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Relationship between the pharmacological activity of antitumor drugs Ametantrone and mitoxantrone (Novatrone) and their ability to condense nucleic acids

(intercalation/rRNA/light scattering/nucleolar segregation)

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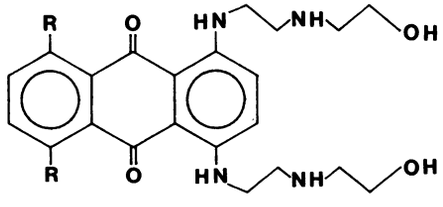
ABSTRACT **Ametantrone (HAQ) and mitoxantrone (DHAQ) are structurally similar antitumor drugs of the anthracenedione class. The cytostatic, cytotoxic, and antitumor activities of these drugs are different, with DHAQ being 10–100 times more potent, per molar basis. Both drugs are strong intercalators and intercalative modes of binding are suspected as relevant to their pharmacological activity. No significant differences, however, that could explain the differences in pharmacological activity are observed in their intercalative properties with respect to base specificity and binding affinity. A correlation, however, is evident between their potency and ability to condense nucleic acids inasmuch as DHAQ condenses nucleic acids at concentrations that are lower by a factor of 5–40 than those of HAQ and these effects can be observed at their pharmacological concentrations. The condensation is base- and sugar-specific and the long purine sequences of single-stranded RNA are the most sensitive. Electron microscopy of L1210 cells exposed a short time (90 min) to 0.21–21 μM DHAQ reveals segregation of nucleoli; the segregated granular portion shows increased electron opacity. In some preparations patchy areas of nuclear chromatin characterized by increased electron opacity can be seen. The results are compatible with the possibility that pharmacological effects of these antitumor drugs could involve condensation of nucleic acids, primarily of RNA in nucleoli.**

Ametantrone (HAQ) and mitoxantrone (DHAQ) are anticancer agents of the anthracenedione class (for review, see refs. 1 and 2). They have similar molecular structure, differing only by two hydroxy groups at positions 5 and 8 of the aromatic system of anthracenedione.



R = H, HAQ; R = OH, DHAQ. Despite structural similarity, the drugs differ markedly in potency; DHAQ is approximately 10-fold more potent *in vivo* and 100-fold more potent *in vitro*, relative to HAQ (2–8). At the same time, however, DHAQ also exhibits higher genotoxicity (9).

Molecular mechanisms and intracellular targets associated with antitumor effects of these drugs are unclear (1, 2, 8). Both drugs bind to DNA by intercalation (5, 10–12). The correlation, however, between their affinity to double-stranded (ds) DNA and antitumor potency is not apparent (5, 11–14). Also, if the intercalative mode of binding is considered, no clear base specificity is evident (10, 12, 13). The latter would explain the difference in the drugs' potency—e.g., under an assumption that intercalation into DNA of the specific base sequences is the primary binding site responsible for the antitumor activity. The possibility that DNA is the drugs' target cannot, however, be disregarded in light of the evidence that DHAQ in comparison with HAQ induces increased DNA strand breaks in proportion to the antitumor activity (15) and that both drugs exhibit cell-cycle effects (G_2 arrest) typical of the DNA-binding drugs (7, 10). Because the relationship between binding to DNA and biological activity of these drugs is unclear, other mechanisms, such as free radical formation, or cell membrane effects were also considered (reviews, see refs. 1, 2, 8, and 16).

In previous studies we observed that certain intercalators can denature and condense nucleic acids in solution (17–21) and in viable cells *in situ* (22, 23). These effects were observed in the cases of acridine orange (17–22) and ellipticine or adriamycin (20) and also were observed at pharmacological concentrations of DHAQ (21). This phenomenon is of great interest inasmuch as it shows a high degree of specificity with respect to the primary and secondary structure of nucleic acids (21). To resolve whether or not the condensation of nucleic acids may be associated with antitumor activity, in the present paper we have compared the propensity of DHAQ and HAQ to condense DNA and RNA with their known pharmacological potencies.

MATERIALS AND METHODS

Nucleic Acids. Phage MS2 RNA was obtained from Miles Laboratories, calf thymus DNA, type I, was from Sigma, and poly(dG)·poly(dC) was from Boehringer Mannheim; all other synthetic polymers were from P-L Biochemicals. The concentration of nucleic acids was determined by UV absorption measurement as described (12).

Drugs. DHAQ (American Cyanamid, Pearl River, NY) was provided by Z. A. Arlin of the N.Y. Medical College in Valhalla. HAQ was obtained through the Investigational Drug Branch, Cancer Therapy Evaluation Program, Division of Cancer Treatment, National Cancer Institute. The concentration of the drugs was determined colorimetrically, at the isosbestic point of the monomer–dimer system, at $\lambda = 682$ nm (molar extinction coefficient $\varepsilon_\lambda = 8.36 \times 10^3\ M^{-1}\cdot cm^{-1}$)

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Abbreviations: HAQ, ANT, NSC-287513, Ametantrone, salt of 1,4-bis({2-[(2-hydroxyethyl)amino]ethyl}amino)-9,10-anthracenedione; DHAQ, NSC-279836 and NSC-301739, mitoxantrone (Novatrone), salts of 1,4-dihydroxy-5,8-bis({2-[(2-hydroxyethyl)amino]-ethyl}amino)-9,10-anthracenedione; ds, double-stranded; ss, single-stranded; I_0, I_b, I_s, intensities of the scattered light, subscript: 0 = initial, b = blank, s = sample; ϕ, increase in light scattering; C, concentration, subscripts: T = total, M = midpoint, B = bound, p = polymer; C_c, critical free ligand concentration.

and $\lambda = 645$ nm ($\varepsilon_\lambda = 7.05 \times 10^3$ $M^{-1} \cdot cm^{-1}$) for DHAQ and HAQ, respectively (10, 12).

Buffers and Solutions. Nucleic acids were dissolved in 0.15 M NaCl/5 mM Hepes, pH 7.0; Triton X-100 (Sigma) was added (final concentration, 0.1%) into samples containing >1 μM concentration of nucleic acids. EDTA (0.5 mM) was added optionally as described in the text; the presence of Triton X-100 or EDTA does not significantly alter the intercalator-induced condensation (21). The buffer was filtered through 0.45-μm pore Millipore filter.

Cell Cultures. The murine leukemia L1210 cells were grown as suspension cultures at 37°C in RPMI 1640 medium with 2 mM L-glutamine, 100 units of penicillin, 100 μg of streptomycin per ml (all from GIBCO), and 10% fetal calf serum (Flow Laboratories), as described (7).

RESULTS

Titration of nucleic acids with DHAQ or HAQ results in the formation of the condensed products that scatter light. Fig. 1 illustrates the increase in light scatter during titration of RNA and denatured and native calf thymus DNA with DHAQ. The curves representing the light-scatter increase are characteristic of the cooperative reaction and transitions occur at different concentrations of the ligand, depending on the structure of the nucleic acid. The most sensitive is MS2 RNA, whereas the most resistant is native DNA. The derivative profiles of the light-scatter increase (Fig. 1*B*), being a more convenient method of data presentation and analysis, were obtained for all combinations of nucleic acids and drugs listed in Table 1. Based on the numerically integrated areas under the derivative profile, the critical free ligand (drug) concentration (C_c) has been established. The method of C_c calculation was described elsewhere (21) and is briefly presented in the legend to Fig. 1.

Table 1 lists C_c values of DHAQ and HAQ for a variety of natural and synthetic nucleic acids. It is evident that DHAQ is much more potent in condensing nucleic acids than HAQ. Thus, for instance, whereas DHAQ at a concentration of 7.8 μM condenses denatured DNA or at 16 μM condenses native DNA, no condensation of denatured or native DNA could be observed even at 100 and 200 μM HAQ, respectively. MS2 RNA is more than five times more sensitive to DHAQ than to HAQ.

The differences in C_c values between DHAQ and HAQ for various synthetic polymers vary between 5 and 40 times, with DHAQ being a more efficient inducer of the condensation in every case. Analysis of the data in Table 1 yields other interesting correlations. Thus, lower concentrations of the drugs were required to condense ss nucleic acids than the ds polymers (e.g., native vs. denatured DNA). However, when one of the components of the ds polymer was exceptionally sensitive to condensation, this polymer underwent condensation at the intermediate drug concentration—i.e., lower than that needed to condense the more resistant component [e.g., poly(rA)·poly(rU) vs. poly(rU)].

Whereas the majority of ribopolymers were more sensitive to condensation than their deoxyribo- counterparts [e.g., poly(rI) vs. poly(dI) or poly(rA) vs. poly(dA)], the reverse was also observed [e.g., poly(rC) vs. poly(dC)]. Of all the polymers, the most sensitive to condensation were ribopolymers, poly(rA,rG), poly(rI), and poly(rA).

Analyzing data for random RNA copolymers shown in Table 1, we observed a correlation between the proportion of purine-nucleotides in nucleic acids and their sensitivity to condensation. When the percentage of adenine plus guanine bases was plotted vs. C_c, the correlation became more evident (Fig. 2). Thus, both drugs preferentially induced condensation of RNA with increasing adenine plus guanine content.

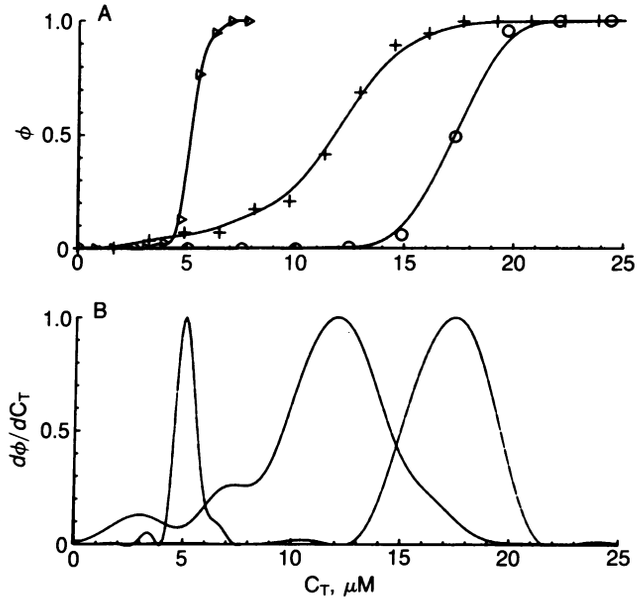

FIG. 1. DHAQ-induced condensation (collapse) of nucleic acids monitored by light scattering. Aliquots containing 2 ml of nucleic acids in buffer were placed in a quartz cuvette in the thermostatic holder (25 ± 0.1°C) of an SLM 4800 spectrofluorimeter (SLM Instruments, Urbana, IL). Light scattering was measured at 90° to the incident light. Both monochromators were set to the same wavelength (350 nm in the experiments with DHAQ and 430 nm when HAQ was used); the bandwidth was 2–4 nm. The sample and the blank (buffer only) were treated with small volumes (5–10 μl) of the drug stock solution (0.1–1 mM); after the addition, the contents of the cuvettes were gently mixed and incubated for 10 min [the time necessary to obtain a stable reading (20)] prior to the light-scattering measurements. The results were corrected for the effect produced by the drug alone $I_s = I_s/I_b$, where I_s and I_b are intensities of the scattered light by the sample and blank, respectively, and expressed as increase of light scattering $\phi = (I_s - I_0)/I_0$, where I_0 is the intensity of scatter light by the sample before addition of the ligand (buffer and nucleic acid only). The data were then processed by computer as described (20, 21). The results of the titration can be expressed as *critical free-ligand concentration* (C_c)—i.e., the concentration of free drug in the mixture corresponding to the point in which one-half of the polymer underwent condensation (20, 21): $C_c = C_M - C_B$, where C_M is the total concentration of the drug at the midpoint of the transition (determined at the point corresponding to one-half of the numerically integrated area under the derivative curve) and C_B is the portion of bound drug at the midpoint. The value of C_B was determined from the known concentration of the polymer (C_p) and the stoichiometry of the drug–polymer interaction. For single-stranded (ss) nucleic acids $C_B = 0.5\ C_p$ (20, 21). All other details are described in previous publications (20, 21). (*A*) Experimental points: △, MS2 RNA; +, denatured calf thymus DNA; and ○, native calf thymus DNA. The initial polymer concentrations were 1, 5.5, and 5 μM, respectively. C_T, total drug concentration in the sample. The solid lines are transition profiles drawn by computer, normalized to 1. (*B*) Derivative light-scatter curves of the transition profiles shown in *A* normalized to 1.

We have shown (22, 23) that the intercalator-induced condensation of nucleic acids *in situ* in viable cells can be detected by electron microscopy of those cells due to the fact that the condensed products are electron opaque. In the present studies we have investigated whether such products can be seen in cells exposed to DHAQ. To this end, L1210 cells were treated with 0.02, 0.21, 2.1, and 21 μM (0.01–10 μg/ml) DHAQ, fixed, and analyzed by electron microscopy. The treatment with DHAQ was of rather short duration (90 min) to exclude secondary changes—e.g., related to cytostatic or cytotoxic drug effects. Whereas more detailed studies will be the subject of a separate publication, we present here the representative micrographs illustrating the

Table 1. Condensation of nucleic acids by DHAQ and HAQ in 0.15 M NaCl/5 mM Hepes, pH 7.0, at 25°C

Nucleic acid	C_c, μM DHAQ	C_c, μM HAQ
Natural*		
Native calf thymus DNA	16.0	>200
Denatured calf thymus DNA	7.8	>100
Phage MS2 RNA	4.8	21.5
Ribosomal (16S + 23S) RNA	2.7	—
Homodeoxyribopolymer†		
poly(dA)	5.2	36.4
poly(dG)	1.1	—
poly(dI)	1.4	21.5
poly(dC)	2.4	>100
poly(dU)	4.4	38.9
poly(dT)	4.0	21.6
Homoribopolymer‡		
poly(rA)	0.55	—
poly(rG)	1.23	—
poly(rI)	0.45	—
poly(rC)	6.30	—
poly(rU)	3.65	—
Ribopolymer pair		
poly(rA)·poly(rU)	2.2	—
poly(rI)·poly(rC)	3.3	—
poly(rG-rC)·poly(rG-rC)	48.4	—
Random RNA copolymer‡		
poly(rC,rU)	4.4	23.1
poly(rU,rG)	1.9	12.1
poly(rA,rG)	0.4	3.7
poly(rA,rC,rU)	2.0	17.3
poly(rA,rU,rG)	1.4	7.0

*Data for DHAQ and natural DNA are taken from ref. 12. DNA concentration, <5 μM; RNA concentration, 1 μM.
†DNA concentration, ≤1 μM; RNA concentration, ≤5 μM.
‡RNA concentration, 0.1–0.2 μM. According to the information supplied by the vendor, the copolymers are ss polyribonucleotides in which the bases indicated in each name are situated randomly in each polymer and are present in approximately equal proportions.

main findings—namely, that short treatment with DHAQ affected the primary structure of the nucleoli (Fig. 3). The effects were seen at 0.21 μM (0.1 μg/ml) and higher drug concentration. The granular portion of the segregated nucleoli exhibited increased electron opacity. In some cells, localized areas of nuclear chromatin also showed increased electron density.

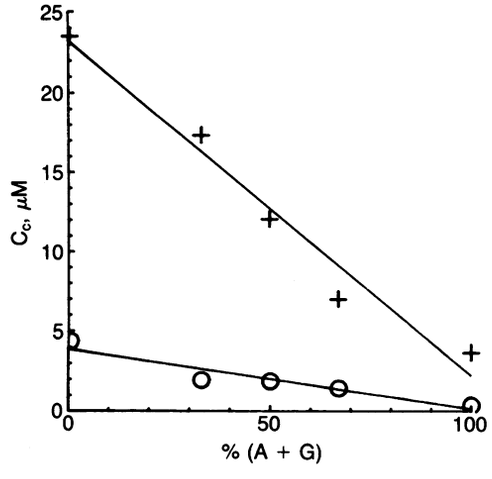

FIG. 2. Relationship between C_c of HAQ (+) or DHAQ (○) and purine-base content of five random ribocopolymers listed in Table 1.

DISCUSSION

Johnson *et al.* (5), while studying a number of substituted alkylaminoanthraquinones, including DHAQ and HAQ, observed that all compounds, regardless of their activity as antitumor agents, are potent inhibitors of DNA and RNA synthesis *in vitro* and bound strongly to DNA. The antitumor activity of these agents, therefore, is due to some mechanism other than DNA binding and inhibition of nucleic acid synthesis (5). Our previous (12) and present studies confirm this conclusion. Data presented in Table 2 indicate that DHAQ and HAQ have similar affinity to bind to ds nucleic acids by intercalation.*

The present data indicate that DHAQ and HAQ can condense nucleic acids. DHAQ, however, is more potent in this respect and the effects can be seen at concentrations that are lower by a factor of 5–40 than those of HAQ. Thus, in contrast to intercalation, pharmacological activity of these drugs correlates with their propensity to condense nucleic acids. Changes in ultrastructure of cells treated with DHAQ (Fig. 3) are also compatible with the assumption that condensation of nucleic acids may be the primary effect of this drug, inasmuch as during the short treatment—i.e., prior to any changes that may be due to drug cytotoxicity (6, 7, 26)—the most affected subcellular components are nucleoli. Nucleolar segregation as well as increased electron density and homogeneity of the granular component may be a consequence of condensation of ribosomal precursor RNA. Appearance of the patchy condensation areas in chromatin may indicate localized condensation of DNA, perhaps of the DNA sections rich in base sequences that are the most sensitive.

Changes in nucleoli (segregation of fibrillar and granular components, nucleolar disintegration) and in chromatin (localized condensation) were also observed in other studies of HAQ (26) and DHAQ (27, 28). However, because in these studies (26–28) cells were incubated with drugs for longer times (2–18 hr), the secondary effects associated with loss of cell viability or cytostasis complicate the interpretation.

The observed correlation between the condensation of nucleic acids and the antitumor potency of the investigated drugs is not, of course, an indication of a cause–effect relationship. Such a relationship, however, should be considered and in the following portion of the *Discussion* we analyze the evidence in favor and discuss alternative explanations.

Table 3 presents a summary of findings in which DHAQ and HAQ are compared with respect to their pharmacological activities and interactions with cellular constituents. As is evident, the only parameter that correlates with their different antitumor properties is DNA damage and, as presently observed, the ability to condense nucleic acids. The correlation between DNA damage and antitumor potency of DHAQ vs. HAQ is striking. Little is known about whether or not the DNA damage is a direct consequence of drug binding, is a result of its reaction with DNA via free radicals (15, 16, 30), or is secondary to DNA condensation induced by these drugs. In the latter case, the primary lesions (condensation) may undergo repair that could involve the transient formation of strand breaks. It would be of obvious interest to study

*The recent report by Lown *et al.* (25) on the contrary cannot be directly compared with our results because in their studies (*i*) the binding was studied at high ionic strength (0.5 M NaCl), (*ii*) uncertainty of the binding site size estimate for HAQ (25) makes its association constant value also uncertain, and (*iii*) the reverse titration method was used (drug titrated with DNA) in which, from the onset of the titration, a high drug/DNA ratio exists, which may cause immediate condensation of the most sensitive sections of DNA (12).

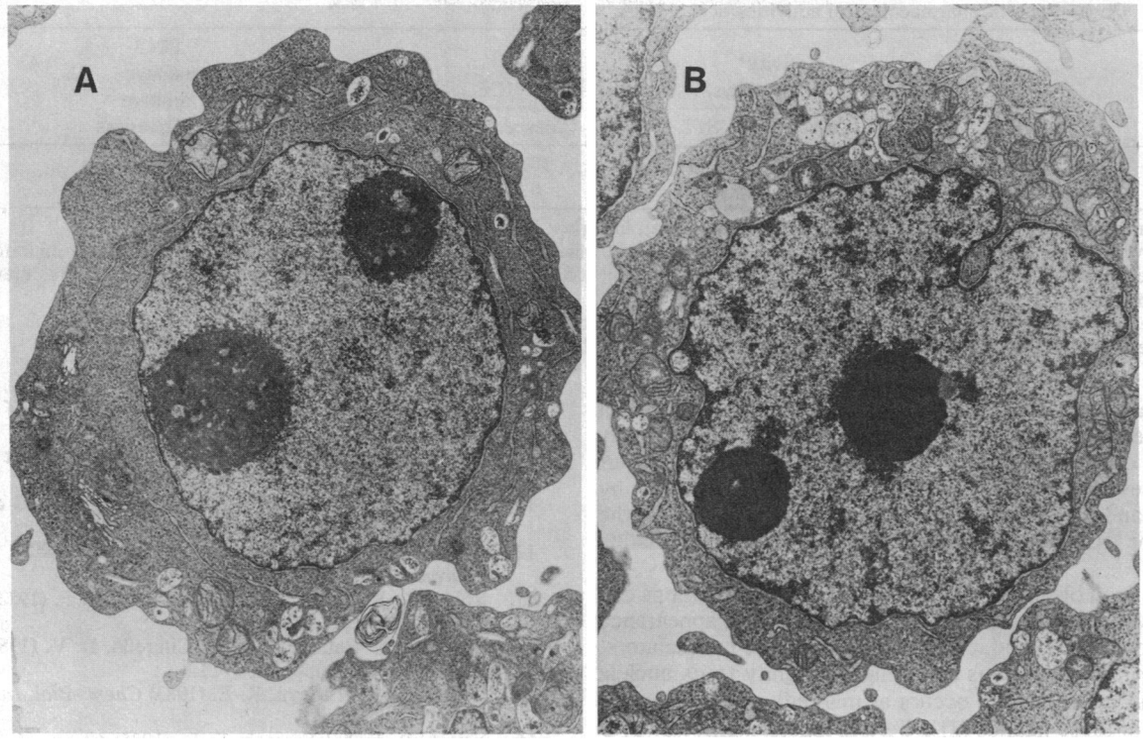

FIG. 3. Electron microscopy. Viable cells suspended in Hanks' balanced salt solution (HBSS) were exposed to DHAQ at concentrations of 0.02, 0.21, 2.1, and 21 μM (0.01–10 μg/ml). This treatment did not affect the immediate cell viability, which was tested by the ability of cells to exclude trypan blue at the end of the incubation period in parallel cell suspensions. Following the incubation with the drug, the cells were centrifuged and rinsed once with HBSS; cell pellets were fixed with 2.5% glutaraldehyde in Pipes buffer (pH 7.2) for 2 hr at 4°C and postfixed with 2% osmium tetraoxide in Pipes buffer (pH 7.2) for 1 hr at 4°C. The sample was dehydrated with ethanol and propylene oxide and embedded in freshly prepared Epon 812, which was polymerized by heating at 37°C overnight, 60°C for 2 days, and 90°C for 5 hr. Thin sections were cut on a MT-2 Porter-Blum ultramicrotome and stained with uranyl acetate followed by lead nitrate. The sections were examined and photographed in a JOEL 10B electron microscope operated at 60 kV. Other details are presented elsewhere (22, 23). (*A*) Electron micrograph of L1210 cell from the control, untreated culture. (×7550.) Note two nucleoli, each containing three different components: (*i*) pale-staining areas (DNA from nucleolar organizer region of chromosome); (*ii*) a dense fibrillar component around the pale regions (immediate RNA transcripts); (*iii*) a granular component, quantitatively the most prominent (ribosomal precursor particles). The nucleoplasm is uniformly dispersed. (*B*) Electron micrograph of typical L1210 cell exposed in culture to 0.21 μM (0.1 μg/ml) DHAQ for 90 min. (×7550.) The following changes characterized the drug-treated cells: (*i*) segregation of fibrillar and granular components of nucleoli; (*ii*) uniform, increased electron density appearance of the segregated granular component; (*iii*) decreased nucleolar size; (*iv*) increased contrast and smoothness of the nucleolar–nucleoplasmic borderline; (*v*) appearance of patchy regions of condensed chromatin, often in association with nucleoli.

whether indeed DNA condensation is followed by repair and what type of the repair mechanism may be involved.

The data presented in this paper suggest that the condensation of nucleic acids may be one of the mechanisms, if not the major one, by which the pharmacological activity of DHAQ and HAQ is manifested. Considering the higher sensitivity of ribopolymers rather than deoxyribopolymers, preferential localization of the drug in nucleoli (10), and the evidence of segregation of nucleoli with an increase in electron density of the granular portion, it is likely that nucleolar RNA may be one of the primary targets of the drug. Its condensation may be associated with cell progression through the cell cycle (cytostatic effect), which, in turn, affects cell viability. Condensation of RNA is expected to interfere with its turnover, thus leading to RNA content increase, and also be reflected by a decrease in protein synthesis rate. Indeed, the data of Traganos *et al.* (7) indicate an increase in RNA content, and observations of Safa & Tseng (28) indicate a suppression of protein synthesis by DHAQ.

Table 2. Affinity of the drugs to ds DNA (intercalative mode of binding) in 0.15 M NaCl/5 mM Hepes, pH 7.0, at 25°C

DNA	Binding site size, base pairs		Intrinsic association constant × 10^{-5}, M^{-1}	
	DHAQ	HAQ	DHAQ	HAQ
Calf thymus DNA	2.6	2.6	2.5	4.5
Poly(dA)·poly(dT)	2.9*	2.0	1.7*	2.6
Poly(dA-dT)·poly(dA-dT)	2.4	2.3	2.7	5.2
Poly(dG)·poly(dC)	7.6*	3.0	1.5*	3.4
Poly(dG-dC)·poly(dG-dC)	2.0	2.0	2.9	3.4

Binding site size and intrinsic association constant were calculated for the noncooperative mode of the polymer–ligand interaction according to McGhee–von Hippel (24), data from ref. 12.

*These data may be affected by the secondary changes in the DHAQ–DNA complexes and, therefore, should be treated with caution (12).

Early changes in nucleoli typical of the segregation induced by several intercalators, including DHAQ, were recently reported by Jensen *et al.* (31). The authors compared the cell killing potency of these drugs with their ability to induce the nucleolar lesions; no correlation was evident between these two activities. It is possible that different affinity of these intercalators to DNA vs. RNA or their base specificity is responsible for the presence or absence of the nucleolar effects. Thus, for instance, only the intercalators that preferentially condense either rRNA (or its precursor) or DNA sections coding for rRNA (nucleolar organizer regions) or otherwise interfere with rRNA synthesis may result in

Table 3. Comparison of pharmacological activities of the drugs

Drug	Optimal dose,* mg/kg	Activity* (P-388, 30 days)		ICF_{50},† μg/ml		DNA damage‡ relative effectiveness	Uptake§ of drugs by intact cells, μM
		% ILS	S/T	Quiescent	Cycling		
HAQ	25.0	>200	5/5	10.0	0.7	1	2
DHAQ	1.6	>186	10/11	0.2	0.006	250	5

*P-388, murine leukemia model; % ILS, % increase in median life span; S/T, survival-to-total ratio; data from ref. 8.
†Cell survival is expressed in terms of ICF_{50}, the concentration in which colony formation (Chinese hamster ovary cells) is inhibited by 50% compared to control cultures. Cycling cells were from the exponentially growing cultures; quiescent cells were from cultures maintained at confluence for 2 days; data from refs. 6 and 7.
‡Relative effectiveness for inducing strand scission in DNA; data from ref. 15.
§Measured colorimetrically; calculated based on the data from ref. 29.

nucleolar segregation. Other intercalators may have no effect on nucleoli at cytostatic or cytotoxic doses and yet may impede other vital cell functions—e.g., coded by DNA sequences that are the most sensitive to condensation by these ligands. These mechanisms can explain diversity of the effects or tissue (tumor) specificity of different intercalating agents.

It has been observed that polyvalent inorganic cations can condense nucleic acids resulting in the torus-like appearance of the condensed products (32). In contrast to intercalators, however, simple cations show higher affinity to ds nucleic acids and condensation occurs at markedly higher concentrations of these ligands (21). Certain mechanisms, however (e.g., cooperativity of the reaction and the polymer charge neutralization step), may be common in both phenomena.

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