Biophysical Chemistry, 35 (1990) 191–202
Elsevier

BIOCHE 01423

Biophysical chemistry of the daunomycin-DNA interaction

Jonathan B. Chaires

Department of Biochemistry, University of Mississippi Medical Center, 2500 North State St., Jackson, MS 39216-4505, U.S.A.

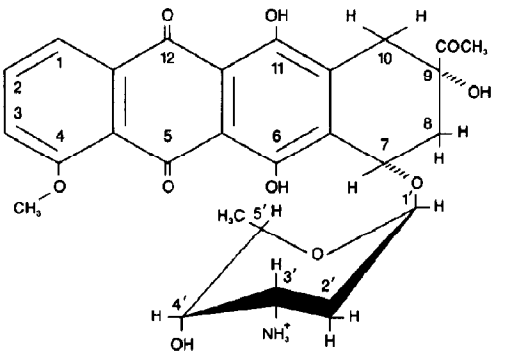
Received 12 April 1989
Accepted 19 July 1989

Daunomycin; Thermodynamics; Kinetics; Sequence specificity; DNA; Intercalation

Daunomycin (daunorubicin) is a potent anticancer antibiotic that binds to DNA by the process of intercalation. Fundamental aspects of the physical chemistry of the daunomycin-DNA interaction are reviewed here, including the thermodynamics and kinetics of the binding reaction, and recent work that indicates that daunomycin binds preferentially to certain sites along the DNA lattice. The solution studies reviewed here combine with recent theoretical and crystallographic investigations to make the daunomycin-DNA interaction one of the best-characterized intercalation reactions. The molecular interactions that stabilize the daunomycin-DNA complex, and which contribute to its sequence preference, are discussed.

1. Introduction

Daunomycin (daunorubicin) (fig. 1) is the prototype anthracycline antibiotic, an important class of compounds that has found wide use in cancer chemotherapy [1,2]. Interaction of daunomycin with DNA is thought to be a crucial step in the molecular mechanism by which it inhibits DNA replication and transcription both in vivo and in vitro. Daunomycin is a potent inhibitor of topoisomerase II, and forms a cleavable ternary complex with the enzyme and DNA. Daunomycin is rapidly accumulated in the nuclei of sensitive cells, and reaches a final concentration that is 5-times higher than the corresponding level in resistant cells [3]. A direct relationship between affinity toward DNA and biological activity has been established for a series of 26 anthracycline derivatives [4]. Daunomycin binds to DNA by the process of intercalation, and its interaction with DNA has been extensively studied for over two decades. Earlier work on this interaction has been the subject of several reviews [5–11]. Since the publication of these reviews, major advances have been made in the understanding of the daunomycin-DNA interaction. Firstly, the structure of a daunomycin-d(CGATCG)$_2$ complex is now known to 1.2 Å resolution [12,13], which makes daunomycin the only monointercalator for which the crystal structure of its complex with DNA is



DAUNOMYCIN

Fig. 1. Chemical structure of the anthracycline antibiotic daunomycin (daunorubicin).

Correspondence address (until 31 July 1990): J.B. Chaires, Max-Planck-Institut für Biophysikalische Chemie, Abteilung Molekulare Biologie, Postfach 2841, 3400 Göttingen, F.R.G.

known in atomic detail. Secondly, detailed thermodynamic and kinetic studies have clarified the reaction mechanism of the daunomycin-DNA interaction in solution. Finally, theoretical and solution studies have clarified the preferred DNA sequences to which daunomycin binds, a question that has previously been particularly controversial. This brief review will focus primarily on recent progress that has been achieved toward understanding the physical chemistry of the daunomycin-DNA interaction in solution and toward defining the sequence preference of daunomycin binding.

2. Thermodynamics of the daunomycin-DNA interaction

2.1. Equilibrium binding constants

The binding of daunomycin to DNA is most appropriately described by the neighbor exclusion model [14,15], which can fully account for the nonlinearity apparent in daunomycin-DNA binding isotherms. Earlier estimates of binding constants and 'site sizes' based on conventional Scatchard analysis (tabulated in ref. 8) failed, with some exceptions, to account for neighbor exclusion effects, resulting in an overestimation of the intrinsic binding constant. McGhee and Von Hippel [15] have provided an analytical expression which embodies the neighbor exclusion principle, and which may conveniently be used to extract the binding constant and the exclusion parameter from experimental data by nonlinear least-squares fitting procedures. In the absence of cooperative interactions,

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

where r denotes the molar ratio of bound drug per total DNA base-pair, C_f the free drug concentration, K the binding constant for the interaction of drug with an isolated site and n the exclusion parameter [15]. Table 1 summarizes the results from several groups of researchers who have determined the binding constant for the interaction of daunomycin with calf thymus DNA under comparable ionic conditions and who have used eq. 1 for a proper analysis of binding data. The agreement between the various groups is excellent, and the data yield an average value of $K = 6.7 (\pm 0.9) \times 10^5$ M^{-1}. The average value of the exclusion parameter n is 3.25 ± 0.4 base-pairs (bp).

Table 1

Equilibrium binding constants for the interaction of daunomycin with calf thymus DNA

Estimates for the binding constant K and the exclusion parameter n were obtained in all cases by nonlinear least-squares fit to the McGhee-von Hippel model, eq. 1. Solution conditions for the data listed were pH 7.0, 0.2 M Na^+, 20 °C.

K ($\times 10^5$) (M^{-1})	n (bp)	Reference
6.2	2.9	16
7.0	3.5	17,18
5.7	3.0	19
8.1	2.9	5
7.5	3.5	20
5.9	3.7	21

The value of 3–4 bp found for the exclusion parameter distinguishes daunomycin from simple intercalators like ethidium and proflavin, for which $n = 2.0$ bp. The physical interpretation of this value is that each bound daunomycin molecule removes 3 bp as potential binding sites, perhaps by physically covering 3 bp. The latter notion is fully consistent with the observed crystal structure [12], in which daunosamine moiety is seen to extend down the minor groove from the actual intercalation site to cover physically a third base-pair. Recent ^{19}F-NMR experiments have independently verified that the exclusion parameter is near 3 bp [22].

An unresolved issue in the equilibrium binding of daunomycin to DNA is whether or not cooperative interactions need to be included in the analysis of the data. Graves and Krugh [19], using a phase partition method, reported positive cooperativity at low binding ratios for the interaction of daunomycin with calf thymus DNA. Other groups have not observed such cooperativity, although the optical methods generally used to monitor binding may not be sufficiently sensitive to detect the phenomenon at low binding ratios. The un-

derlying physical basis for possible cooperative interactions in calf thymus DNA is not clear. Positive cooperativity was observed for the binding of daunomycin to poly(dGdC) in the left-handed Z conformation [23,24] and to poly(dA)-poly(dT) under standard solution conditions [25]. In both of these cases, the positive cooperativity can be quantitatively accounted for by application of the model of Crothers et al. [26] for DNA allosteric interactions, in which drug binding is postulated to be coupled to a conformational transition in the polynucleotide. These studies indicate that daunomycin binds with higher affinity to DNA in the standard B form, and that daunomycin can exert long-range effects on DNA conformation. The apparent positive cooperativity arises from the coupling of binding to the Z-to-B transition in the one case [23,24], or from that to the transition of poly(dA)-poly(dT) from a nonstandard right-handed conformation to a more normal B-form state [25].

Tsuchiya [27] has argued that it is necessary to include terms that account for anticooperativity (negative cooperativity) in the analysis of daunomycin-DNA binding isotherms. The proposed requirement of an anticooperativity term has been carefully tested by the Jena group [11,16], who found that inclusion of the additional fitting parameter did not lead to a statistically significant improvement in the fit for daunomycin binding data. Therefore, the inclusion of negative cooperativity terms in the analysis of daunomycin binding isotherms appears to be unwarranted.

2.2. *Thermodynamic profile of the daunomycin-DNA interaction*

The thermodynamic profile for the daunomycin-DNA interaction may be obtained by studying the temperature dependence of the binding constant or, more directly, by calorimetric measurements. The Gibbs free energy is defined by the standard relation

$$\Delta G^\circ = -RT\ln K = \Delta H^\circ - T\Delta S^\circ$$

from which the enthalpic and entropic contributions to the binding free energy may be discerned. Table 2 summarizes representative results for a variety of DNA and deoxypolynucleotide samples. The general point to emerge from these data is that the favorable free energy for daunomycin binding to natural DNA and synthetic deoxypolynucleotides of alternating purine-pyrimidine sequence is derived largely from the contribution of a large, negative enthalpy. Daunomycin binding to these DNAs is thus energetically driven, rather than entropically. The thermodynamics of daunomycin binding to deoxypolynucleotides of nonalternating sequence is considerably more complex, due to the coupling of drug binding to a conformational transition in the polynucleotide, and the original literature should be consulted for a complete discussion of these cases [25,28]. Possible molecular interactions that contribute to the binding enthalpy and entropy have been discussed [18,29]. The major contributors to the endothermic enthalpy are likely to be hydrogen-bonding interactions, a notion that correlates well with the crystal structure of a daunomycin-DNA complex in which extensive hydrogen-bonding interactions have been observed.

2.3. *Ionic strength dependence of daunomycin binding to DNA*

The dependence of the equilibrium binding constant (K) for an antibiotic-DNA interaction

Table 2

Thermodynamic profiles for the interaction of daunomycin with DNA and synthetic deoxypolynucleotides

DNA	ΔG° (kcal/mol)	ΔH° (kcal/mol)	ΔS° (cal/K per mol)	Reference
Calf thymus [a]	−7.8	−12.8 [b]	−17.0	17,18
	−7.7	−10.6 [b]	−9.7	20
Salmon testes [c]	−9.0	−9.9 [d]	−3.0	28
$[Poly(dAdT)]_2$	−7.9 [a]	−9.1 [b]	−4.1	25
	−9.4 [c]	−8.9 [d]	+2.0	28
$[Poly(dGdC)]_2$ [e]	−8.7	−10.0 [b]	−4.2	21
Poly(dAdC)-poly(dGdT) [e]	−9.4	−11.0 [b]	−5.4	21

[a] 10 mM sodium phosphate, 0.185 M NaCl, 1 mM Na_2-EDTA, pH 7.0.
[b] Obtained by van't Hoff analysis.
[c] 10 mM sodium phosphate, 1 mM EDTA, pH 7.0.
[d] Obtained by calorimetry.
[e] 10 mM sodium phosphate, 150 mM NaCl, 1 mM EDTA, pH 7.4.

on ionic strength can provide valuable insight into the contribution of ionic interactions to the overall binding free energy. For a positively charged antibiotic molecule, the dependence of K on the ionic strength arises from the coupling of drug and cation binding to DNA. Daunomycin carries a single positive charge at neutral pH, located on the daunosamine moiety ($pK \approx 8.2$). Record and co-workers [30] have shown that

$$\frac{\delta \ln K}{\delta \ln M^+} = -Z\psi$$

where M^+ denotes the monovalent cation concentration, Z the charge on the ligand (daunomycin in this case), and ψ the fraction of counterions associated with each DNA phosphate. For double-stranded DNA, $\psi = 0.88$, thus a ligand with a single positive charge (like daunomycin) is predicted from the theory of Record et al. to have $(\delta \ln K)/(\delta \ln M^+) = -0.88$. Experimentally, $-Z\psi$ has been determined to be 0.84–0.88 [17–20], in excellent agreement with the theoretical predictions. The thermodynamic equilibrium constant (K_t°) may be quantitatively assessed once $Z\psi$ is known. K_t° is the equilibrium constant corrected for the free energy of coupled ion release, and refers to a standard state in which all reactants, including ions, have unit concentration. Record et al. [30] show that

$$\ln K_{obs} = \ln K_t^\circ + Z\xi^{-1} \ln(\gamma_\pm \delta) - Z\psi \ln[M^+] \quad (2)$$

where K_{obs} is the observed equilibrium constant at monovalent cation concentration $[M^+]$, $Z\psi$ is defined above, $\gamma_\pm$ is the mean activity coefficient of M^+, and the remaining terms are constants for double-stranded DNA ($\xi = 4.2$; $\delta = 0.56$). Over the range of Na^+ concentration of 0.05–1.0 M, $\ln K_t^\circ$ was determined to be 12.33 [17], corresponding to $K_t^\circ = 2.3 \times 10^5$ and a Gibbs free energy of -7.3 kcal mol^{-1}. The large magnitude of K_t° implies that the daunomycin-DNA complex is stabilized by extensive nonionic interactions. It follows from this that ionic interactions involving the charged daunosamine moiety are rather nonspecific, and contribute little to the overall binding free energy. Early models of the daunomycin-DNA complex (reviewed in ref. 8) featured specific electrostatic interactions between the daunosamine and DNA phosphates. Such interactions are not observed in the high-resolution crystal structure [12], and need not be invoked to account quantitatively for the salt dependence of the daunomycin-DNA binding constant, as the above analysis shows.

A puzzling aspect of the ionic strength dependence of daunomycin binding to DNA remains unexplained. The magnitude of the enthalpy for daunomycin binding was observed to decrease by a factor of two over the range of NaCl concentrations of 0.05–1.0 M [18]. Such behavior is in conflict with the prediction of Record's theory, in which the variance of K with ion concentration arises predominantly from entropic effects. The variation of ΔH with NaCl concentration has been verified for daunomycin, and the same behavior found for adriamycin, a closely related anthracycline antibiotic [20]. It is notable that no ionic strength dependence of ΔH was found for the simple intercalators ethidium and propidium [31]. A possible explanation of the result in the case of daunomycin is that it stems from specific ion interactions in the drug-DNA complex. In the crystal structure [12], a hydrated Na^+ is observed to be coordinated in the major groove to N7 of a guanine and the O4 and O5 constituents on the intercalated daunomycin. A considerable enthalpic contribution could arise from the formation of this coordination complex, the magnitude of which may reasonably be expected to vary with total Na^+ concentration. If this explanation is correct, the apparent agreement of the ionic strength dependency of daunomycin binding to DNA with Record's theory may be in part fortuitous, and arises from the unusual enthalpy-entropy compensation found in this system [18,20]. Further studies of this phenomenon, particularly using direct calorimetric methods, are essential in order to clarify the situation.

2.4. Base composition and sequence effects on the binding constant

The question as to whether or not the binding constant for the daunomycin-DNA interaction,

depends on base composition or sequences has been particularly vexing and the early literature concerning this subject is confusing and contradictory (reviewed in refs. 8, 32 and 33). Recent results have clarified the situation. When carefully prepared and well characterized deoxypolynucleotides were used in binding studies, daunomycin was found to show a marked preference for alternating purine-pyrimidine sequences [33]. The preference for binding to poly(dAdT) over poly(dA)-poly(dT) was particularly striking [25,28,33]. Daunomycin binding to a more extensive series of synthetic deoxypolynucleotides has recently been examined [34]. That study confirmed the general preference of the drug for alternating purine-pyrimidine sequences, and thoroughly characterized the thermodynamics of daunomycin binding to polynucleotides. The use of synthetic deoxypolynucleotides to probe the site and sequence specificity of the binding reaction may, however, be complicated by the unusual conformations that some of the simple repeating sequences adopt. Further, it is possible, regardless of how extensive a set of model sequences is used, that the most preferred binding site is not represented within that set. The use of natural DNA sequences, containing all possible binding sites, is therefore necessary for the full exploration of the issue, in spite of the added complexity introduced. Preferential binding of antibiotics to sites within natural DNA sequences gives rise to variation in the observed binding constant according to the fractional GC content, a dependence which may be quantitatively analyzed in terms of simple models for the base composition at the binding site [35,36]. Daunomycin binding to DNA shows a marked dependence on the overall base composition [17,37]. Fig. 2 illustrates the dependence of the apparent binding constant K on the fractional GC content, derived from studies of the interaction of daunomycin with a series of natural DNA samples of varying base composition [37]. The magnitude of K increases with increasing GC content, a clear indication that daunomycin interacts preferentially with sites containing GC base-pairs. The functional dependence of K on the fraction of GC base-pairs is nonlinear, which indicates that the site preference is complex and involves more than

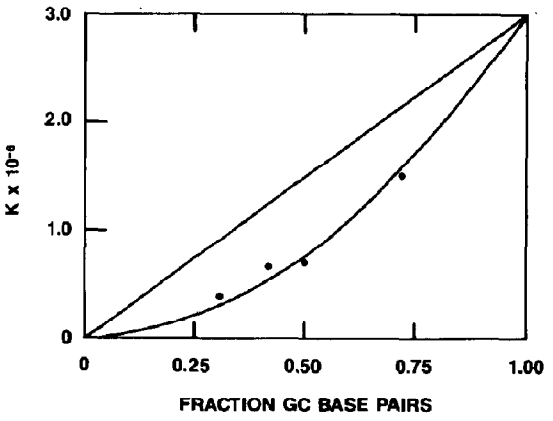


Fig. 2. The apparent equilibrium binding constant K as a function of the fractional GC content for the binding of daunomycin to five DNA samples. The points correspond to K values determined for daunomycin binding to DNA from *C. perfringens* (31% GC), *E. coli* (50% GC), calf thymus (42% GC) and *M. lysodeikticus* (72% GC). A K value for poly(dGdC) (100% GC) was also determined. The straight line represents the predicted behavior for the preferential binding of drug to one side of a GC base-pair. The line passing through the experimental points is calculated assuming that daunomycin binds to a site containing two adjacent GC base-pairs.

a single base-pair. The data shown in fig. 2 may be further analyzed by expressing the conditional probabilities for specific types of binding sites in terms of the fractional GC content, f. Table 3 lists the probability terms for several types of sites. The

Table 3

Probability factors for specific types of antibiotic-binding sites

The probability factors (P) expressed in terms of the fractional GC content (f) were derived for the various types of possible antibiotic recognition sites using the approach outlined in refs. 35 and 36

Type of site	Probability factor (P)
One side, GC bp	f
One side, AT bp	$1-f$
Two adjacent GC bp	f^2
Three adjacent GC bp	f^3
GA, AG or GG	$2f-f^2$
AGG, GGA, or GAG	$3f^2-3f^3$
AGG, GGA, or GGG	$2f^2-f^3$

apparent equilibrium may then be expressed as

$$K_{app} = K_0 P$$

where P is the probability function as listed in table 3 and K_0 the binding constant when $f = 1.0$ [35,36]. The best fit of the data in fig. 2 is provided by the equation $K_{app} = (3.1 \times 10^6) f^2$. The binding constant thus depends on the *square* of the fraction of GC base-pairs, suggesting as a minimal model that adjacent GC base-pairs are present at the preferred daunomycin-binding site. An independent demonstration of the preference of daunomycin for binding to GC base-pairs was provided by a careful nearest-neighbor analysis of CD data [37].

2.5. Conclusions

The thermodynamics of the daunomycin-DNA interaction are now well characterized, and provide a description of the binding equilibrium over a wide range of temperatures and ionic strengths. Daunomycin binding to DNA is energetically favored, and is driven by a large, negative enthalpy term. The pattern of the thermodynamic profile for the daunomycin-DNA interaction suggests that hydrogen-bonding interactions are major contributors to the stability of the drug-DNA complex, in good agreement with the known crystal structure in which at least four hydrogen bonds are evident. The charged daunosamine moiety participates in nonspecific ionic interactions that contribute little to the overall binding free energy, a conclusion that again is consistent with the crystallographic results in which no specific molecular interactions involving the daunosamine are found. Daunomycin binding to DNA is strongly dependent on base composition, and equilibrium data clearly suggest that the drug interacts preferentially with sites that contain adjacent GC base-pairs.

3. Kinetics of the daunomycin-DNA interaction

Kinetic studies are essential for a thorough understanding of antibiotic-DNA interactions for at least two reasons. First, pharmacological activity is often more readily correlated with kinetic rather than equilibrium properties [39], and it is therefore of fundamental importance to determine the lifetime of the antibiotic-DNA complex. Second, kinetic studies are necessary to understand the detailed reaction mechanism governing the antibiotic-DNA interaction. While many compounds intercalate into DNA, the kinetic pathway leading to the formation of the final intercalation complex differs among antibiotics, and may be surprisingly complicated. Comparatively little attention has been paid to the kinetics of antibiotic-DNA binding reactions in comparison to equilibrium studies, perhaps because of the specialized instrumentation required to monitor moderately fast reactions. The kinetics of daunomycin binding to DNA has now been studied in some detail using a variety of methods, leading to insight into the underlying reaction mechanism of its binding process.

3.1. Dissociation studies

The first studies to characterize the lifetime of the daunomycin-DNA complex used the SDS dissociation method developed by Mueller and Crothers [39]. In this procedure, the daunomycin-DNA complex is disrupted by the addition of SDS, which sequesters antibiotic in a presumed fast step. The observable rate-limiting step is the dissociation of drug from the complex. By this method, early studies indicated that daunomycin dissociated from DNA through a simple first-order process [40–42]. Dissociation from salmon testes or calf-thymus DNA was characterized by first-order rate constants of 0.7 and 1.4 s^{-1}, respectively [40,41], implying a lifetime for the daunomycin-DNA complex of about 1 s. Daunomycin dissociation from synthetic deoxypolynucleotides was also reported to be a simple first-order process, characterized by a rate constant of about 4 s^{-1} [42].

3.2. Temperature jump studies

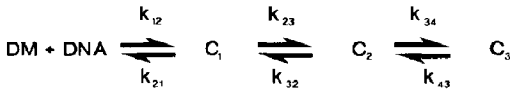
Indication of a more complex reaction mechanism for the daunomycin-DNA interaction came

from careful temperature-jump relaxation studies performed by the Jena group [11,43,44]. Two relaxation times were required to fit data obtained over a time interval of 0–200 ms. The interpretation of this kinetic complexity was that it arose from nearest-neighbor interactions, and not from transient intermediate species [11,44]. Using a kinetic theory that takes into account these nearest-neighbor interactions, estimates for association and dissociation rate constants for the binding of daunomycin to an isolated, a singly contiguous, and a doubly contiguous lattice site were obtained [44]. The dissociation rates yielded by this approach are nearly an order of magnitude larger that those reported in stopped-flow dissociation experiments, perhaps because of the very different time scales used in the two types of experiments.

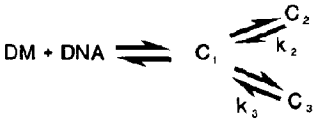
3.3. *Stopped-flow studies*

Even further complexity in the daunomycin-DNA binding reaction was revealed by stopped-flow kinetic studies [45]. Association studies in which DNA-binding sites were in large excess, conditions that should minimize complications arising from nearest-neighbor interactions, required three exponentials to fit the time course over the range 0–300 ms. Kinetic studies over a wide range of reactant concentrations were performed, allowing for the analysis of the concentration dependence of the three relaxation times. The simplest mechanism consistent with the kinetic behavior was a three-step sequential model, in which daunomycin first binds to DNA in a bimolecular step, which is followed by two sequential isomerization reactions (scheme 1, mechanism 1). Rate constants for this mechanism were extracted from the experimental data (table 4), and may be used to calculate an overall equilibrium constant in excellent agreement with values obtained from binding studies. The sequential model is the simplest mechanism that can fit the data, although it was freely acknowledged that alternate multistep models (such as the branched model (scheme 1, mechanism 2)) could probably fit the data as well [45]. Distinguishing among these models requires

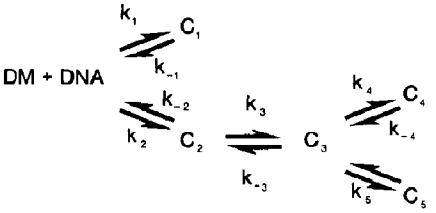
1. Sequential



2. Branched



3. Multiple Branched



Scheme 1. Proposed reaction mechanisms for the interaction of daunomycin with calf thymus DNA from stopped-flow kinetic studies. DM, daunomycin; DNA, DNA-binding sites; C_i, species of drug-DNA complexes. Values for the indicated rate constants are listed in table 4.

Table 4

Rate constants determined for the interaction of daunomycin with calf thymus DNA for the reaction mechanisms in scheme 1

(1) Sequential model [45]	
$k_{12} = 3.0 \times 10^6\ M^{-1}\ s^{-1}$	$k_{21} = 168\ s^{-1}$
$k_{23} = 92.5\ s^{-1}$	$k_{32} = 11.2\ s^{-1}$
$k_{34} = 4.2\ s^{-1}$	$k_{43} = 1.2\ s^{-1}$
(2) Branched model	
$k_2 = 3.5\ s^{-1}$	$k_3 = 0.83\ s^{-1}$ [46]
$k_2 = 3.5\ s^{-1}$	$k_3 = 0.54\ s^{-1}$ [48]
(3) Multiple branched [49]	
$k_1 = 5 \times 10^5\ M^{-1}\ s^{-1}$	$k_{-1} = 270\ s^{-1}$
$k_2 = 8 \times 10^5\ M^{-1}\ s^{-1}$	$k_{-2} = 140\ s^{-1}$
$k_3 = 60\ s^{-1}$	$k_{-3} = 15\ s^{-1}$
$k_4 = 3.2\ s^{-1}$	$k_{-4} = 2.5\ s^{-1}$
$k_5 = 0.9\ s^{-1}$	$k_{-5} = ?$

independent experimental evidence beyond simply the concentration dependence of relaxation times.

Additional stopped-flow dissociation kinetic studies provided evidence to favor the branched mechanism over the sequential type. The effect of ionic strength changes on the two slowest resolved dissociation rates was examined, resulting in each rate showing an identical ionic strength dependence, with $(\delta \ln k/\delta \ln Na^+) = -0.4$ [46]. This result, it was argued, could only arise from a branched mechanism (scheme 1, mechanism 2) since the observed slopes were identical to the predicted result for the dissociation of a monocation from DNA [47]. For a sequential mechanism one rate should be independent of ionic strength [47]. A second dissociation study also favors the branched mechanism [48]. Only one dissociation rate was observed for daunomycin bound to synthetic deoxypolynucleotides, whereas two were observed for drug bound to a series of natural DNA samples of varying GC content. This behavior was proposed to be consistent with the branched mechanism (scheme 1, mechanism 2) in which each branch now may be considered to represent intercalation into two classes of binding sites, differing in sequence or conformation.

Further complexity in the daunomycin-DNA reaction mechanism was introduced by a very recent fluorescence stopped-flow kinetic study [49]. Reactant concentrations were again chosen to minimize neighbor exclusion effects. Both association and dissociation reactions nonetheless required three exponential terms to fit the time course of the reactions. Analysis of the reciprocal relaxation times as a function of concentration required a five-step, multiply branched mechanism (scheme 1, mechanism 3) to provide an adequate fit to the experimental data. One of the additional steps was assigned to the bimolecular formation of a nonintercalated, weakly bound species. The mechanism also incorporates a branched pathway that possibly arises from intercalation into two classes of sites.

3.4. Conclusions

The most general conclusion from the combined kinetic studies is that daunomycin binding to DNA is slow and complex. The lifetime of the daunomycin-DNA complex is comparatively long, a fact that correlates well with the clinical effectiveness of the drug. The slow and complicated kinetics of daunomycin binding distinguishes the drug from simple intercalators like ethidium and proflavin, which tend to bind to DNA more rapidly and via simpler reaction mechanisms. Plausible explanations for the kinetic complexity of the daunomycin binding reaction are that it arises from neighbor exclusion effects [43,44] or from a complex reaction mechanism containing transient intermediate species [45–49]. While these two points of view are difficult to distinguish experimentally, recent studies seem to favor the latter, for two reasons. First, the detailed stopped-flow studies [46,49] were designed to minimize complications arising from the neighbor exclusion phenomenon, yet still revealed multiple relaxation times. Second, multiple exponentials are observed for daunomycin binding to natural DNA [45,46,48,49], but *not* for binding of the drug to synthetic deoxypolynucleotides of simple repeating sequence [48]. If multiple exponentials were to arise solely from neighbor exclusion effects, these simpler polynucleotides ought to show the same complexity as the natural DNA sequences. The kinetic complexity of daunomycin thus probably arises from transient intermediates and multiple binding pathways (scheme 1). Branched mechanisms (scheme 1, mechanisms 2 and 3) are most consistent with current data, and may arise from daunomycin binding to classes of sites that differ in sequence or conformation.

4. Preferred sequences for daunomycin binding

Whether or not daunomycin preferentially binds to particular sequences has been perhaps the most confusing aspect of its interaction with DNA. The more recent equilibrium binding studies, reviewed above, suggest specificity toward GC base-pairs, and the kinetic complexity of the binding process might arise from daunomycin binding to specific classes of sites. In crystallographic studies, daunomycin intercalated between two GC base-pairs, even though there were four types of

dinucleotide intercalation sites to select from in the oligonucleotide to which the drug bound. Whether this selectivity was fortuitous, or resulted from the peculiar conditions within the crystalline state, or a true reflection of sequence preference, was unclear. Recent theoretical and solution studies have clarified the sequence preference of daunomycin binding. Daunomycin indeed binds preferentially to particular sites. The elucidation of the properties of these sites has been perhaps the major advance in studies of the daunomycin-DNA interaction over the last several years.

4.1. Theoretical studies

The key insight into the sequence preference of daunomycin came from a theoretical investigation from the laboratory of Pullman [50]. Computations were performed on the energetics of daunomycin binding to six double-stranded hexanucleotides of varying sequence. The surprising result to emerge was that daunomycin preferentially recognized a *triplet* sequence. The order of sequence preference, from most to least preferred, was: 5′-TCG ≥ 5′-ACG > 5′-ATA ≥ 5′-ACI ≫ 5′-GCG > 5′-GTA. For the first time, an intercalator was predicted to show preferential site binding beyond the simple dinucleotide level. Given that daunomycin binds to a site containing 3 bp, as established from equilibrium binding studies, the proposal that the drug recognizes a triplet sequence is both reasonable and attractive. The mixed base composition of the preferred triplet sequence was a further novelty. The pronounced dependence of the equilibrium binding constant on GC content, discussed earlier, is readily explained by these theoretical calculations. Further, the most preferred triplet sequences are the very sites to which daunomycin is observed to be bound in the crystal studies [12,13], suggesting that those sites are indeed energetically more favorable than the other possible intercalation sites available in the hexanucleotide used in the structural studies.

4.2. Footprinting studies

'Footprinting' methods, using either chemical [51–53] or enzymatic [54,55] cleavage reagents, offer a high-resolution procedure for mapping preferred antibiotic-binding sites on DNA in solution. In the footprinting experiment, a DNA fragment of known sequence is cleaved under stringently controlled conditions such that each molecule is cleaved only once, on average, and at random position. High-resolution gel electrophoresis is used to separate the products of the cleavage reaction, producing a 'ladder', in which each rung corresponds to a single nucleotide step along the known sequence. If antibiotic is bound to the DNA prior to the cleavage reaction, positions occupied by antibiotic will be protected from cleavage, and gaps, or footprints will appear in the ladder. Since the DNA sequence is known, the exact sequence at the position occupied by ligand may be immediately inferred from the location of the gaps. Footprinting experiments using de-

Table 5

Catalog of sequences protected from DNase I digestion by bound daunomycin

Data taken from ref. 37. Protection data were obtained for both strands for the DNA fragment containing the tyrosine tRNA operator (except where indicated by an asterisk) by selectively end labeling each strand. Protection data were obtained for only one strand for the two restriction fragments from pBR322 DNA. The putative triplet recognition sequence is indicated by a dashed line.

(A) Restriction fragment containing tyrosine tRNA operator	
5′*******	5′CGCAA
3′ATGCCTA	3′GCGTTGGTCCA
5′AGCG	5′ TGCCGCCCC
3′TCGC	3′ACTACGC
5′ACGTAACACT	5′GGGAGCAGGCCAGT
3′TGCATTGTGA	3′CCCTCGT
5′TTACCC	
3′******	
(B) 61 bp restriction fragment from pBR322 DNA	
5′GCTACC	5′CCT
5′TAGC	5′CGA
5′CGGTGAAGCC	
(C) 63 bp restriction fragment from pBR322 DNA	
5′TGGTGGCA	5′GCC
5′GTGGC	5′GGG

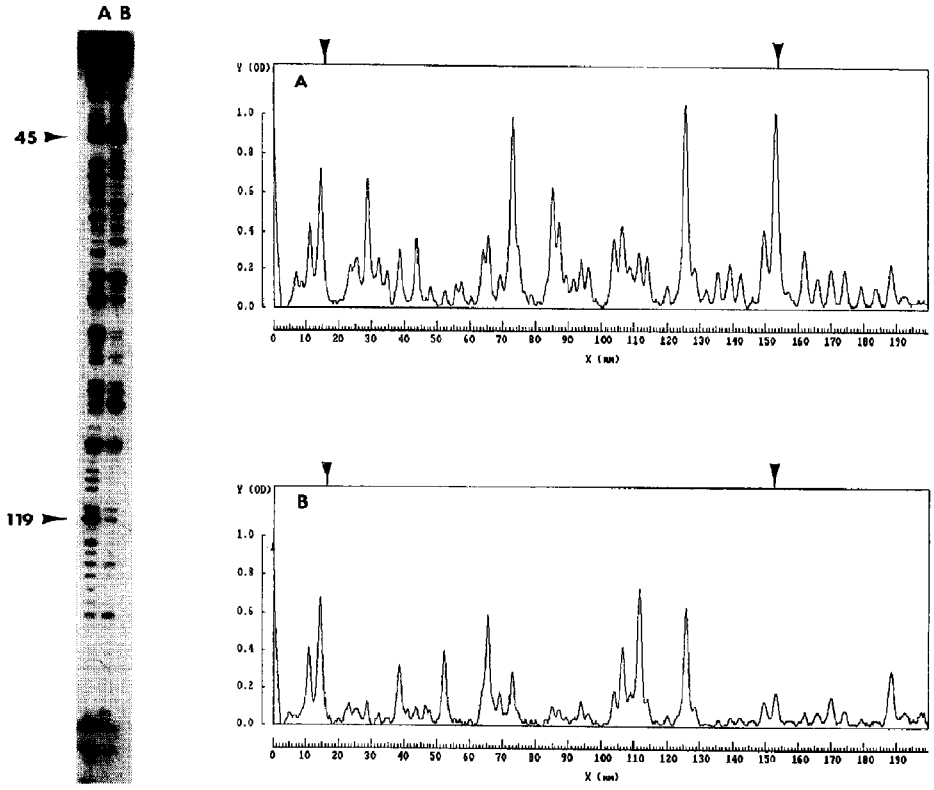


Fig. 3. Example of a deoxyribonuclease footprinting experiment. On the left is an image of an autoradiogram obtained for a ^{32}P labeled restriction fragment following limited digestion by deoxyribonuclease in the absence (A) or presence (B) of daunomycin. On the right side are densitometric scans of the autoradiogram.

oxyribonuclease I as a cleavage reagent were performed to study the sequence preference of daunomycin [37]. The result of a footprinting experiment is shown in fig. 3. Particular regions of the DNA are seen to be protected from DNase I attack in the presence of daunomycin, indicating that the drug indeed preferentially binds to certain sequences. These sequences are cataloged in table 5, along with other protected sequences that have been determined using additional DNA fragments. Inspection of table 5 reveals that all protected sequences observed contain a common triplet sequence of the type predicted by theoretical studies to be a preferred daunomycin-binding site [37]. Additional footprinting studies have appeared that differ from this interpretation [56,57]. However, a more quantitative footprinting procedure has recently been implemented in our laboratory that confirms that daunomycin preferentially recognizes the triplet sequence 5′TCG (J.B. Chaires, J.E. Herrera and M.J. Waring, manuscript in preparation). The concentration dependence of daunomycin protection was examined in these studies. The nine most highly protected sites were identified by this more refined footprinting procedure. Seven of these nine sites were within or immediately adjacent to the sequences 5′-TCG or 5′-TGC. These are the very sequences predicted by the theoretical studies to be preferred binding sites.

4.3. Conclusions

Theoretical studies predicted that daunomycin preferentially interacts with a triplet sequence $5'^{T}_{A}CG$, a prediction that has been verified by footprinting studies in solution. Daunomycin does indeed recognize preferred sites. The preferred site is unusual in that it is a triplet sequence and of mixed base composition.

5. Summary

Structural, theoretical and solution studies have converged to provide a coherent and detailed picture of the interaction of daunomycin with its DNA-binding site. The daunomycin-DNA interaction is now perhaps the most thoroughly characterized intercalation reaction. A novel aspect of daunomycin binding to DNA is its apparent preference for a triplet sequence containing an AT base-pair flanked by adjacent GC base-pair. Structural and theoretical studies show that the origin of this preference arises from a combination of specific hydrogen bonds and the stereochemical fit of the daunosamine moiety within the minor groove [12,37,50]. The fact that daunomycin binding is stabilized by a combination of intercalation and minor groove interactions is unique, which may, in turn, contribute to the unique type of sequence preference exhibited by the drug. The detailed understanding of the interaction of this important anticancer drug with its DNA-binding site should contribute in a fundamental way to the rational design of new chemotherapeutic compound with enhanced potency.

Acknowledgement

Research on the daunomycin-DNA interaction in this laboratory is supported by U.S. Public Health Service Grant CA35635, awarded by the National Cancer Institute.

References

1 F. Arcamone, Doxorubicin, (Academic Press, New York, 1981).
2 S.T. Crooke and S.D. Reich, Anthracyclines: Current status and new developments (Academic Press, New York, 1980).
3 M. Gigli, S.M. Doglia, J.M. Millot, L. Valentini and M. Manfait, Biochim. Biophys. Acta 950 (1988) 13.
4 L. Valentini, V. Nicolella, E. Vannini, M. Menozzi, S. Penco and F. Arcamone, Il Farmaco 40 (1985) 377.
5 L. Gianni, B.J. Corden and C.E. Meyers, Rev. Biochem. Toxicol. 5 (1983) 1.
6 S. Neidle, in: Topics in antibiotic chemistry, vol. 2, ed. P.G. Sammes (Halsted Press, New York, 1978) p. 240.
7 J.W. Lown, Mol. Cell. Biochem. 55 (1983) 17.
8 S. Neidle and M.R. Sanderson, in: Molecular aspects of anti-cancer drug action, eds. S. Neidle and M.J. Waring (Verlag-Chemie, Weinheim, 1983) p. 35.
9 G. Aubel-Sadron and D. Londos-Gagliardi, Biochimie 66 (1984) 333.
10 J.R. Brown and S.H. Imam, Prog. Med. Chem. 21 (1984) 169.
11 H. Fritzsche and H. Berg, Gazz. Chim. Ital. 117 (1987) 331.
12 A.H.-J. Wang, G. Ughetto, G.J. Quigley and A. Rich, Biochemistry 26 (1987) 1152.
13 S.R. Holbrook, A.H.-J. Wang, A. Rich and S.-H. Kim, J. Mol. Biol. 199 (1988) 349.
14 D.M. Crothers, Biopolymers 6 (1968) 575.
15 J.D. McGhee and P.H. von Hippel, J. Mol. Biol. 86 (1974) 469.
16 H. Shutz, F.A. Gollmick and E. Stutter, Stud. Biophys. 75 (1979) 147.
17 J.B. Chaires, N. Dattagupta and D.M. Crothers, Biochemistry 21 (1982) 3933.
18 J.B. Chaires, Biopolymers 24 (1985) 403.
19 D.E. Graves and T.R. Krugh, Biochemistry 22 (1983) 3941.
20 F. Barcelo, J. Mortorell, F. Gavilanes, and J.M. Gonzalez-Ros, Biochem. Pharmacol. 37 (1988) 2133.
21 L.E. Xodo, G. Manzini, J. Ruggiero and F. Quadrifoglio, Biopolymers 27 (1988) 1839.
22 B.C. Hammer, R.A. Russell, R.N. Warrener and J.G. Collins, Eur. J. Biochem. 178 (1989) 683.
23 J.B. Chaires, Biochemistry 24 (1985) 7479.
24 J.B. Chaires, J. Biol. Chem. 261 (1986) 8899.
25 J.E. Herrera and J.B. Chaires, Biochemistry 28 (1989) 1993.
26 N. Dattagupta, M. Hogan and D.M. Crothers, Biochemistry 19 (1980) 5998.
27 T. Tsuchiya, Biopolymers 22 (1983) 1967.
28 K.J. Breslauer, D.P. Remeta, W.-Y. Chou, R. Ferrante, J. Curry, D. Zaunczkowski, J.G. Snyder and L. Marky, Proc. Natl. Acad. Sci. U.S.A. 84 (1987) 8922.
29 L.A. Marky, J. Curry and K.J. Breslauer, in: Molecular basis of cancer, Part B: Macromolecular recognition, chemotherapy and immunology, ed. R. Rein (A.R. Liss, New York, 1985) p. 155.
30 M.T. Record, Jr, C.F. Anderson and T.M. Lohman, Q. Rev. Biophys. 11 (1978) 103.

31 H.P. Hopkins, Jr and W.D. Wilson, Biopolymers 26 (1987) 1347.
32 S. Neidle and Z. Abraham, CRC Crit. Rev. Biochem. 17 (1984) 73.
33 J.B. Chaires, Biochemistry 22 (1983) 4204.
34 D.P. Remeta, C.P. Mudd, R.L. Berger and K.J. Breslauer, Biophys. J. 53 (1988) 480a.
35 W. Mueller and D.M. Crothers, Eur. J. Biochem. 54 (1975) 267.
36 G. Dougherty and W.J Pigram, CRC Crit. Rev. Biochem. 12 (1982) 103.
37 J.B. Chaires, K.R. Fox, J.E. Herrera, M. Britt and M.J. Waring, Biochemistry 26 (1987) 8227.
38 M.B. Jones, U. Hollstein and F.S. Allen, Biopolymers 26 (1987) 121.
39 W. Mueller and D.M. Crothers, J. Mol. Biol. 35 (1968) 251.
40 E.J. Gabbay, D. Grier, R.E. Fingerle, R. Reimer, R. Levy, S.W. Pearce and W.D. Wilson, Biochemistry 15 (1976) 2062.
41 M. Grant and D.R. Phillips, Mol. Pharmacol. 16 (1979) 357.
42 B.M. Gandecha, J.R. Brown and M.R. Crampton, Biochem. Pharmacol. 34 (1985) 733.
43 E. Stutter and W. Forster, Stud. Biophys. 75 (1979) 199.
44 W. Forster and E. Stutter, Int. J. Biochem. 6 (1984) 114.
45 J.B. Chaires, N. Dattagupta and D.M. Crothers, Biochemistry 24 (1985) 260.
46 C.R. Krishnamoorthy, S.-F. Yen, J.C. Smith, J.W. Lown and W.D. Wilson, Biochemistry 25 (1986) 5933.
47 W.D. Wilson, C.R. Krishnamoorthy and Y.H. Yang, Biopolymers 24 (1985) 1941.
48 D.R. Phillips, P.C. Greif and R.C. Boston, Mol. Pharmacol. 33 (1988) 225.
49 V. Rizzo, N. Sacchi and M. Menozzi, Biochemistry 28 (1989) 274.
50 K.-X. Chen, N. Gresh and B. Pullman, J. Biomol. Struct. Dyn. 3 (1985) 445.
51 M.W. Van Dyke, R.P. Hertzberg and P.B. Dervan, Proc. Natl. Acad. Sci. U.S.A. 79 (1982) 5470.
52 D.S. Sigman, Acc. Chem. Res. 19 (1986) 180.
53 T.D. Tullius, B.A. Domboski, M.E.A. Churchill and L. Kam, Methods Enzymol. 155 (1987) 537.
54 M.J. Lane, J.C. Dabrowiak and J.N. Vournakis, Proc. Natl. Acad. Sci. U.S.A. 80 (1983) 3260.
55 K.R. Fox and M.J. Waring, Nucleic Acids Res. 12 (1984) 9271.
56 A. Skorobogaty, R.J. White, D.R. Phillips and J.A. Reiss, FEBS Lett. 227 (1988) 103.
57 A. Skorobogaty, R.J. White, D.R. Phillips and J.A. Reiss, Drug Design Delivery 3 (1988) 125.