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Comparative Study in Mice of the Toxicity, Pharmacology, and Therapeutic Activity of Daunorubicin-DNA and Doxorubicin-DNA Complexes

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Summary. *We have compared the toxicologic, pharmacologic, and therapeutic properties of the DNA complexes of daunorubicin and doxorubicin, after intravenous (IV) administration into mice. The overall toxicity of doxorubicin is significantly reduced after IV injection as a DNA complex while daunorubicin-DNA is as toxic as free daunorubicin. On hemopoietic stem cells, daunorubicin-DNA was found to be more cytotoxic than daunorubicin, while the opposite was observed with doxorubicin and doxorubicin-DNA. Both complexes are more effective than the corresponding free drugs on the L 1210 murine leukemia, when given IV at equitoxic doses. The tissue uptake in mice, after IV administration, is generally lower when the drugs are given bound to DNA. The stability of the two DNA complexes is very different in the bloodstream: daunorubicin-DNA behaves more like a prodrug of daunorubicin, while doxorubicin-DNA, remaining stable in the bloodstream, meets much more the requirements of an ideal drug-macromoleculare carrier entity.*

Introduction

When daunorubicin (DNR) and doxorubicin (DOX) are associated to high molecular weight DNA, they acquire distinctive properties with regard to the corresponding free drugs [9, 10]. When bound to DNA, DNR and DOX become more active against experimental L 1210 leukemia in mice while the toxicity of DOX only is decreased. In order to better understand the different toxicities of DNR-DNA and DOX-DNA in view of the drug-carrier concept, we have compared in mice the toxicologic, pharmacologic, and therapeutic properties of DNR and DOX both as free and DNA-bound drugs.

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Materials and Methods

Hydrochlorides of daunorubicin (DNR) and doxorubicin (DOX) were provided by Rhône-Poulenc, Paris, France. DNA is extracted from herring sperm (type VII, Sigma Chemicals, St. Louis, USA. The drug-DNA complexes were prepared as described previously [10], using a nucleotide/drug molar ratio of 20.

Female mice (average weight, 20 g) were injected in the tail veins. The following strains were used: NMRI, C_{57} black (Proefdierencentrum, KUL, Heverlee, Belgium), and DBA_2 (Charles River, St-Aubin-lès-Elbeuf, France).

The toxicity of the free or DNA-bound drugs was determined in DBA_2 mice in terms of LD_{50} (lethal dosis for 50% of the treated animals). The drugs were given IV on two consecutive days. After 30 days of observation, the LD_{50} values were calculated by linear regression (on probit scale) of the data obtained with at least five different dosages per drug form and an average of ten mice in each dose series.

The myelotoxicity studies were performed on C_{57} black mice. Five mice were given IV injections of each dosage. CFU-S (in vivo colony-forming units) were assayed, according to the method of Till and McCulloch [8]. CFU-C (granulocytic progenitor cells) were assayed in soft agar culture as described by Quesenberry et al. [7], and at a final cell concentration of 0.75 to 3.10^5 cells $\times$ ml^{-1}. Linear regressions were fitted according to the method of least squares, $D_{1/2}$ were calculated from the slopes. The values were compared by the Fisher's two-tailed F test for analysis of covariance.

Plasma levels of drugs and high molecular weight DNA were followed in DBA_2 mice after IV injection of the free or DNA-bound drugs at 7 mg/kg. After various times, the blood was collected, during sacrifice, from the femoral vein, on EDTA as anticoagulant and centrifuged at 4° C for 10 min at 2300 rpm (Damon/IEC International, Needham Hts., Massachusetts, USA, model PR 6000, rotor 259). The drugs were detected in the plasma by high-pressure liquid chromatography (HPLC) and fluorometry according to the method described by Baurain et al. [1, 2]. For the complexes, ^{125}I-labeled DNA was used; the labeling was done according to the method of Commerford [3], modified by Orosz and Wetmur [6]. The total radioactivity was measured in a Packard model 5130 autogamma scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Illinois, USA). The amount of TCA-soluble ^{125}I-DNA was determined by adding 0.01 ml of bovine serum albumin (Poviet producten, N.V., Amsterdam, Holland) at 10%, and 0.2 ml of ice-cold TCA at 40% to 0.1 ml of plasma. After at least 1 h, the tubes were centrifuged in an 'International' centrifuge (Damon/IEC International, Needham Hts., Massachusetts, USA, model PR 6000, rotor 259)

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at 4° C for 45 min at 2400 rpm and the radioactivity measured in the supernatant. The amount of TCA-insoluble DNA was calculated by difference. The labeled DNA used contained a maximum 1.4% of TCA-soluble fragments.

Determination of drug concentrations in tissues was performed in NMRI mice, 30 and 120 min after IV administration of free or DNA-bound drugs at 7 mg/kg. Several tissues, blood, bile, and urine were taken and immediately kept at 0° C. Tissue aliquots were homogenized using glass Potter-Elvehjem homogenizers (maximum volume: 7 ml), containing 1 ml of saline. After rinsing the homogenizer with 1 ml of saline, the pooled suspensions were sonicated for 30 s at 50 W (Branson Sonicator B-12, Branson Sonic Power Co., Danbury, Connecticut, USA). Blood was collected on EDTA as anticoagulant and sonicated in the same conditions. The drugs were analyzed by HPLC (see above) on 0.1 ml sample. The tissular and blood proteins were measured by the Lowry method [4]. The amount of blood contaminating each tissue sample was carefully measured using the immunologic method of Mancini [5] to estimate the concentration of serum albumin present in the tissue. Rabbit antiserum directed against mouse serum albumin was kindly supplied by J. P. Vaerman (Laboratoire de Médecine Expérimentale, ICP, Brussels, Belgium). The drug and protein values of each sample were corrected for the amount of contaminating blood. Experiments with perfused mice livers, using the same immunologic method, indicated that the amount of albumin present in the hepatocytes is negligible as compared with the albumin due to contaminating blood. The volume of bile, collected from the gall bladder, was estimated by weighing and assuming a specific density of 1.

The chemotherapeutic activity of free or DNA-bound drugs was evaluated using the experimental L 1210 leukemia. This leukemia was obtained in 1972 in its intraperitoneal (IP) form from Dr. C. Gosse and J. Morizet at Villejuif, France. These cells were then propagated in our laboratory by IP inoculation into DBA_2 mice. In these experiments, the leukemic cells, harvested from a 6 to 8 days-old ascitic form of L 1210 leukemia, were inoculated into DBA_2 mice on day 0, either intravenously (IV) or subcutaneously (SC). In both cases, the treatment was administered IV on days 1 and 2 following the inoculation of the cells. The drug dosages given for each drug form were chosen on an equitoxicity basis.

Results

The overall toxicity of DNR and DOX, free and as DNA complexes, was evaluated after two consecutive IV injections into DBA_2 mice, and the results, expressed as LD_{50} values, determined after 30 days of observation, are given in Table 1. DNR-DNA is as toxic as DNR in these conditions, while DOX-DNA shows a significantly reduced toxicity with regard to free DOX.

Table 1. LD_{50} of free and DNA-bound drugs in DBA_2 mice after administration on two consecutive days

LD_{50}[a] (mg/kg per day)		
Drug form	Daunorubicin	Doxorubicin
Free (F)	19.1	14.2
Bound to DNA (B)	20.0	23.4
Ratio B/F	1.05	1.65

[a] Obtained after 30 days of observation

The cytotoxic effects of free or DNA-bound DNR and DOX on the survival of pluripotent and committed hemopoietic stem cells have been studied, using the CFU-S and CFU-C assays. The fractions of CFU-S surviving per tibia as a function of the injected drug dose are shown in Fig. 1. For DOX- and DNR-treated mice, the dose survival curves of CFU-S were exponential with similar shapes. CFU-S in DNR-DNA-treated mice were more depressed than in mice receiving free DNR. In contrast, CFU-S are less sensitive to DOX-DNA than to DOX and moreover much less sensitive to DOX-DNA than to DNR-DNA.

We have estimated the $D_{1/2}$ values which are the doses of drug required to reduce the cell population by 0.5 [11]. For DNR, the $D_{1/2}$ values for the CFU-S population were found to be 3.4 mg × kg^{-1} and 2.5 mg × kg^{-1} after administration of free and DNA complex, respectively ($P < 0.05$). These values were 3.1 mg × kg^{-1} in mice treated with DOX and 3.6 mg × kg^{-1} in the DOX-DNA-treated group. The difference observed be-

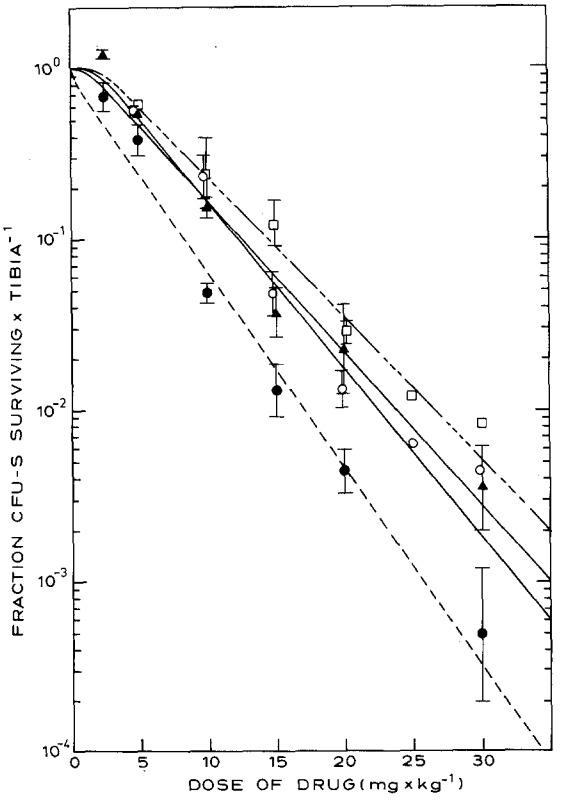

Fig. 1. Dose survival curves for pluripotent stem cells (CFU-S). Groups of five mice were given different doses of DNR (▲—▲), DNR-DNA (●- - -●), DOX (○—○), and DOX-DNA (□- - -□). Their tibia marrows were assayed 24 h later for their content in CFU-S. The values shown are normalized to the untreated controls. Each point represents the geometric mean ± SE of 2–3 separate experiments from a pool of five tibias per experiment

tween DNR-DNA and DOX-DNA was significant ($P < 0.01$).

The fractions of CFU-C surviving per tibia 24 h after the administration of different doses are presented in Fig. 2. Free DOX showed a greater effect than free DNR toward CFU-C; in the same way, DNR-DNA affected more the CFU-C population than DNR alone. The $D_{1/2}$ values of free DNR and DNR-DNA on the CFU-C populations were, respectively, 3.9 mg × kg^{-1} and 3.1 mg × kg^{-1} ($P < 0.01$). DOX and DOX-DNA gave equal values: 2.5 mg × kg^{-1}. The difference observed between DOX and DNR was significant at the level of $P < 0.01$.

Plasma levels of drug and high molecular weight ^{125}I-DNA have been measured after IV administration of free or DNA-bound DNR and DOX into DBA_2 mice. These results are shown in Fig. 3. Free DNR rapidly disappears from the plasma while DNR-DNA is characterized by a much slower decrease (Fig. 3A). The same observations can be made for DOX and DOX-DNA (Fig. 3B). DOX-DNA, however, permits significantly higher sustained plasma levels in comparison with DNR-DNA. Moreover, there is an important difference in stability between the two complexes: DNR, indeed, dissociates in a major part rapidly from high molecular weight DNA, while DOX disappears from the plasma more slowly and almost in parallel to the TCA-insoluble DNA.

Accumulation of free or DNA-bound DNR and DOX have been also followed in several tissues and bio-

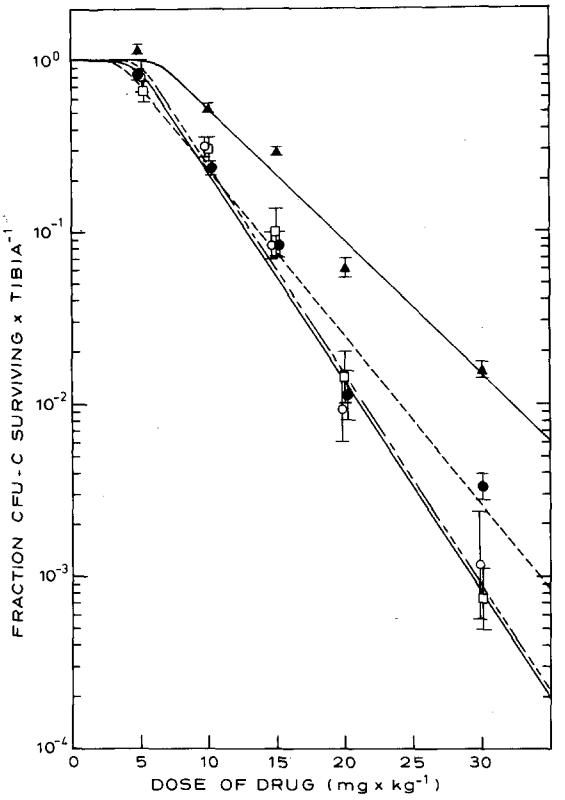

Fig. 2. Dose survival curves for committed myeloid stem cells (CFU-C). Groups of five mice were given different doses of DNR (▲—▲), DNR-DNA (●- - -●), DOX (○—○), and DOX-DNA (□- - -□). The tibia marrows were assayed 24 h later for their content in CFU-C. The values shown are normalized to the untreated controls. Each point represents the geometric mean ± SE of three separate experiments from a pool of five tibias per experiment

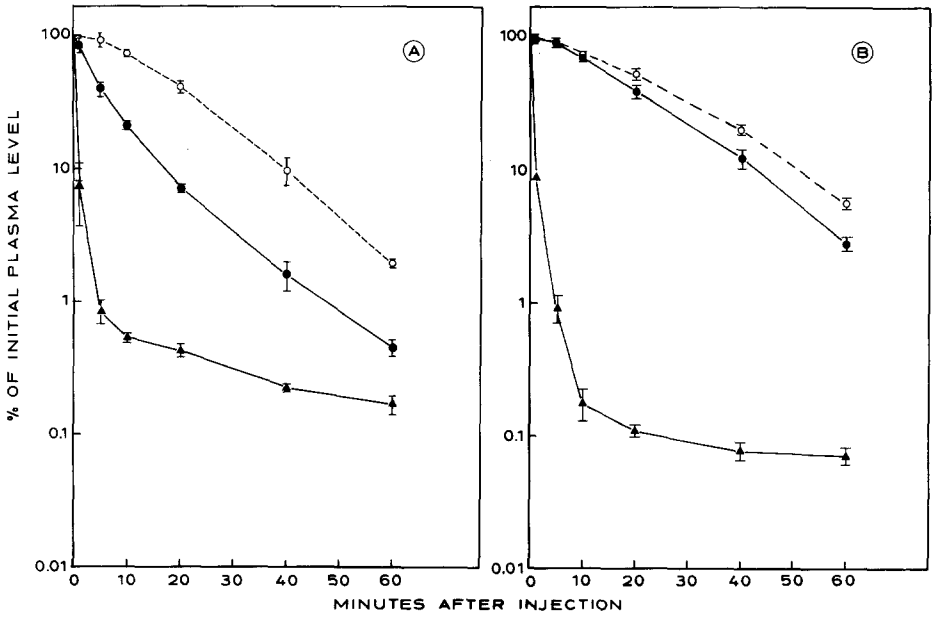

Fig. 3. Plasma levels of DNR, DOX, and DNA after IV administration of free and DNA-bound drugs into DBA_2 mice. (A) DNR and DNR-DNA were injected IV at 7 mg/kg. DNR (▲—▲) after DNR administration; DNR (●—●) and high molecular weight DNA (○- - -○) after DNR-DNA administration. (B) DOX and DOX-DNA were injected IV at 7 mg/kg. DOX (▲—▲) after DOX administration; DOX (●—●) and high molecular weight DNA (○- - -○) after DOX-DNA administration. The results are expressed in percentage of the initial plasma levels. Mean ± SD of three individual assays are given

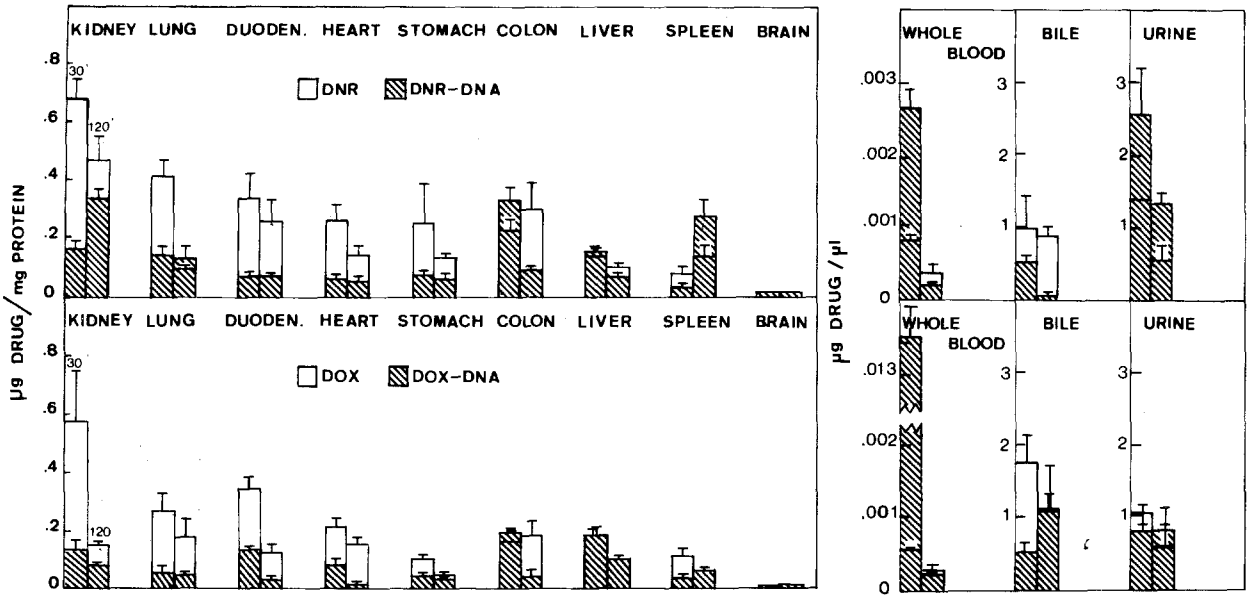

Fig. 4. Tissue distribution of free and DNA-bound drugs after IV administration into NMRI mice. The drugs were given IV at 7 mg/kg and the mice were sacrificed after 30 and 120 min. Total fluorescence (parent drug and fluorescent metabolites) was measured in the samples. Mean ± SE of at least three individual assays are given

logical fluids, 30 and 120 min after IV injection into NMRI mice, at 7 mg/kg. The total fluorescence, i.e., corresponding to the parent drug plus its fluorescent metabolites, recorded in the various tissues, in blood, bile, and urine is given in Fig. 4. DNR and DOX accumulate in the organs very similarly after 30 min. After 120 min, however, the levels of DOX in most of the tissues are lower than those obtained with DNR. When both drugs are associated with DNA, there is a general decrease in the tissue uptake as compared with free DNR or DOX. Significantly lower quantities of drugs and metabolites are found 30 min after administration of both complexes in kidney, lung, duodenum, heart, stomach, and spleen. In contrast, at the same time, the amounts measured in liver and colon are about equal for the free or DNA-bound drugs. Two hours after injection of the complexes, lower levels, with regard to the free drugs, are still observed in kidney, duodenum, heart, and also in the colon, in contrast with the data obtained after 30 min. After 120 min, the drug accumulation in the

Table 2. Chemotherapeutic activity on L 1210 leukemia of free and DNA-bound drugs administered on two consecutive days in DBA_2 mice

L 1210 cells		Drug			Increase in life-span (%)	Number of survivors on day 30/ Total number of mice	Average tumor size (mm^3)	
Number	Route of inoculation	Form	Route of administration	Doses mg/kg per day			On day 10	On day 15
10^4	IV	DNR	IV	10	42	0/17	–	–
				12	110	19/153	–	–
10^4	IV	DNR-DNA	IV	10	100	2/8	–	–
				12	260	31/65	–	–
10^4	IV	DOX	IV	6	58	0/8	–	–
10^4	IV	DOX-DNA	IV	9	344	10/16	–	–
10^5	SC	–	–	–	0	0/18	346	–
10^5	SC	DNR	IV	12	70	0/10	0.4	57.9
10^5	SC	DNR-DNA	IV	12	81	1/20	0	58.6
10^5	SC	DOX	IV	6	139	4/9	0	4.9
10^5	SC	DOX-DNA	IV	9	448	16/19	0	0.05

liver is similar after administration of free or complexed drugs. This is also observed in the spleen for DOX-DNA and DOX, whereas DNR-DNA is present after 2 h at higher levels in the spleen, as compared with free DNR. As expected, only trace amounts of drugs were recovered in brain.

Finally, in two different experimental models, we have compared the chemotherapeutic activity of free or DNA bound DNR and DOX on L 1210 leukemia in DBA_2 mice (Table 2). In a first series of experiments, leukemic cells and drugs were both administered IV. When comparing equitoxic doses for free and DNA-bound drugs, it appears that the complexes are much more active, not only in increasing the life-span of the treated animals, but also in allowing a greater number of long-term survivors. The best therapeutic effect, however, is obtained with DOX-DNA.

In a second model, the L 1210 cells were inoculated SC; the treatment was done IV, using the same schedule as above. In those experiments, DNR-DNA was as effective as DNR: we observed a similar increase in life-span, practically no long-term survivors, and the same evolution of the primary tumor. In contrast, the therapeutic results obtained with DOX were significantly better. Free DOX allowed a twice longer survival of the treated mice, with 44% of the mice still alive after 30 days and a slow development of the tumor. The DOX-DNA complex was strikingly more active than DOX: the life-span was increased more than three times, the percentage of long-term survivors reached 85%, and the average tumor size remained negligible even after 15 days.

Discussion

Confirming our results reported previously [9, 10], DOX-DNA is less toxic than DOX when given IV on two consecutive days, while in contrast, the toxicity of DNR is not decreased when administered as a DNA complex. The chemotherapeutic activity of both DNR and DOX against the IV form of L 1210 leukemia is significantly increased when the drugs are given as DNA complexes; this increase, however, is the most striking for DOX, which can be given at higher doses without increased toxicity and which is also more effective against the subcutaneously implanted form of L 1210 leukemia.

The differences observed between the toxic and chemotherapeutic activities of DNR-DNA and DOX-DNA are more easily understood in view of their pharmacokinetic properties. The DNR-DNA complex dissociates rapidly indeed after IV injection into mice since DNA and DNR disappear at a very different rate after injection. DOX-DNA is, in contrast, much more stable, and DOX is eliminated from the plasma in parallel to high molecular weight DNA. DNR-DNA behaves thus much more like a prodrug being released in the bloodstream without needing an activation step in the extracellular fluid or in the target cells. As a high molecular weight prodrug, the plasma levels of DNR will be increased, and this can by itself explain the better chemotherapeutic results obtained with DNR-DNA. On the other hand, the tissue distribution of DNR will be changed, since the drug will circulate a longer time through the bloodstream and its tissular uptake will be regulated by the tissular distribution of its high molecular weight carrier. Tis would be enough to modify the toxicity of DNR, but we have to take into account that the higher plasma levels could induce a higher toxicity for those cells which are in direct contact with the bloodstream. Such is the case for the hemopoietic stem cells, which are more sensitive to DNR-DNA than to DNR. The combination of altered tissue distribution and higher hemopoietic toxicity explains most probably the similar overall toxicity of DNR and DNR-DNA.

The DOX-DNA complex comes much more close to the ideal drug-carrier entity. The closely related plasma elimination of DOX and high molecular weight DNA suggests strongly that DOX leaves the bloodstream as a DNA complex. This means that its distribution will be more regulated than that of DNR-DNA by the accessibility of the different tissues to high molecular weight DNA, and also that DOX must be released from DNA, either in the interstitial space by the action of enzymes or by dilution, or by lysosomal enzymes after endocytosis in the target cells. This is very well illustrated by the lower toxicity of DOX-DNA for the hemopoietic stem cells; even in presence of much higher plasma levels of DOX as a DNA complex, these cells are less sensitive, most probably because the rate at which they can activate DOX-DNA lies beneath the rate at which they would accumulate the free drug.

The higher chemotherapeutic activity of DOX-DNA on L 1210 leukemia indicates that these cells, inoculated IV or SC, can activate DOX-DNA in a very significant manner. The exact mechanism of this activation is still unclear but could occur by endocytosis of the complex and its intralysosomal digestion.

For both DNR and DOX-DNA complexes, we should stress that the heart concentration of DNR and DOX is significantly reduced. If, as one could expect, the cardiotoxicity of anthracyclines is related to their uptake by heart tissue, we provide here strong experimental evidence in favor of the lower cardiotoxicity of anthracycline-DNA complexes as already suggested and reported elsewhere.

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