS ATP

Data were obtained in 50 mM Hepes buffer (pH 7.0)/5 mM EDTA/200 mM NaCl.

	λ_{max} (nm)	ε_{503} ($M^{-1} \cdot cm^{-1}$)	$\Delta\varepsilon_{544}$ [a] ($M^{-1} \cdot cm^{-1}$)	$\Delta\varepsilon_{463}$ [a] ($M^{-1} \cdot cm^{-1}$)
Daunomycin	480	11400	-	-
Daunomycin/ATP	490	11400	1900	-1200

[a] Differences in molar extinction coefficient referred to that determined in the presence of excess ATP (200 mM) minus that observed for daunomycin alone.

tween 'free' and 'nucleotide-associated' daunomycin. An isosbestic point at 503 nm is observed during titration of daunomycin solutions with ATP. Also, difference spectra indicate that differences between the two absorbing species are maximal at 544 and 463 nm. The concentrations of 'free' and 'ATP-associated' daunomycin are calculated as follows. When using 5 cm path cuvettes and for each concentration of added ATP, the total concentration of drug present is calculated from the absorbance at the isosbestic point, $C_t = A_{503}/5 \times \varepsilon_{503}$. This quantity is used to calculate the expected absorbance at either 463 or 544 nm, and then the differences between expected (exp) and observed (obs) absorbances at, for instance, 544 nm, are used to calculate the concentration of 'bound' (b) drug, $C_b = (A_{obs} - A_{exp})/5(\varepsilon_b - \varepsilon_f)$. Concentration of 'free' drug in each case, is calculated as the difference between total and 'bound' drug.

Treatment of these data as a simple equilibrium:

$$\text{drug} + \text{ATP} \rightleftharpoons \text{(drug-ATP)}$$

where (drug-ATP) represents the 'nucleotide-associated' species at a concentration C_b, allows estimation of an equilibrium constant for the association process (K_{eq}) which, in several different titrations, ranged from 95 to 140 M^{-1}.

Circular dichroism

The CD spectrum of daunomycin shows the presence of two positive bands at 470 nm and 350 nm (Fig. 2). Upon the addition of ATP, the main spectral changes occur at the 470 nm peak. A red shift of approx. 10 nm and a decrease of the molar ellipticity are observed at this wavelength (Fig. 2A). These changes are different from those observed upon binding of daunomycin to DNA. Under conditions at which all the drug is bound to DNA, a red shift of 30–40 nm is observed for both peaks (Fig. 2B). Moreover, the molar ellipticity of the 470 nm peak significantly increases as a consequence of binding to DNA.

Values of $[\theta]$ at 470 nm were used to calculate the concentration of free and ATP-associated daunomycin. At each ATP concentration:

$$[\theta] = [\theta]_f(C_f/C_t) = [\theta]_b(C_b/C_t)$$

where $[\theta]_f$ and $[\theta]_b$, equal to 4100 and 2600

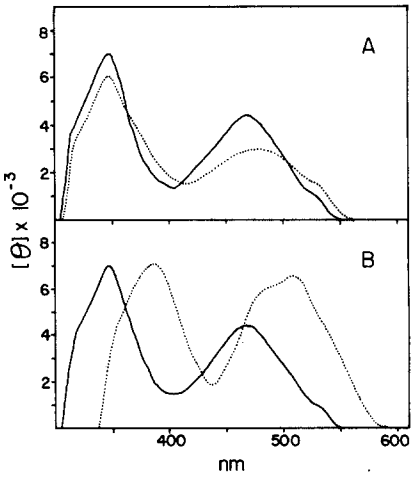

Fig. 2. Circular dichroism spectra of daunomycin in the absence and in the presence of ATP and DNA. (A) 7.5 μM daunomycin in buffer B (solid line) and the same solution containing 50 mM ATP (dotted line). (B) 7.5 μM daunomycin in buffer B (solid line) and the same solution containing 50 μg/ml of DNA (dotted line). Spectra were taken at 25°C.

degrees · cm^2 · $dmol^{-1}$, respectively, correspond to the molar ellipticities of the free and bound forms of daunomycin. $[\theta]_b$ was determined by adding ATP to daunomycin solutions until no further change in the CD spectrum was observed. From the calculated C_f and C_b values and considering the equilibrium described above, the association constant was estimated to be between 80 and 130 M^{-1}, values which are in accordance with those determined by visible absorbance.

Fluorescence studies

The fluorescence emission spectra of daunomycin in the presence of added ATP are shown in Fig. 3. As indicated, increasing additions of ATP cause an increased quenching of the emitted fluorescence, without any modification of the spectral shape. Similar effects are observed when other purine-derived nucleotides, nucleosides or free bases are added, instead of ATP, to daunomycin solutions. Conversely, addition of comparable amounts of pyrimidine derivatives to drug solutions produces only minor effects on the emission intensity.

Quenching of anthracycline fluorescence by nucleotides and related compounds can be treated as a dynamic, collisional process according to the Stern-Volmer equation. Fig. 4 shows Stern-Volmer plots using ATP as a quencher at two different ionic strength conditions. Straight lines are obtained for ATP, as well as for all other purine or pyrimidine-derived compounds we have tested (data not shown).

Efficiency of a collisional quenching agent is usually described by the reciprocal of the Stern-Volmer quenching constant, which represents the quencher concentration required to reduce the fluorescence emission of the unquenched fluorophore to 50% of its initial value. Table II shows the reciprocal values of the Stern-Volmer constant obtained for a variety of nucleotides and related compounds. K_Q^{-1} values obtained for ATP are well within the range of intracellular concentration for this compound. Also, estimations of K_Q^{-1} for the low-efficiency quenchers, pyrimidine

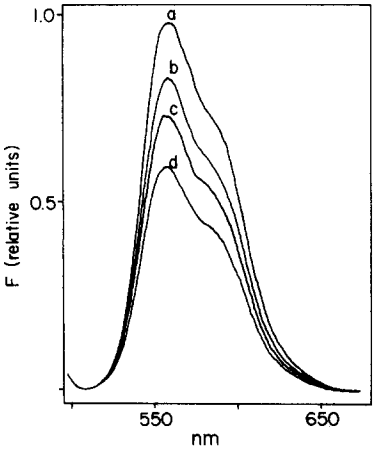

Fig. 3. Uncorrected fluorescence emission spectra of a 7.1 μM solution of daunomycin in the absence (a) and in the presence of ATP at (b) 0.5, (c) 1.0 and (d) 1.8 mM. Daunomycin and ATP (20 mM) stock solutions were freshly prepared in 50 mM Hepes buffer (pH 7.0)/200 mM NaCl/5 mM EDTA. Temperature of operation was 20 °C. An excitation wavelength of 473 nm was used.

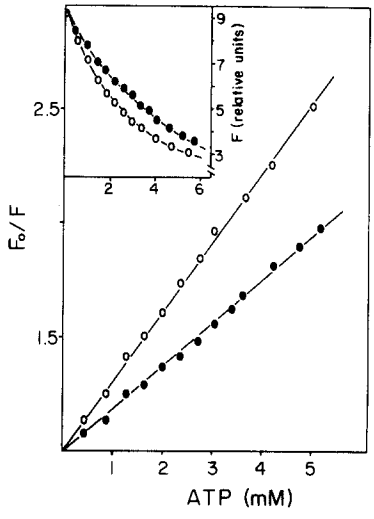

Fig. 4. Representative Stern-Volmer plots of the quenching of daunomycin fluorescence by ATP at 20 °C. The buffers used were (○) 50 mM Hepes (pH 7.0)/5 mM EDTA, and (●) 50 mM Hepes (pH 7.0)/5 mM EDTA/200 mM NaCl. Daunomycin concentration was 7.1 μM. Fluorescence emission was monitored at 557 nm, while exciting at 473 nm. For the experiments shown in this figure, stock solutions of quencher (20 mM) were prepared by dissolving ATP in a volume of 50 mM Hepes (sodium salt) and 5 mM EDTA, so that the final pH value for the solution was 7.0. The inset shows the decrease in relative fluorescence emission observed during the titration with ATP for the daunomycin solutions indicated above. Fluorescence data used in Stern-Volmer plots were appropriately corrected for fluorophore dilution caused by the addition of quencher solutions.

TABLE II

DYNAMIC TREATMENT OF FLUORESCENCE QUENCHING OF ANTHRACYCLINES BY NUCLEOTIDES AND RELATED COMPOUNDS

Only data obtained at 22–23°C from 7.1 μM solutions of anthracyclines were used for calculations. Values for Stern-Volmer constants are independent of drug concentration up to 11 μM. See Materials and Methods for a definition of buffers A, B and D. K_Q^{-1} represents the reciprocal value of the Stern-Volmer constant. This indicates the quencher concentration at which 50% of the initial fluorescence intensity is quenched.

Compound	Buffer	K_Q^{-1} (mM)	
		daunomycin	adriamycin
ATP	A	2.3	2.1
	B	4.1	4.4
	D	3.8	3.7
ADP	B	4.15	
	D	4.1	
AMP	B	5.0	
	D	4.9	
dAMP	B	5.7	
	D	5.2	
GMP	B	6.2	
	D	6.2	
CMP	B	LQ [a]	
	D	LQ	
UMP	B	LQ	
	D	LQ	
Adenosine	B	5.0	
Adenine	B	4.4	

[a] LG indicates low quenching efficiency. Estimated K_Q^{-1} values for the indicated LQ compounds were in the 85–100 mM range.

nucleotides, indicate values exceeding those for purine nucleotides by more than one order of magnitude.

Fluorescence lifetimes values obtained for daunomycin at several concentrations and in different buffers, are shown in Table III. For collisional processes, the Stern-Volmer constant for a given quencher and the fluorophore's lifetime in the absence of quencher are related through the second-order rate constant for collisions between fluorophore and quencher. Calculations for k_{2app} yield values of about $(2–3) \cdot 10^{11}$ and about $1 \cdot 10^{10}$ $M^{-1} \cdot s^{-1}$, respectively, for purine and pyrimidine derivatives.

TABLE III

OBSERVED FLUORESCENCE LIFETIME VALUES OF DAUNOMYCIN IN PRESENCE AND ABSENCE OF ADDED FLUORESCENCE QUENCHERS

Lifetime values shown represent the average of four different determinations. See Materials and Methods for a definition of buffers B and C. 'Hepes' buffer refers to 10 mM Hepes (pH 7.4)/100 mM $NaNO_3$.

Quencher added and concentration		Buffer used	Fluorescence lifetime (ns)		
			[Daunomycin] (μM):		
			1.8	3.5	8.9
None		C	1.06	1.08	1.21
		B	1.04		1.12
		Hepes			1.20
ATP	1.6 mM	C			1.21
	3.2 mM	C			1.15
	4.7 mM	C			1.19
DNA	15 μg/ml	B			1.14
Tl^+	20 mM	Hepes			1.35

Discerning between dynamic or static quenching mechanisms is best established on the basis of the effect (or lack of effect) of the added quencher on the fluorescence lifetime of the fluorophore. Table III shows the observed fluorescence lifetime values upon addition of ATP, as well as dynamic (Tl^+) and static (double stranded DNA) quenchers of anthracycline fluorescence. No significant changes in the drug's initial lifetime are observed upon the addition of any of the quenchers we attempted. Because of the partly inconclusive nature of our fluorescence lifetime quenching measurements, we decided to use other approaches to investigate the mechanism of anthracycline fluorescence quenching by nucleotides (Fig. 5). A test of the dominance of collisional quenching over static quenching is the proportionality of the quenching rate constant with T/η [19–21], where T is the absolute temperature and η the viscocity of the media. Since the quenching rate constant is equal to the Stern-Volmer constant divided by the lifetime of the unquenched fluorophore, it follows that the Stern-Volmer constant should also be proportional to T/η. Thus, the proportionality

shown in Fig. 5, should be taken as evidence for a collisional quenching process. Also, as expected from a predominantly collisional quenching mechanism, the efficiency of quenching of daunomycin fluorescence by purine nucleotides is dependent upon ionic strength. K_Q^{-1} values obtained for quenching of daunomycin by ATP in buffer A increase from 2.3 to 4.7 mM as the concentration of NaCl in the media is increased from 0 to 300 mM.

^{31}P-NMR

To assess a possible interaction between daunomycin and the phosphate group(s) of nucleotides, ^{31}P-NMR experiments were conducted. Characteristically, ATP exhibits a ^{31}P-NMR spectrum where resonance signals corresponding to the alfa, beta and gamma phosphates are well resolved [22]. Initially, we used conditions, including drug and ATP concentrations, ionic strength and pH of the media, similar to those used in previous sections (Table IV). However, those conditions are highly unfavorable for the detection of ionic interactions involving the nucleotide's phosphate groups. Therefore, we decided to optimize the possible occurrence of such an interaction by (i) increasing the drug/ATP ratio, (ii) using low ionic strength, and (iii) using buffered media at a pH which, first of all, is closer to the phosphate's pK, but also favors full protonation of the amine group at the daunosamine moiety of daunomycin. Results obtained under these conditions are also shown in Table IV. No significant differences are produced in the characteristic chemical shifts for the resonance signals corresponding to any of the phosphate groups of ATP. Table IV shows only chemical shift values for the doublet corresponding to the γ-phosphate resonance.

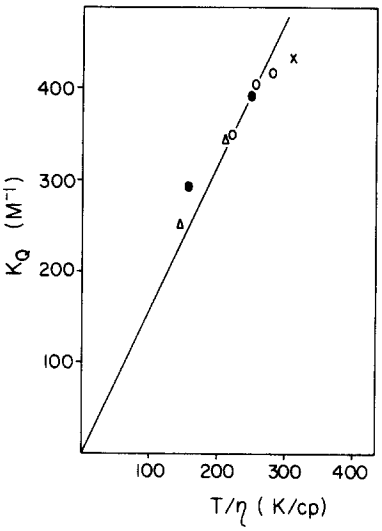

Fig. 5. Proportionality of the ATP quenching constant with T/η. Stern-Volmer quenching constants were determined in buffer A (×, 22°C) and in comparable samples containing either sucrose (○) 4% (21°C), 9% (23.5°C) and 16% (26°C) (w/v); glycerol (△) 10% (25°C) and 20% (25°C) (w/w) or ethylene glycol (●) 10% (25°C) and 20% (22°C) (w/w). Daunomycin concentration was 7.1 μM for all samples. T/η values for the different solutions were calculated from Scheeler [30] and the Handbook of Tables for Applied Engineering Science [31].

TABLE IV

OBSERVED CHEMICAL SHIFTS FOR RESONANCES OF THE GAMMAPHOSPHATE OF ATP IN THE PRESENCE AND IN THE ABSENCE OF DAUNOMYCIN

10% ^2H_2O was added to the buffer as an internal frequency lock. See Materials and Methods for a definition of buffer B. pH is uncorrected for 10% ^2H_2O present. Indicated values were measured in the NMR tubes upon termination of the spectra. Chemical shift values for an observed doublet corresponding to gamma-phosphate resonances are given, separated by commas.

ATP (mM)	Daunomycin (μm)	Buffer	pH	Chemical shift (ppm)
10.0	none	B	7.0	−6.46, −6.64
10.0	8.9	B	7.0	−6.44, −6.62
0.5	none	Mes [a]	6.11	−10.56, −10.74
0.5	13.0	Mes	6.11	−10.55, −10.73
0.5	210.0	Mes	6.14	−10.49, −10.67

[a] 10 mM Mes/1 mM EDTA at an initial pH of 6.1 prior to addition of daunomycin.

Discussion

Spectrophotometric data on the interaction of ATP and daunomycin are indicative of an association-dissociation equilibrium between these two molecules. The occurrence of a single isosbestic point maintained throughout a wide range of concentration of added ATP lead us to think of this process as an equilibrium between two absorbing species, i.e., nucleotide-associated daunomycin and free drug. Possibly, the simplest reaction scheme to account for this process corresponds to what we have indicated under Results. Calculation of the association constant corresponding to that reaction scheme, yields values within the 10^2 M^{-1} range, which might explain why high concentrations of ATP are needed to detect significant absorbance and CD changes. This, however, is only one of several possibilities to be considered. For instance, one might assume that the drug is able to interact preferentially with aggregated forms of ATP resulting from base-stacking phenomena. The limiting step for the interaction to occur then would be the formation of the right size stacks as a function of nucleotide concentration. However, calculated values for the corresponding association constants when ATP dimers, trimers, etc. are considered would not change significantly (same order of magnitude) up to and including 'aggregates' of at least 8–10 ATP molecules. Furthermore, at ATP concentrations below 30–50 mM, which are higher than those used for most of our data points, the nucleotide is expected to exist primarily as dimers [23]. Therefore, we conclude that our estimation of the association constant for the interaction of daunomycin and ATP, is reasonably correct. Such an association constant is significantly lower than that calculated by Chaires et al. [12] for the first reversible step in the proposed binding mechanism between daunomycin and calf thymus DNA ($K_{12} = 2 \cdot 10^4$ M^{-1}). Thus, it seems unlikely that the non-intercalated drug-DNA complex could be explained in terms of the observed interaction between daunomycin and free nucleotides. The hypochromic effect induced by ATP indicates a stacking interaction of daunomycin molecules in the low affinity drug-nucleotide complex (see Ref. 24 and references therein). However, as indicated by the CD spectra, the stacking induced by ATP is different to that produced by intercalation of the drug between base pairs of DNA.

Formation of an 'external' drug-DNA complex has also been proposed to occur through electrostatic interactions between the protonated amine group on the daunosamine moiety of daunomycin and the phosphate groups on the DNA [11]. Our ^{31}P-NMR data using daunomycin and ATP under conditions in which possible interactions involving the phosphate group(s) should be maximized, appear to negate such an interaction, at least, when free nucleotides (as different from nucleic acids) are used. Nonetheless, daunomycin at the concentrations used for NMR experiments is in the form of aggregates [18], which may or may not be representative of monomeric daunomycin regarding its interaction with ATP. Also, it is possible that even under conditions favoring interactions between daunomycin and ATP, the mole fraction of nucleotide present as a drug complex, might be too small for ^{31}P-NMR.

Interaction of anthracycline with nucleotides and related compounds exhibits different patterns when purine or pyrimidine derivatives are considered. Our visible absorbance data indicate that interactions between daunomycin and purine-derived compounds, are relatively stronger than those observed for pyrimidine derivatives. Previous reports by Dalmark and Johansen [26,27], using an octanol-aqueous buffer partition technique, also indicate that the association between purine compounds and anthracycline is stronger than that between the drug and pyrimidine derivatives. This base 'selectivity' is best illustrated through our fluorescence quenching studies. Quenching of the intrinsic anthracycline fluorescence by purine derivatives exhibit apparent Stern-Volmer quenching constants which are about 20-fold higher than those corresponding to pyrimidine derivatives (Table II). The observed quenching patterns do not change substantially when mono-, di- or triphosphate derivatives, deoxy-forms or nucleosides are considered. It is also of interest that values for the apparent K_Q^{-1} for ATP are well within the range of the intracellular concentration for this nucleotide. Considering the high intracellular concentration of ATP as well as other purine-derived compounds such as ADP, GTP, nicotinamide-adenine

dinucleotides, etc., it is expected that intracellular quenching of anthracycline fluorescence by these compounds would be a very significant process and therefore should be taken into account when 'in vivo' fluorescence detection methods are used for quantitation of anthracycline uptake into cultured cells [25].

Quenching of anthracycline intrinsic fluorescence by nucleotides and related compounds follows a predominantly, but not exclusively, collisional mechanism. The linearity of Stern-Volmer plots, as well as the effects of increasing viscosity or ionic strength, seems to favor a dynamic, collisional process. On the other hand, fluorescence lifetime determinations in the presence of quencher were not conclusive, since the lifetime value of the unquenched drug did not change upon the addition of any of the different quenchers we attempted. Prior to every fluorescence lifetime determination, we checked the proper functioning of the instrumentation by determining the fluorescence lifetimes of a well known fluorophore (1,3,6,8-pyrene tetrasulfonate) [28], and found agreement between experimental and reported values. Also, our observed fluorescence lifetime for daunomycin in solution is identical to that reported recently by other group [32]. Therefore, we attribute the lack of variation of daunomycin lifetimes upon addition of ATP to intrinsic limitations of the SLM instrument for short-lived fluorophores. In either case, assuming that the fluorescence lifetime values determined for daunomycin in the absence of quencher are correct, the apparent bimolecular collisional rate constant (k_{2app}) can be calculated. Depending upon the buffer used, values of k_{2app} around $(2-3) \cdot 10^{11}$ $M^{-1} \cdot s^{-1}$ are obtained for purine derivatives, which are at least one order of magnitude higher than typical values for pure collisional, diffusion controlled processes ($k_{2app} \approx 10^9-10^{10}$ $M^{-1} \cdot s^{-1}$), therefore indicating formation of 'static' drug/nucleotide complexes. Calculations of the second-order collisional rate constant for pyrimidine derivatives, yield values of approximately $1 \cdot 10^{10}$ $M^{-1} \cdot s^{-1}$, thus indicating practical absence of static complex formation. The different k_{2app} values obtained for the interaction of daunomycin with purine and pyrimidine derivatives are consistent with the daunomycin/purine nucleotide association suggested by our visible absorbance and CD data. Predominance of collisional processes and simultaneous occurrence of low-affinity complexes could perhaps be explained on the basis of a rapid exchange between free and 'bound' forms of the drug. Actually, a decrease in the rate of drug exchange has been observed in NMR studies, as oligonucleotides or polynucleotides of increasing length were used as model compounds to study anthracycline association [29].

Considering the above processes at the cellular level, the association between anthracyclines and small molecular weight cytosolic compounds with high intracellular concentrations, such as purine nucleotides and related compounds, might play an important role in retaining the anthracycline in the cell, thus contributing to cellular uptake of these clinically important drugs.

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References

1 Arcamone, F. (1978) in Topics in Antibiotic Chemistry, (Sammes, P.G., ed.), Vol. 2, pp. 99–231, Ellis Horwood, Chichester
2 Arcamone, F. (1981) In Doxorubicin: Anticancer Antibiotics (Sartorelli, A.C., ed.), Academic Press, New York
3 Neidle, S. (1978) in Topics in Antibiotic Chemistry (Sammes, P.G., ed.), Vol. 2, p. 230 Ellis Horwood, Chichester
4 Calendi, E., Di Marco, A., Reggiani, M., Scarpinato, B. and Valentini, L. (1965) Biochim. Biophys. Acta 103, 25–33
5 Zunino, F., Gambetta, R., Di Marco, A. and Zaccara, A. (1972) Biochim. Biophys. Acta 277, 489–498
6 Zunino, F., Di Marco, A. and Zaccara, A. (1979) Chem.-Biol. Interact. 24, 217–222
7 Chaires, J.B., Dattagupta, N. and Crothers, D.M. (1982) Biochemistry 21, 3933–3940
8 gabbay, E.J., Grier, D., Fingerle, R.E., Reyner, R., Levy, R.

Pearce, S.W. and Wilson, W.D. (1976) Biochemistry 15, 2062–2070
9 Plumbridge, T.W. and Brown, J.R. (1977) Biochim. Biophys. Acta 479, 441–449
10 Plumbridge, T.W. and Brown, J.R. (1978) Biochem. Pharmacol. 27, 1881–1887
11 Zunino, F., Gambetta, R., Di Marco, A., Velcich, A., Zaccara, A., Quadrifoglio, F. and Crescenzi, V. (1977) Biochim. Biophys. Acta 476, 38–46
12 Chaires, J.B., Dattagupta, N. and Crothers, D.M. (1985) Biochemistry 24, 260–267
13 Kersten, W., Kersten, H. and Szybalski, W. (1966) Biochemistry 5, 236–244
14 Plumbridge, T.W. and Brown, J.R. (1979) Biochim. Biophys. Acta 563, 181–189
15 Quigley, G.J., Wang, A.H.J., Ughetto, G., Van der Marel, G., Van Boom, J.H. and Rich, A. (1980) Proc. Natl. Acad. Sci. USA 77, 7204–7209
16 Spencer, R.D. and Weber, G. (1969) Ann. N.Y. Acad. Sci. 158, 361–369
17 Gonzalez-Ros, J.M., Farach, M.C. and Martinez-Carrion, M. (1983) Biochemistry 22, 3807–3811
18 Chaires, J.B., Dattagupta, N. and Crothers, D.M. (1982) Biochemistry 21, 3927–3932
19 Vavilov, S.I. (1929) Z. Phys. 53, 665–671
20 Vaughan, W.M. and Weber, G. (1970) Biochemistry 9, 464–473
21 Lehrer, S.S. (1971) Biochemistry 10, 3254–3263
22 Cohn, M. and Hughes, T.R. (1962) J. Biol. Chem. 237, 176–182
23 Gallego, E., Rodriguez de Bodas, A. and Peral, F. (1981) Ann. Quim. 77, 98–104
24 Devoe, H. and Tinoco, I., Jr. (1962) J. Mol. Biol. 4, 518–523
25 Yanovich, S. and Taub, R. (1982) Cancer Res. 42, 3583–3588
26 Dalmarck, M. and Johansen, P. (1981) Proc. Am. Assoc. Cancer Res. 22, 31
27 Dalmarck, M. and Johansen, P. (1982) Mol. Pharmacol. 22, 158–165
28 Haugland, R.P. (1985) Molecular Probes Handbook of Fluorescence Probes, p. 77, Junction City, OR
29 Jardetzky, O. and Roberts, G.C.K. (1981) NMR in Molecular Biology p. 525, Academic Press, New York
30 Scheeler, P. (1981) Centrifugation in Biology and Medical Sciences, p. 236, John Wiley & Sons, New York
31 Handbook of Tables for Applied Engineering Sciences, 2nd Edition (1973) C.R.C. Press, Boca Raton, FL
32 Burke, T.G. and Tritton, T. (1985) Biochemistry 24, 5972–5980