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The peculiar binding properties of 4′-deoxy,4′-iodo-doxorubicin to isolated DNA and 175 bp nucleosomes

C.Cera[1] and M.Palumbo[1,2]*
[1]Department of Pharmaceutical Sciences, University of Padova, via Marzolo 5, 35131 Padova and
[2]Department of Pharmacy, Chemistry and Technology, University of Cagliari, Italy

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ABSTRACT

The thermodynamic parameters governing the interaction of 4′-deoxy,4′-iododoxorubicin (4′-IAM) to double stranded DNA or 175 bp nucleosomes have been evaluated at different ionic strength and temperature conditions by means of fluorescence techniques. The iodo-anthracycline exhibits quite different characteristics from the parent compounds adriamycin (AM) and daunomycin (DM) and other second generation derivatives. In fact, the contribution of electrostatic interactions to the total free energy of binding is rather poor and the changes in enthalpy, usually high and negative, are low and eventually positive. Unlike other members of its family, 4′-IAM exhibits preference for the nucleosomal structure. In addition, its binding to isolated DNA is remarkably cooperative. Circular dichroism studies show changes in the geometry of the intercalation complex when the drug binds to nucleosomes. The possibility for the iodo-sugar moiety to act as an alkylating or free-radical producing species was also considered as an alternative mechanism of action. However, no evidence was obtained to support these hypotheses. Thus the major differences observed in DNA-binding in comparison to parent anthracyclines appear to be mostly related to the lower pK_a and higher lipophilicity exhibited by 4′-IAM.

INTRODUCTION

Anthracyclines represent an important class of compounds, some of which are widely clinically used in anticancer chemotherapy (1–3). The low responsiveness of solid tumor diseases, the onset of resistance and of undesirable side effects, in particular cardiotoxicity, have stimulated the synthesis of new analogues with more favorable pharmacological properties (4, 5). A large number of anthracycline derivatives have been screened for biological activity so far. Among them 4′-deoxy,4′-iododoxo-rubicin (4′-IAM, Fig. 1) is peculiar because of a greater lipophilicity and a reduced basicity of the amino group at position 3′. In addition, it is characterized by elevated and fast cellular uptake, is active against P388 leukemia cells resistant to doxorubicin and against pulmonary metastasis from Lewis lung carcinoma. Moreover, the antitumor activity is retained after oral administration (6). Given the above physico-chemical properties, which set it somewhat apart from its congeners, it appeared of interest to investigate to what extent they could affect binding to DNA and whether or not this had any bearing to the biological response exhibited by the drug. Although a few mechanisms of action were proposed for this class of antitumor agents (7–10), it is now widely accepted that anthracyclines act as poisons for Topoisomerase II enzymes (11, 12). In this connection the extent of formation of a ternary complex (including the protein) and the distribution of the drug along the genome have to be related to the ability of anthracyclines to bind to DNA and to their preference for different structural arrangements of the nucleic acid. Thus, in addition to isolated DNA, we focused our attention on the binding of 4′-IAM to 175 bp nucleosomes. In fact the reduced basicity of the amino group might alter the distribution of the drug between more and less structurally organized regions of DNA (which are characterized by different electrostatic potentials) in comparison to the parent compounds AM and DM.

Given the electrophilic character of the carbon atom at position 4′, we also examined the reactivity of 4′-IAM towards aliphatic amines and DNA bases, in particular guanine. The possibility of a photochemical decomposition, leading to reactive radical species, was finally considered.

MATERIALS AND METHODS

4′-IAM (in the hydrochloride form) was a generous gift from Farmitalia (Milano, Italy). Because of its low solubility, stock solutions were prepared dissolving the powder in the smallest volume of ethanol, and then adding buffer to reach the desired concentration, determined by absorption measurements at 485 nm (the molar extinction coefficient value is 10,050 $M^{-1}cm^{-1}$). The absence of changes in the absorption spectra and of precipitates was regularly verified.

Highly purified calf thymus DNA was purchased from Sigma as lyophilized. It was sonicated to average molecular weight of about 250,000, corresponding to a length of ~400 base pairs.

* To whom correspondence should be addressed at the Department of Pharmaceutial Sciences, University of Padova, Via Marzolo 5, 35131 Padova, Italy

Solutions were prepared by swelling it (~1 mg/ml) overnight at 4°C in low ionic strength buffer, then filtering and adjusting the concentration to the desired value. An extinction coefficient of 6800 $M^{-1}cm^{-1}$ has been used.

175 bp calf thymus nucleosomes were prepared as described in the literature (13, 14) and used within a few days after preparation. The length of the DNA fragments and the presence of the histones in the correct ratio were controlled by gel electrophoresis techniques, whereas circular dichroism spectra were recorded to confirm the nucleosomal structure (15).

Calf thymus histones were prepared and purified as previously described (16).

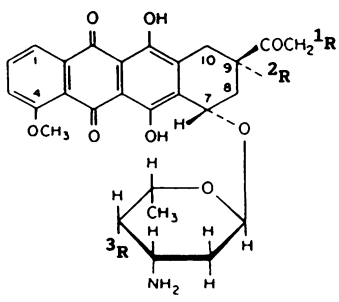
Buffers at three ionic strengths (0.022 M, 0.104 M, 0.355 M) have been used. They were prepared by dissolving the correct amount of sodium chloride, Carlo Erba, in TE buffer (10 mM TRIS, Fluka, 1 mM EDTA, Carlo Erba, pH 7).

Measurements were performed on a Perkin Elmer MPF-66 spectrofluorimeter interfaced with a Perkin Elmer 7300 Data Station, using quartz cells of 1×1 cm or 2×2 mm optical pathway. Titrations were carried out at 11°C, 25°C, 40°C using a Haake F3C thermostat. When operating at low temperature, nitrogen was kept fluxing in the cell compartment in order to avoid moisture condensation on the cuvette walls. All solutions were filtered through 3 μm Millipore filters prior to use. Emission spectra of the samples were recorded using an excitation wavelength of 540 nm (corresponding to the isosbestic region of the absorption spectra for the mixtures anthracycline-DNA). The changes in the absorption spectra are practically superimposable using either nucleosomes or isolated double stranded DNA. In order to obtain a more intense emission signal some of the measurements were also performed at 485 nm (corresponding to the maximum of the drug's absorption spectrum). In all cases was the absorption of the examined solution below 0.1. Using either wavelength value, no appreciable changes were found in the evaluation of the data. Intensity of fluorescence was read at the wavelength of maximum emission, 596 nm.

The titrations were performed starting with a sample containing ~10^{-4} M (on a phosphate basis) nucleic acid and ~10^{-6} M 4'-IAM. After measuring the emission intensity, a weighed amount of this mixture was drawn and the corresponding amount of drug stock solution (at the same concentration as in the starting sample) was added. This procedure was repeated several times to generate DNA/anthracycline ratios ranging between 100 and 1. No corrections were made for drug aggregation as the relationship between concentration and absorption was linear up to well above the maximum drug concentration used in our experiments. The experimental data, corrected for dilution, were elaborated according to McGhee & Von Hippel (17) and represented as Scatchard plots (18).

Circular dichroism studies were performed on a JASCO J 500 spectropolarimeter interfaced with a JASCO mod. 501 N Data Processor, using quartz cuvettes of 1 cm optical pathway. In the visible region (650–320 nm) every sample (drug concentration ~10^{-5} M) was scanned four times with 1 m°/cm sensitivity, whereas in the UV range (320–200 nm) two accumulations and 2 m°/cm sensitivity were used (DNA concentration ~10^{-4} M). All spectra were corrected for dilution.

For irradiation experiments, a Phototechnology Inst. Model 200-LPS optical apparatus provided with a Osram 150 W Lamp was used. Irradiation intensity was in the range 50–55 joule/sec/m^2. The excitation wavelength was chosen at 300–330 nm, in agreement with Murray and Martin (19); in a second set of measurements the whole light spectrum was used,



ANTHRACYCLINE	^1R	^2R	^3R
AM	OH	OH	OH
DM	H	OH	OH
4'-IAM	OH	OH	I

Figure 1. Chemical structures of 4'-IAM and the parent compounds AM and DM.

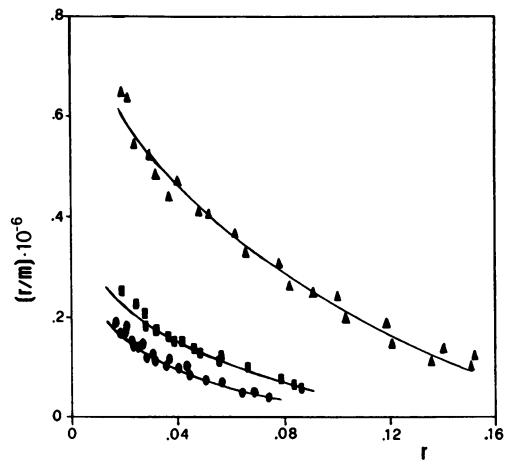


Figure 2. Scatchard plot for the interaction between 4'-IAM and nucleosomes at 25°C, pH 7 and 0.022 M (▲), 0.105 M (■), 0.355 M (●) ionic strength.

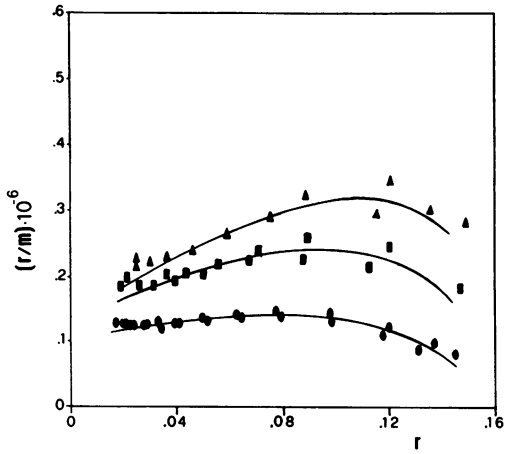


Figure 3. Scatchard plot for the interaction between 4'-IAM and ds DNA at 25°C, pH 7 and 0.022 M (▲), 0.105 M (■), 0.355 M (●) ionic strength.

after filtering off frequencies corresponding to wavelengths shorter than 290 nm to avoid direct damage to DNA.

TLC measurements were performed according to literature data on the parent anthracyclines (4), using n-butanol:acetic acid:water =4:1:5 as the elution system. Mixtures of 4′-IAM and guanine, guanosine, ethanolamine, 3-amino-propane-1-ol, incubated up to 1 day at different temperatures (25°−60°C) were tested.

A 21 bp double stranded oligonucleotide, corresponding to the wild type Arc operator (20), was prepared on an Applied Biosystem DNA Synthesizer mod. 380B. After irradiation in the presence and absence of 4′-IAM it was run on a native as well as denaturing 20% polyacrylamide gel at 180 V for 4 hours. The gels were then stained with 0.5 mg/ml ethidium bromide and photographed under UV light.

RESULTS

The intense fluorescence signal of 4′-IAM is almost completely quenched when the chromophore is inserted between the base pairs of the nucleic acid. No difference was observed in the spectroscopic properties of bound drug using either naked DNA or nucleosomes. In addition no change in absorption was detected incubating 4′-IAM with excess amount of histones. Thus the same experimental conditions were chosen to study complex formation with either DNA structure.

The binding of ethidium to nucleosome core particle was recently examined in detail (21−22). The authors pointed out the possibility of dissociation of the nucleic acid from the histone core induced by the drug. Gel electrophoresis experiments performed with 4′-IAM and 175 bp nucleosomes up to bound-drug/DNA ratios of about 0.1 did not show evidence for appreciable dissociation in our case. The experimental data were accordingly elaborated considering the complex with nucleosomes as a single species. The Scatchard plots obtained from fluorescence quenching experiments at different salt concentration using ds DNA or 175 bp nucleosomes are presented in Fig. 2 and 3.

The intrinsic binding constant (K_i) and the exclusion parameter (n) are reported in Table I. The corresponding data for the parent compounds AM and DM are also included for comparison. The exclusion parameter is somewhat affected upon increasing ionic strength only for the binding of 4′-IAM to nucleosomes. In addition, a positive cooperativity is found when using the isolated macromolecule.

Table I. Thermodynamic parameters for the interaction of 4′-IAM, AM and DM to free DNA or nucleosomes at 25°C, pH 7 and different ionic strength.

		Ionic Strength (M) 0.022	0.104	0.355
4′-IAM-DNA[a]	$K_i(\times 10^{-5})$	1.8±0.2	1.7±0.2	1.2±0.2
	n	5.1±0.3	5.0±0.3	5.0±0.3
AM-DNA	$K_i(\times 10^{-5})$	120 ±8	17 ±0.7	4.6±0.4
	n	6.0±0.3	6.0±0.3	6.2±0.3
DM-DNA	$K_i(\times 10^{-5})$	93 ±3	17 ±0.6	4.4±0.3
	n	8.9±0.3	9.0±0.3	9.0±0.3
4′-IAM-nucleosomes	$K_i(\times 10^{-5})$	6.2±0.5	3.0±0.3	2.3±0.2
	n	4.5±0.3	6.9±0.3	8.0±0.3
AM-nucleosomes	$K_i(\times 10^{-5})$	17.8±0.7	4.4±0.4	1.4±0.2
	n	7.1±0.3	6.9±0.3	7.0±0.3
DM-nucleosomes	$K_i(\times 10^{-5})$	5.5±0.4	1.4±0.2	0.5±0.2
	n	10.0±0.3	10.1±0.3	10.0±0.3

[a] Cooperativity factors are 15, 8 and 9 respectively. In no other case were cooperativity effects observed.

In this class of compounds K_i is expected to decrease at the increasing of salt concentration, because of the reduced electrostatic binding of the charged amino-sugar to the phosphate backbone (13, 23). Using 4′-IAM a very moderate salt dependence is observed for ionic strength in the range 0.022−0.355 M (Fig. 4A). This finding indicates a rather poor charged interaction between the anthracycline derivative and the nucleic acid, which is further confirmed using nucleosomes.

Peculiar to 4′-IAM is also its relative preference for structurally organized DNA rather than for the free nucleic acid. This fact had not been observed before within this class of antibiotics which usually exhibit a substantial preference for isolated DNA (13, 23). As a matter of fact, the binding of 4′-IAM to ds DNA is characterized by remarkably lower values in comparison to AM and DM. Instead, K_i for nucleosomes is comparable for the parent anthracyclines under physiological conditions.

The binding parameters obtained at different temperatures are summarized in Table II. The dependence of ln K_i vs. 1/T is shown in Fig. 4B. The enthalpy value for complex formation between isolated DNA and 4′-IAM is positive and very low. The

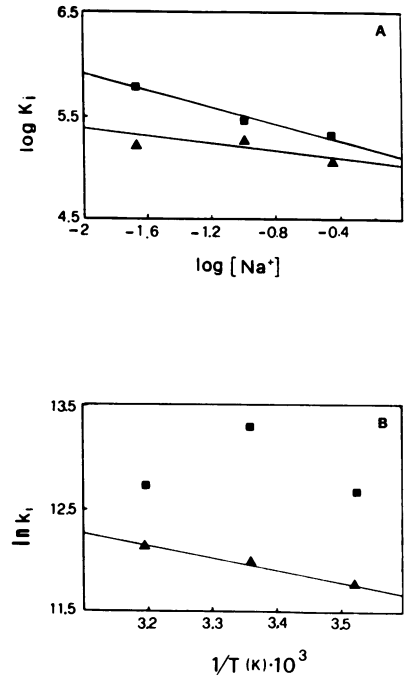


Figure 4. Dependence of the intrinsic interaction constant for the systems 4′-IAM-isolated DNA (▲) and 4′-IAM-nucleosomes (■) upon ionic strength at 25°C (A), and temperature at 0.022 M ionic strength (B).

Table II. Thermodynamic parameters for the binding of 4′-IAM to ds or nucleosomal DNA at pH 7, 0.022 M ionic strength and different temperatures.

		Temperature (K) 284	298	313
4′-IAM-DNA	$K_i(\times 10^{-5})$	1.3±0.2	1.6±0.2	1.9±0.2
	n	4.1±0.3	5.1±0.3	4.1±0.3
	w[a]	8	15	10
4′-IAM-nucleosomes	$K_i(\times 10^{-5})$	3.2±0.3	6.2±0.3	3.5±0.3
	n	4.5±0.3	4.5±0.3	5.0±0.3
	w[a]	4	1	1

[a] cooperativity factor

data relative to 175 bp nucleosomes are somewhat scattered, possibly due to the variation in cooperativity as a function of temperature. Thus a safe evaluation of ΔH is not possible. In any event its absolute value appears to be remarkably lower than for the parent compounds. In general, intercalation complexes of anthracyclines are characterized by large negative enthalpy changes (13, 23). The above findings are therefore quite unusual and might imply the presence of alternative interaction mechanisms for 4'-IAM.

Taking this into consideration, we tried in particular to reveal possible covalent binding of the drug to DNA bases or DNA degradation occurring upon cleavage of the halogen-carbon bond. The first hypothesis was tested by chromatographic analysis of mixtures of 4'-IAM with different primary amines or guanosine and guanine. The systems were incubated up to 24 hours at temperatures ranging between 25° and 60°C. In no case were we able to detect modified species of the anthracycline.

To investigate the cleavage hypothesis, 4'-IAM was irradiated as such and in the presence of a synthetic (21 bp) duplex oligonucleotide. Neither DNA single-/double-strand fragmentation, nor drug decomposition were detectable following these experiments, as proven by polyacrylamide gel electrophoresis or TLC.

Circular dichroism measurements give further insight into the geometry of complex formation between 4'-IAM and the nucleic acid. The effects in the UV region are comparable to those observed for the parent anthracyclines derivatives (23) and confirm that very similar structural modification of DNA and nucleosomes are occurring upon drug binding (24).

Circular dichroism changes in the visible region are diagnostic of the orientation of the intercalated moiety within the base pair pocket (25). Our data with isolated DNA are in agreement with literature reports (26), and indicate a perpendicular orientation of the drug transition moment with reference to the base-pair longest dimension. On the other hand a dramatic increase in ellipticity is observed for the system 4'-IAM-nucleosomes at comparable extent of complex formation (Fig. 5). These findings suggest a different stereochemical arrangement of the anthraquinone chromophore when bound to DNAs having different levels of structural organization.

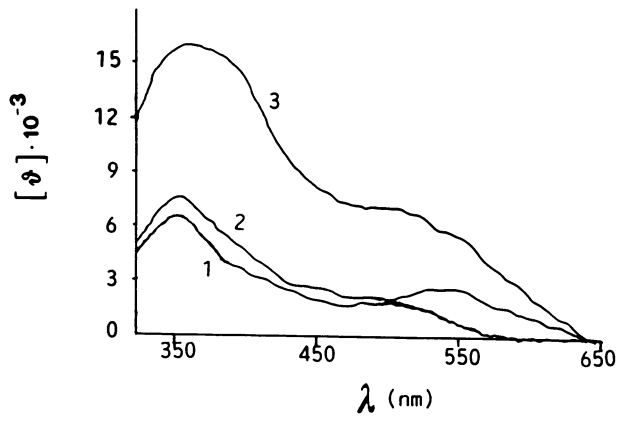


Figure 5. Circular dichroism in the visible region for the system 4'-IAM-nucleosomes at 25°C, 0.1 M ionic strength, and pH 7. Molecular ratio between nucleic acid and drug (R) is: 1) R=0, 2) R=5.2, 3) R=10.2.

DISCUSSION

4'-IAM is a very interesting compound among anthracyclines of second generation not only for its pharmacological characteristics, but also for its DNA-binding properties.

In particular, it prefers the nucleic acid in the nucleosomal arrangement rather than in the isolated B form, the electrostatic contribution to the binding to both structures is very moderate, and the enthalpy values for complex formation are low and eventually positive. All of this is rather unusual for drugs of the anthracycline structural type. The rationale for such a dramatic change must be due only to the substitution of the hydroxyl group with iodine at position 4' of the sugar moiety, the rest of the molecule being identical to AM. Such a substitution produces an increase in lipophilicity, a decrease in basicity, changes in steric hindrance and loss of hydrogen bonding ability. All of them could play a role in affecting DNA-binding. In particular, the pK_a of the amino group in the sugar drops to 6.4, against a value of 8.4 for AM (27). Thus, while the parent anthracyclines are almost fully protonated at physiological pH, 4'-IAM is predominantly uncharged under the same conditions: hence the lower dependence of K_i upon ionic strength and the reduced affinity of the drug for isolated DNA at corresponding salt concentrations.

The binding to nucleosomes, on the other hand, is in general much less stabilized by electrostatic interactions, since a large fraction of negative charges on the nucleic acid is neutralized by histones . Indeed, the affinities of 4'-IAM, AM and DM for nucleosomal regions are quite comparable, the iodinated drug being even a little more effective at physiological ionic strength.

A consequence of the above facts is the relative preference for organized over isolated DNA when using 4'-IAM. On the other hand, positive cooperativity characterizes binding to ds-DNA, but not to nucleosomes under physiological conditions. A balance of these factors leads to a more even distribution of the drug along the genome.

The structural organization of DNA plays a role not only in the efficiency of electrostatic binding of the iodo-sugar moiety, but also in the stereochemistry of the intercalated chromophore. In fact, as shown by circular dichroism measurements, a somewhat distorted binding geometry is observed when the double helix folds into the nucleosomal structure. This is again at variance with the results obtained when using AM or DM as the anthracycline (23).

The substantially lower value of the enthalpy of 4'-IAM binding to DNA or nucleosomes deserves some further comments. It cannot be accounted for solely by the reduced charged interactions between the sugar moiety and the negative potential of DNA. In fact the enthalpy associated with the formation of ionic contacts is usually close to zero, as, for example, in the case of lysine oligomers binding to DNA (28). Thus, a perturbation of the non-electrostatic forces stabilizing the intercalated complex is another consequence of 4' substitution. Lack of the OH group at this position should not be of basic relevance, as it points away from the DNA molecule in the complex (29). Probably more affected is the hydrogen bond between 3'-N of daunosamine and N_3 of adenine via a bridging water molecule (29), which is closest to iodine and should be destabilized both electronically and, possibly, sterically.

The electrophilic character of the carbon-halogen bond seems to be reduced in the anthracycline derivative, probably by the presence of the adjacent amino group. In fact covalent binding

to DNA bases or aliphatic amines is not occurring under our experimental conditions. Homolytic cleavage of the iodo-sugar residue has also to be ruled out as a possible mechanism of nucleic acid damage.

A comparison between biological activity and DNA-binding data is worth a final comment. Our previous work (23) indicated that a better relationship existed between anticancer activity and binding to nucleosomes rather than to free DNA for a number of first and second generation anthracyclines. Interestingly, the same holds for 4′-IAM, which exhibits outstanding anticancer activity, yet it binds poorly to isolated DNA. Again, if the comparison is made considering K_i for nucleosomes at physiological salt concentration, then 4′-IAM appears to be as effective in the binding process as the most potent congeners. Of course additional important factors, such as drug distribution and metabolism, are responsible for the overall biological properties. Nonetheless, binding affinity for nucleosomes appears to represent a parameter more reliable than affinity for free DNA to rank the effectiveness of an anthracycline derivative as an anticancer drug.

REFERENCES

1. Young, R. C., Ozols, R. F. and Myers, C. E. (1981) *New Engl. J. Med.*, **305**, 139–153.
2. Favaudon, V. (1982) *Biochimie*, **64**, 457–475.
3. Arcamone, F. (1984) *Med. Res. Rev.*, **4**, 153–158.
4. Arcamone, F. (1981) 'Doxorubicin Anticancer Antibiotics', Academic Press, New York.
5. Di Marco, A., Arcamone, F. and Zunino, F. (1975) 'Antibiotics, Vol. III, Mechanism of Action of Antimicrobial and Antitumor Agents' (J. W. Corcoran and F. E. Hann, Eds.), p. 101–128.
6. Barbieri, B., Giuliani, F. C., Bordoni, T., Casazza, A. M., Geroni, C., Bellini, O., Suarato, A., Gioia, B., Penco, S. and Arcamone, F. (1987) *Cancer Research*, **47**, 4001–4006.
7. Quigley, G. L., Wang, A. M. J., Ughetto, G., van der Marel, G., van Boom, J. H. and Rich, A. (1980) *Proc. Natl. Acad. Sci. U.S.A.*, **77**, 7204–7208.
8. Hickman, J. A., Scanlon, K. J. and Tritton, T. R. (1984) *Trends Pharmacol. Sci.*, **5**, 15–17.
9. Tanaka, M. and Yoshida, S. (1980) *J. Biol. Chem.*, **87**, 911–918.
10. Muindi, J. R. F., Sinha, B. K., Gianni, L. and Myers, C. E. (1984) *FEBS Letters*, **172**, 226–230.
11. Capranico, G., Zunino, F., Kohn, K. W. and Pommier, Y. (1990) *Biochemistry*, **29**, 562–569.
12. Tewey, K. M., Rowe, T. C., Yang, L., Halligan, B. D. and Liu, L. F. (1984) *Science*, **226**, 466–468.
13. Chaires, J. B., Dattagupta, N. and Crothers, D. M. (1983) *Biochemistry*, **22**, 284–292.
14. Palù, G., Valisena, S., Bertoloni, G., Cera, C. and Palumbo, M. (1988) *Microbiologica*, **11**, 307–319.
15. Rill, R. L., Ramsay Show, B. and Van Holde, K. E. (1978) *Meth. Cell. Biol.*, **18**, 69–103.
16. Stein, A. and Mitchell, M. (1988) *J. Mol. Biol*, **203**, 1029–1035.
17. McGhee, J. D. and Von Hippel, P. H. (1974) *J. Mol. Biol.*, **86**, 469–489.
18. Scatchard, G. (1949) *Ann. N. Y. Acad. Sci.*, **51**, 660–672.
19. Murray, V. and Martin, R. F. (1989) *Nucleic Acids. Res.*, **17**, 2675–2691.
20. Vershon, A. K., Kelley, R. D. and Sauer, R. T. (1989) *J. Biol. Chem.*, **264**, 3267–3273.
21. McMurray, C.T. and van Holde, K.E. (1991) *Biochemistry*, **30**, 5631–5643.
22. McMurray, C.T., Small, E.W. and van Holde, K.E. (1991) *Biochemistry*, **30**, 5644–5652.
23. Cera, C., Palù, G., Marciani Magno, S. and Palumbo, M. (1991) *Nucleic Acid Res.*, **19**, 2309–2314.
24. Montecucco, A., Pedrali-Noy, G., Spadari, S., Zanolin E. and Ciarrocchi, G. (1988) *Nucleic Acids Res.*, **16**, 3907–3918.
25. Lyng, R., Hard, T. and Norden, B. (1987) *Biopolymers*, **26**, 1327–1345.
26. Rizzo, V., Penco, S., Menozzi, M., Geroni, C., Vigevani, A. and Arcamone, F. (1988) *Anti-Cancer Drug Design*, **3**, 103–115.
27. Frezard, F. and Garnier-Suillerot, A. (1990)*Biochim. Biophys. Acta*, **1036**, 121–127.
28. Lohman, T. M., deHaseth, P. L. and Record, M. T. (1980) *Biochemistry*, **19**, 3522–3530.
29. Wang, A.H.J., Ughetto, G., Quigley, G.J. and Rich, A. (1987) *Biochemistry*, **26**, 1152–1163.