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DNA-Binding Parameters of Daunorubicin and Doxorubicin in the Conditions Used for Studying the Interaction of Anthracycline-DNA Complexes with Cells in vitro

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Summary. *Affinity constants of daunorubicin and doxorubicin for DNA at 37° C and in presence of 10% serum were determined by an optical method and calculated from Scatchard plots. Values from 0.10 to 0.12 and from 0.13 to 0.16 × 10^6 M^{-1} were obtained for daunorubicin and doxorubicin, respectively. According to these affinity constants, the amounts of free drugs were calculated for various concentrations of daunorubicin-DNA or doxorubicin-DNA and for various molar ratios. In a large range of concentrations there is rather stable concentration of both free drugs, and these concentrations are inversely proportional to the nucleotides/drug ratio.*

The amount of free drug present in the medium is low as long as the concentration of daunorubicin-DNA or doxorubicin-DNA is higher than 1 μg/ml (expressed as drug concentration). At lower concentrations, however, the percentage of free drug increases very sharply.

Introduction

Daunorubicin (DNR) and doxorubicin (DOX) form noncovalent complexes with DNA and are used as such in experimental [10, 11] and clinical [3, 5] cancer chemotherapy. The exact mechanism of action of these complexes at the cellular level is still not clear. Several studies have been reported on the action of anthracycline-DNA complexes on cells in vitro [1, 8, 9]. Interpretation of these results is somewhat difficult because of the uncertainty regarding the amount of drug being released from the DNA complexes into the culture medium. In an attempt to promote the understanding of in vitro experiments, we have determined the affinity constants of DNR and DOX for DNA in conditions as close as possible to those used in experiments with cultured cells. According to these affinity constants, we have calculated the amounts of drugs which could become free in various conditions.

List of Abbreviations

DNR	daunorubicin
DOX	doxorubicin
D	drug
D_t	total concentration of D
D_f	concentration of free D
$(D\text{-}DNA)_b$	concentration of D-DNA complex
DNA_t	total concentration of DNA nucleotides
DNA_f	concentration of free DNA nucleotides
FCS	fetal calf serum
Ka	affinity constant
NBCS	newborn calf serum
PBS	phosphate buffered saline

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Materials and Methods

Daunorubicin (DNR) hydrochloride and doxorubicin (DOX) hydrochloride were supplied by Rhône-Poulenc, S.A., Paris, France. Phosphate buffered saline (PBS) at pH 7.4 has the following composition: NaCl 140 mM, KCl 3 mM, and KH_2PO_4 8 mM in de-ionized water. Fetal calf serum (FCS) and newborn calf serum (NBCS) were purchased from Gibco-Biocult, Paisley, Scotland. Herring sperm DNA (highly polymerized, type VII, Sigma Chemical Co., St. Louis, USA) is dissolved in sterile 0.15 M NaCl to a concentration of 2.34 mg/ml, autoclaved for 15 min at 120° C, and cooled slowly [11].

The binding parameters were determined by an optical method, measuring for various DNA nucleotides/drug ratios the absorbances of the drugs at 475 nm in a Gilford spectrophotometer thermostated at 37° C (Gilford, Inc., Ohio, USA). The concentraions of free and bound drugs were calculated from those values.

To 2.00 ml of drug solution (200 μg/ml in PBS), 0.05–2.00 ml of DNA (2.34 mg/ml) were added; the volume was completed to 4.00 ml by PBS and/or FCS or NBCS. Final concentration of calf serum was 10%.

Results and Discussion

The relation to be studied is:

$$D_f + DNA_f \rightleftarrows (D\text{-}DNA)_b . \quad (1)$$

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Let D_t, $(D\text{-}DNA)_b$, and D_f be the total, bound, and free drug concentrations, respectively. Let DNA_t be the total DNA concentration in moles of nucleotides per liter (mean nucleotide molecular weight used: 327); n_x, the number of sites occupied at the DNA concentration P_x; n_{max}, the number of strong binding sites per nucleotide; and Ka, the affinity constant. Then the binding parameters are given by the Scatchard equation:

$$n_x = n_{max} - \frac{1}{Ka}\frac{n_x}{D_f}. \quad (2)$$

The spectral titration used to determine the binding parameters is based on the fact that DNA induces progressive hypochromic shifts in the visible absorption spectra of both DNR and DOX as the molar ratio of nucleotides to drug molecules increases. In addition, the maximum absorption shifts to greater wavelengths upon binding to DNA, and approach a constant value as the ratio of DNA nucleotides to drug becomes greater than 10 : 1. For increasing concentrations in DNA (P_x), the absorbance of the drug at 475 nm is read (A_x). The extinction coefficient of free drug (ε_f), at 475 nm is measured directly, and the extinction coefficient, at the same wavelength, of drug bound to DNA (ε_b) is calculated using the relationship [2]:

$$\frac{1}{(\varepsilon_x - \varepsilon_f)} = \frac{1}{P_x}\,\frac{1}{(\varepsilon_b - \varepsilon_f)\cdot n_{max}\cdot Ka} + \frac{1}{(\varepsilon_b - \varepsilon_f)} \quad (3)$$

where ε_x is the apparent extinction coefficient of the drug at the DNA concentration (P_x). A plot of $1/(\varepsilon_x - \varepsilon_f)$ versus $1/P_x$ yields, at high molar ratios of DNA to bound drug, a straight line with an intercept, $1/(\varepsilon_b - \varepsilon_f)$, from which ε_b is calculated. $(D\text{-}DNA)_b$ and D_f are calculated using the following equations:

$$(D\text{–}DNA)_b = \frac{(\varepsilon_f \cdot D_t) - A_x}{(\varepsilon_f - \varepsilon_b)} \quad (4)$$

and

$$D_f = D_t - (D\text{–}DNA)_b\,. \quad (5)$$

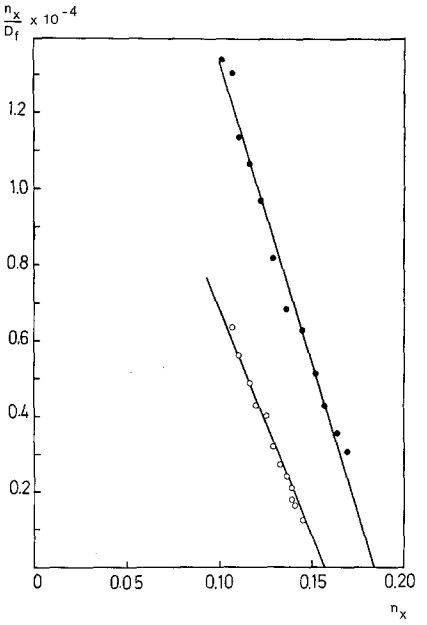

Fig. 1. Scatchard plot of the binding of DNR or DOX and DNA in 10% FCS at 37° C. The ratio of bound drug per DNA nucleotide divided by the free drug concentration (n_x/D_f) is plotted as a function of n_x. The total drug concentration (D_t) was 100 μg/ml in each case. The *solid line* is calculated by linear regression using equation (2). The correlation coefficient was greater than 0.993. ○: DNR; ●: DOX

As $n_x = (D\text{-}DNA)_b/P_x$, a plot of n_x versus n_x/D_f gives, according to equation (2), the binding parameters. Ka can be calculated from the data illustrated in Fig. 1.

The affinity constants (Table 1) we report here for DNR-DNA or DOX-DNA complexes determined at 37° C in presence of 10% calf serum are of the same order of magnitude as those previously reported for complexes using the same type of DNA [4, 12], but determined at 22° C in PBS. They confirm that the DOX-DNA complex is more stable than the DNR-DNA complex; the differences, however, are largely lower at 37° C in presence of serum than at 22° C in PBS.

Table 1. Affinity constants (Ka) of DNR-DNA or DOX-DNA complexes. They were determined at 37 °C and 22 °C in presence of 10% NBCS or FCS, as explained in the text. Ka in PBS were determined at 22 °C [12]

Temperature	Medium	DNR		DOX	
		Ka	n_{max}	Ka	n_{max}
22 °C	PBS	0.31×10^6	0.183	0.54×10^6	0.154
	10% NBCS	0.14×10^6	0.161	0.19×10^6	0.186
	10% FCS	0.18×10^6	0.179	0.23×10^6	0.163
37 °C	10% NBCS	0.10×10^6	0.166	0.13×10^6	0.180
	10% FCS	0.12×10^6	0.155	0.16×10^6	0.184

The value of n_{max} allows, following Huang and Phillips [6], the calculation of the number of base pairs (for each bound drug molecule) that are altered or sterically excluded in such a way that additional drug is unable to bind. Therefore we can calculate that the exclusion distance is approximately two base pairs for each DNR or DOX bound to DNA.

As the values of n_{max} are approximately the same at 22° and 37° C, it seems that there is no change in the conformation of the drug-DNA complex in the temperature range studied.

With a noncovalent complex, such as DNR-DNA or DOX-DNA, we must also take into account that a certain proportion of the drug will be present in its free or noncomplexed form. The following relations are indeed to be considered:

$$Ka = \frac{(D\text{–}DNA)_b}{D_f \cdot DNA_f} \tag{6}$$

$$(D\text{–}DNA)_b + D_f = D_t \tag{7}$$

$$(D\text{–}DNA)_b + DNA_f = DNA_t \tag{8}$$

where Ka is the affinity constant of the D-DNA complex; DNA_t and D_t, the total concentrations (free and complexed) of DNA and D; $(D\text{-}DNA)_b$ and DNA_f, the actual concentrations of DNA present in the complexed (bound) or free forms; $(D\text{-}DNA)_b$ and D_f, the concentrations of D bound or free. Rearranging these relations D_f can be deduced:

$$D_f = \frac{\sqrt{[Ka \cdot (DNA_t - D_t) + 1]^2 + 4\,Ka \cdot DNA_t} - [Ka \cdot (DNA_t - D_t) + 1]}{2 \cdot Ka}. \tag{9}$$

Using an affinity constant of 0.16×10^6 (DOX) and of 0.12×10^6 (DNR), the actual concentrations of D_f were estimated for various D_t ranging from 0.01 to 100 μg/ml, with a molar ratio of 20 nucleotides per drug molecule (DNA_t from 0.12 to 1170 μg/ml) corresponding to the ratio used in many in vitro or in vivo experiments. Results are presented in Fig. 2. They indicate that for D_t from 10 to 100 μg/ml, D_f represents less than 2.5% of D_t for both drugs; one must also notice that in all this concentration range for both complexes, D_f is the same (0.235 μg/ml for DNR and 0.185 μg/ml for DOX). Moreover, for D_t of 1 μg/ml, D_f represents 81% and 89%, respectively, of the level obtained if D_t is 100 μg/ml.

On the other hand, when D_t is further decreased, D_f is not proportionally lowered. If D_t is 0.1 μg/ml, D_f represents 70% (DNR) and 63% (DOX) of D_t; if D_t is 0.01 μg/ml, for both drugs, D_f represents more than 95% of D_t.

In a second step, we calculate D_f for D_t ranging from 0.01 to 100 μg/ml, but using various nucleotides/drug molar ratios. Results for DNR are presented in Fig. 3. With a high molar ratio (80 nucleotides/drug molecule), D_f is largely the same (0.037 if D_t is 0.1 μg/ml; 0.059 if D_t is 100 μg/ml). Difference increases when this ratio is lowered: for a molar ratio of 5, D_f is 0.090 μg/ml for D_t of 0.1 μg/ml; D_f is 1.14 μg/ml for D_t of 100 μg/ml.

From all these calculations it appears that with noncovalent complexes such as DNR-DNA or DOX-DNA, and with the affinity constants determined at 37° C in presence of 10% of serum (conditions used for most of the in vitro experiments with cultured cells), for both drugs D_f is not negligible, expecially at low D_t. The presence of the drug in its free form must be taken into

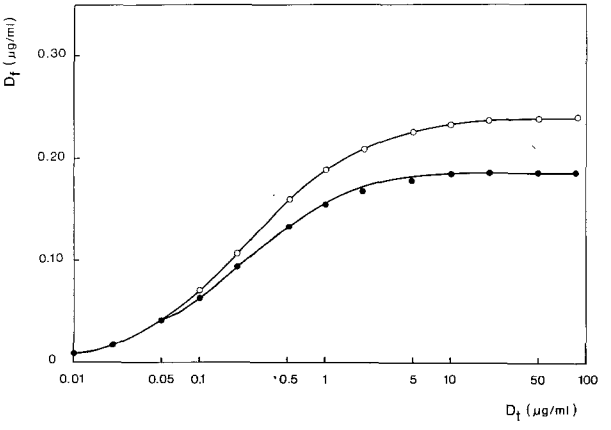

Fig. 2. Concentration of free DNR (○) and free DOX (●) as a function of the concentration of DNR-DNA or DOX-DNA. Calculations were performed according to equation (9), using Ka of 0.12×10^6 (DNR) or 0.16×10^6 (determined at 37° C in presence of 10% FCS) and with a molar ratio of 20 nucleotides/drug molecule

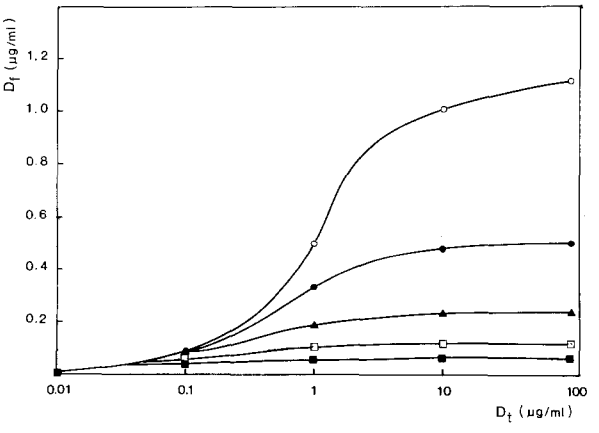

Fig. 3. Concentration of free DNR as a function of the concentration of DNR-DNA at various nucleotides/drug ratios. Calculations were performed according to equation (9), using Ka of 0.12×10^6 (37° C in presence of 10% FCS) and various molar ratios (○ : 5 nucleotides/drug molecule; ●: 10; ▲: 20; □: 40; ■: 80)

account for the interpretation of the experimental results mainly with cultured cells where a stable concentration of free drug will be maintained throughout the incubation period.

Finally, at a molar ratio of 20, for D_t ranging from 1 to 100 μg/ml, D_f is largely similar for both drugs (between 0.15 and 0.24 μg/ml). The difference in the therapeutic activity [12] of DNR-DNA and DOX-DNA cannot be entirely understood by the dissociation of the complexes, but could be explained by the differences in the cellular pharmacology of DNR and DOX [7, 8]. For D_t ranging from 0.1 to 100 μg/ml (where both DOX and DNR are active [12] and with a molar ratio (nucleotides/drug molecule equal or higher than 20) a rather stable D_f (0.04 to 0.024 μg/ml) will be reached and maintained for both drugs. Moreover, this level will not be dramatically affected if DNA concentration is decreased by deoxyribonucleases present in extracellular space.

Our calculations [equation (9)] indicate that using DNA as a carrier for antitumoral drugs (or by extension, any noncovalent complex), a low and stable concentration of free drug can only be maintained if Ka is higher than or equal to 10^6; an excess of the carrier (DNA) will reduce this concentration. In addition, determination of the Ka in the conditions prevailing in cell culture experiments is also important since temperature and presence of calf serum partially destabilize the DNR-DNA and DOX-DNA complexes.

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