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Accumulation, Metabolism and Subcellular Localization of Daunorubicin, Doxorubicin and their DNA-Complexes in Rat Heart Ventricles

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Abstract—*The accumulation in rat heart ventricles, the presence of metabolites and the subcellular localization of daunorubicin (DNR), doxorubicin (DOX) and their DNA complexes were investigated. After intravenous perfusion of free DNR or DOX, equal amounts of the drugs are recovered in the heart, whereas after injection of their DNA complexes, the accumulation levels represent 42% (DNR) and 32% (DOX) of those reached with the free drugs. Daunorubicinol and daunomycinone are detected after administration of DNR or DNR–DNA but only trace amounts of metabolites are found with DOX and DOX–DNA. Subcellular distributions of DNR injected as free or DNA complex are similar. Isopycnic and differential centrifugations indicate that DNR could be associated with nuclear DNA and to a smaller extent with lysosomes. By contrast, after injection of free or complexed DOX the drug is almost exclusively associated with the nuclear DNA. The mechanisms by which these drugs are accumulated by the heart and the relation with their cardiotoxicities are discussed.*

INTRODUCTION

DAUNORUBICIN (DNR) and doxorubicin (DOX) are two anthracycline antibiotics with potent antitumor activity [1–4], which has been related to an interaction with DNA [5, 6].

The clinical use of these drugs is, however, seriously restricted by their cumulative cardiotoxicity [2–8]. Morphological studies performed after long treatments point out the vacuolization of the myocardial cells and mitochondrial lesions [9]. It was proposed that these alterations could result from interaction of the drugs with mitochondrial phospholipids [10] or from inhibition of coenzyme Q10 [11, 12]. Other mechanisms such as peroxidation of cardiac lipids [13], inhibition of DNA [14] or myosin [15] synthesis have also been suggested.

In an attempt to decrease the toxicity of the anthracyclines, Trouet *et al.* [16] have used these drugs complexed to DNA assuming that the uptake of the complexes could be restricted to cells having a high endocytic rate. Experimental [17] and clinical data [18, 19] indicate that the use of DNA complexes reduces the cardiotoxicity without affecting the antitumoral activity. Moreover, recent data from this laboratory [17] indicate that the accumulation of DNR or DOX by the heart is reduced three times when the drugs are injected intravenously to mice as DNA complexes.

In this paper we described the accumulation and the presence of metabolites (detected by the use of high-pressure liquid chromatography) in hearts of rats injected with DNR or DOX free, or complexed to DNA. The subcellular localization of the accumulated drugs was also investigated by differential and isopycnic centrifugation. In view of this we have developed an original fractionation method for rat heart muscle.

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MATERIALS AND METHODS

Materials

Daunorubicin hydrochloride (DNR) was a gift from Rhône-Poulenc (Paris, France). Doxorubicin hydrochloride (DOX) was provided by Farmitalia-Benelux (Bruxelles, Belgium). DNR and DOX were dissolved in 0.15 M NaCl to a concentration of 0.4 mg/ml. The DNR–DNA and DOX–DNA complexes were prepared according to Trouet *et al.* [19] using a nucleotide/drug molar ratio of 20. All chemicals of analytical grade were purchased from E. Merck A. G. (Darmstadt, F.R.G.), Sigma Chemicals Co. (St. Louis, MO, U.S.A.), Koch-Light (Colnbrook, U.K.) or Calbiochem (San Diego, CA, U.S.A.). Male rats of the Wistar strain were bred in a local animal house. They weighed 150–250 g at sacrifice.

Isolation of the heart

After sacrifice by decapitation, the heart was rapidly excised and freed of contaminating blood by perfusion of ice-cold phosphate-buffered saline pH 7.4 through the aorta for about 2 min. The heart was then placed in ice-cold 0.25 M sucrose supplemented at pH 7.4 with 1 mM EDTA or 3 mM imidazole. The auricles, great blood vessels and connective tissue were removed. The ventricles were blotted on a paper filter and weighed after opening and washing with ice-cold sucrose. They were finally minced with scissors. Heart pieces were dispersed (10%:w/v) in 0.25 M sucrose buffered at pH 7.4 with 3 mM imidazole and 20 mM KCl and homogenized in 5 steps. In the two first steps, heart pieces were disrupted in a Vir-Tis blender (Research Equipment Gardiner, NY, U.S.A.). The rotation speed was increased progressively, the medium position of the rheostat being reached in 10 sec, and immediately afterwards decelerated reaching the off position after 10 sec. For the last three steps, the suspension was homogenized in a Dounce homogenizer (Kontes Glass Co., Vineland, NJ, U.S.A.) using the tight pestle. After each step the suspension was centrifuged at 50 ***g*** for 10 min; the pellets were resuspended in the homogenization medium and then further homogenized. All centrifugations were made in a I.E.C. centrifuge (Damon Corp., Needham Heights, MA, U.S.A., Model CRU 5000 head No. 253) with meniscus and bottom of the column fluid respectively at 13.5 and 21.5 cm from the axis. The final pellet contained most of the myofibrils and some organelles: it is indicated as the HP fraction; all supernatants were pooled and formed the HS fraction. The homogenization procedure we adopted allowed recovery of between 60 and 70% of protein, *N*-acetyl-*β*-glucosaminidase, cytochrome *c* oxidase and alkaline phosphodiesterase in the HS fraction; in addition about 50% of the *N*-acetyl-*β*-glucosaminidase activity remained latent.

Fractionation experiments

Cell fractionation was performed on a HS fraction by differential centrifugation according to de Duve *et al.* [20] and by isopycnic centrifugation according to Beaufay and Amar-Costesec [21]. All sucrose solutions used to form the density gradient contained KCl and imidazole at the concentrations used in the homogenization medium.

Enzyme assays and protein

Enzymes were assayed according to published methods. The activities of cytochrome oxidase [22] and catalase [23] were measured in the presence of 0.015% digitonin or 0.22% Triton X-100, respectively, in order to suppress their latency. Activities are expressed in milli-units (nanomoles of substrate degraded per minute); cytochrome oxidase and catalase are expressed in units as defined respectively by Cooperstein and Lazarow [22] and by Baudhuin *et al.* [23]. The deoxyribonucleic acid was measured according to Burton [24]. Protein content was determined by the method of Lowry *et al.* [25].

As previously described [26, 27], *N*-acetyl-*β*-glucosaminidase [28] and cathepsin B [29] can be detected in rat heart homogenates. In fresh homogenate prepared in isotonic medium, these two enzymes show partial latency which can be suppressed by detergent. After isopycnic centrifugation in sucrose gradients (Fig. 1), both enzymes display a bimodal distribution. Part of the activity is detected at the top of the gradient, part equilibrates at higher densities (median: 1.20 g/cm^3). After differential centrifugation (Fig. 2) both enzymes display their highest relative specific activity in L fraction, whereas a large proportion is also recovered in fractions M and S. These properties, analogous with those described for lysosomal enzymes of other tissues or cells [20, 30], allow us to suggest their association to lysosomes. The activity detected at the top of the gradient or in S fraction could be attributed to soluble enzyme escaping from damaged granules, while the other

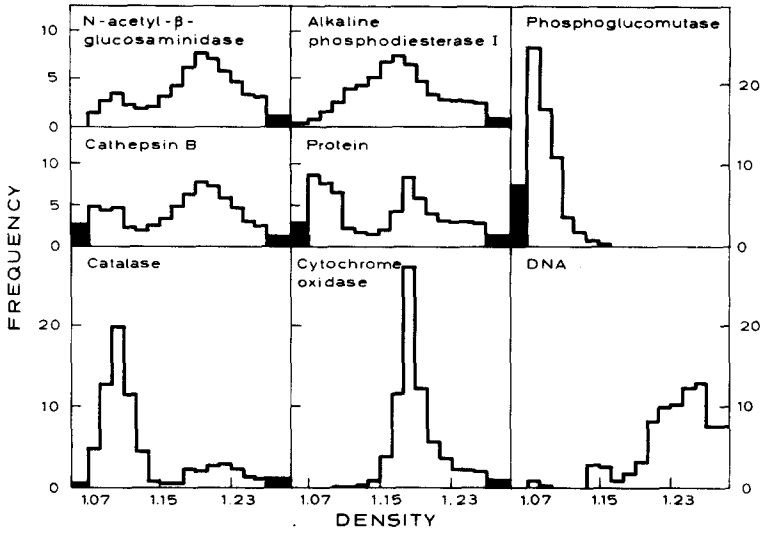

Fig. 1. Distribution patterns of enzymes, protein and DNA after isopycnic centrifugation. Results are presented in the form of normalized histograms [51]. *The HS fraction contained* 67% *of N-acetyl-β-glucosaminidase,* 68% *of cathepsin B,* 74% *of catalase,* 65% *of cytochrome oxidase,* 65% *of DNA and* 99% *of phosphoglucomutase. (Free activity of* N*-acetyl-β-glucosaminidase was* 50%.)

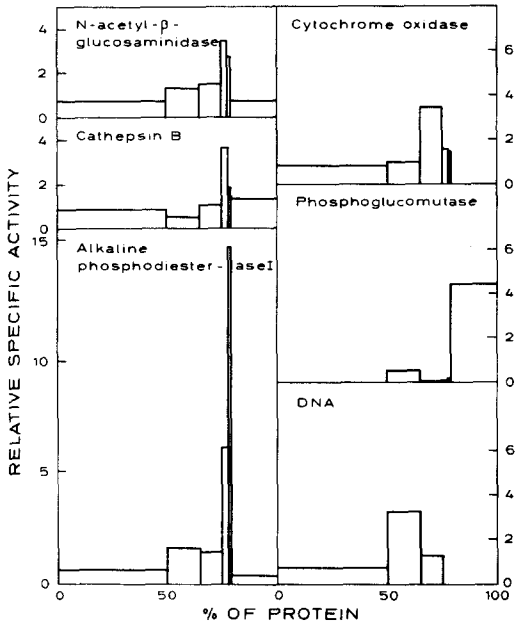

Fig. 2. Distribution patterns of enzymes after fractionation by differential centrifugation. The homogenate was fractionated in six fractions: HP, N, M, L, P and S. These are represented by blocks ordered according to the same sequence on the abscissa where they span a length proportional to their protein content. The ordinate (heights of the blocks) give the relative specific activity (or amount of drug) which is the percentage of activity recovered in each fraction over the percentage of protein of the same fraction. Percentages relate to the sum of activities found in all the fractions.

part of the distribution could be related to the presence of lysosomal granules.

After isopycnic centrifugation alkaline phosphodiesterase I [31] displays a broad somewhat variable distribution (mean median $1.1624\,g/cm^3 \pm 0.0047$) (Fig. 1). After differential centrifugation (Fig. 2.), its highest relative specific activity is in fraction P but large proportions of the activity are also recovered in fractions N, M and L. Our results are similar to those obtained in other cell types [32, 33] and by comparison we can propose this enzyme as a marker of plasma membrane.

The activity of cytochrome *c* oxidase, marker enzyme for mitochondria [34], is recovered by differential centrifugation mainly in the M fraction (Fig. 2). After isopycnic centrifugation enzyme distributes with a sharp peak around a density of $1.18\,g/cm^3$ (Fig. 1). By contrast to most of the other enzymes, cytochrome *c* oxidase appears to be 4.3 times more active in rat heart than in rat liver.

After differential centrifugation, phosphoglucomutase [35] (Fig. 2) is mostly recovered in fraction S and after isopycnic centrifugation (Fig. 1), the enzyme remains at the top of the gradient. This enzyme distribution is similar to that observed with other cells (rat fibroblasts [36], hepatoma cells [37]). As proposed

for these cells, we can also consider phosphoglucomutase as a marker of the cytosol.

As previously described [38] catalase activity was detected in rat heart homogenates. After isopycnic centrifugation (Fig. 1) most of the activity was found in the soluble fraction and a very small part equilibrates at a density of about 1.23 g/cm^3. The soluble activity could result from the destruction of heart peroxisomes during homogenization [38].

After isopycnic centrifugation (Fig. 1), most DNA is detected at high densities around 1.25 g/cm^3. DNA present in the HS fraction is recovered after differential centrifugation mainly in the N and partially in the M fraction (Fig. 2). The presence of the DNA in fractions N and M suggests that the nuclei remains intact during cell fractionation.

Administration of drugs

Under anaesthesia by diethyl-ether, a polyethylene catheter was inserted into the jugular vein of the rat. This catheter was passed under the skin and emerged dorsally from the neck. Drugs (at an anthracycline dosage of 20 mg/kg of rat body weight) were then perfused at a rate of 0.5 ml/min. After 30 min the rat was sacrificed by decapitation.

Drug assays

Drugs were assayed by fluorimetry [39] or by high-pressure liquid chromatography (h.p.l.c.) using a fluorescence detector [40, 41].

RESULTS

Accumulation of the drugs

As shown in Table 1, 0.41 μg of fluorescent drug equivalent is accumulated per mg of heart protein, after perfusion of free DNR or free DOX. After perfusion of DNR–DNA and DOX–DNA, 0.17 μg (DNR) and 0.13 μg (DOX) per mg of protein are found associated with the heart. These values represent 42% (DNR) and 32% (DOX) of the accumulation levels reached with the free drugs.

Metabolism of accumulated drugs

After perfusion of free DNR the parent drug is the main fluorescent material found in the heart (76%) while daunorubicinol and daunomycinone account respectively for 17 and 7%. When DNR–DNA is perfused the parent drug represents 79% of the total fluorescence, daunorubicinol and daunomycinone amounting respectively to 8 and 13%.

When DOX or DOX–DNA are perfused almost all the fluorescent material is identified as DOX.

Subcellular distribution of accumulated drugs

Isopycnic centrifugation. After perfusion of DNR the distribution of the accumulated fluorescent material is rather broad (Fig. 3); important amounts equilibrate at high densities where DNA is also present. Large proportions are, however, also recovered at the top of the gradient and at low densities where *N*-acetyl-β-glucosaminidase, cytochrome oxidase and alkaline phosphodiesterase I are detected. Distribution of DNR as assayed by h.p.l.c. is largely similar to that of the total fluorescent material. Daunorubicinol distributes rather uniformly through all the gradient. Daunomycinone is recovered only in the heaviest fractions of the gradient (not shown) and could represent insoluble or aggregated material. After administration of

Table 1. Accumulation of DNR, DOX and their metabolites in rat heart ventricles (mean of two independent experiments)

Perfused drug (20 mg/kg of body weight)	Accumulation: μg of drug equivalent per mg heart protein	Percentage of the fluorescence* accounted by		
		DNR	Daunorubicinol	Daunomycinone
DNR	0.41	76.1	16.8	7.0
DNR–DNA	0.17	79.2	7.9	12.9
		DOX	Doxorubicinol	Doxorubicinone
DOX	0.41	99.7	0.3	0.0
DOX–DNA	0.13	100.0	0.0	0.0

*Determined by h.p.l.c.

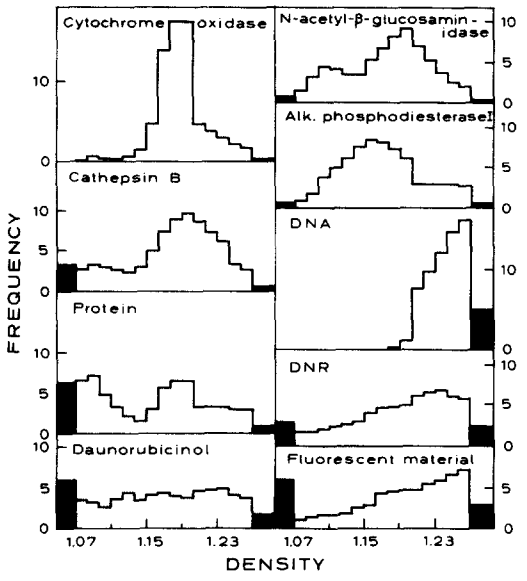

Fig. 3. Isopycnic centrifugation of a HS fraction prepared from the heart of a rat perfused with 20 mg/kg *of DNR* 30 min *before sacrifice. Results are presented as in Fig.* 1.

DOX (Fig. 4), most of the accumulated fluorescent material equilibrates at the same densities as DNA. Only a small proportion is recovered at lower densities.

Similar results to those described above have been obtained after perfusion of DNR–DNA and DOX–DNA.

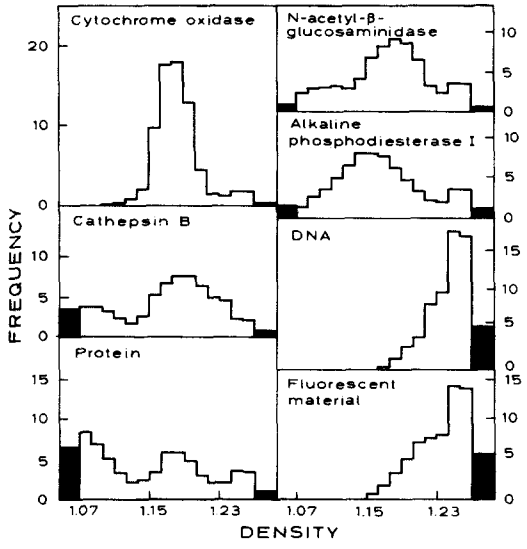

Fig. 4. Isopycnic centrifugation of a HS fraction prepared from the heart of a rat perfused with 20 mg/kg *of DOX* 30 min *before sacrifice. Results are presented as in Fig.* 1.

Differential centrifugation. As shown in Fig. 5, after perfusion of DNR, fluorescent material sediments partially in the HP fraction, where rather similar proportions of all the enzymes and constituents are present. The distribution of DNR is largely similar to that of DNA, but with high relative specific activities in L and P fractions.

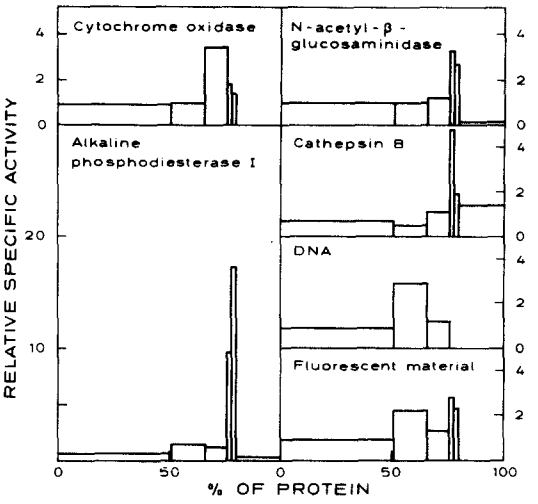

Fig. 5. Fractionation by differential centrifugation of rat heart ventricle. The animal was perfused with 20 mg/kg *of DNR* 30 min *before sacrifice. Results are presented as in Fig.* 2.

After administration of DOX, the distribution of fluorescent material, shown in Fig. 6, is largely similar to that of DNA and low relative specific activities are found in L and P fractions.

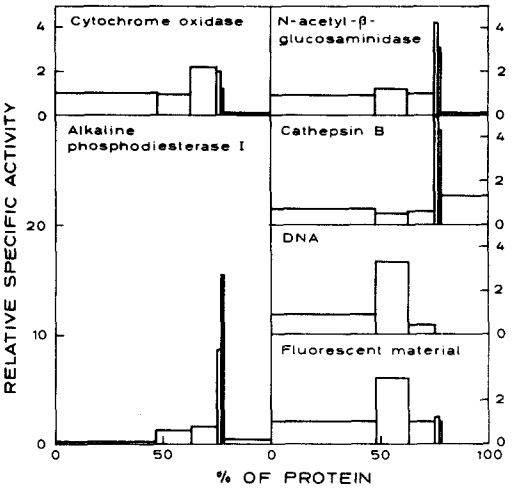

Fig. 6. Fractionation by differential centrifugation of rat heart ventricle. The animal was perfused with 20 mg/kg *of DOX* 30 min *before sacrifice. Results are presented as in Fig.* 2.

DISCUSSION

After perfusion of 20 mg of anthracyclines per kg of body weight, free DNR and DOX are accumulated in rat heart ventricles to the same extent (0.41 μg of fluorescent material/mg of heart protein). These levels are much lower when the drugs are perfused

complexed to DNA (0.17 μg/mg heart protein for DNR and 0.13 μg/mg for DOX).

The drug metabolites were investigated in the heart by high-pressure liquid chromatography and the results indicate that after perfusion with DNR or DNR–DNA, 20–24% of the accumulated fluorescent material consist of metabolites (daunorubicinol or daunomycinone). After perfusion with DOX or DOX–DNA only trace amounts of fluorescent metabolites (doxorubicinol) can be detected. This latter observation agrees with the results of Yesair *et al.* [42] who did not detect metabolites of doxorubicin in rats.

The subcellular distribution of accumulated material was investigated by analytical fractionation techniques. After isopycnic centrifugation, similar results have been obtained if rats were perfused with free DNR or DNR–DNA. The distribution pattern of the fluorescent material is broad but high proportions are detected at the bottom of the gradient (Fig. 3), which suggests that a significant part of the drug could be associated with DNA which is found in the heavy fractions of the gradient, while another part of it can be associated tentatively either with *N*-acetyl-β-glucosaminidase or cytochrome oxidase equilibrating at lower densities. The association of a large proportion of the fluorescent material with DNA is confirmed by the results obtained after differential centrifugation (Fig. 5). The high relative specific activity of the fluorescent material in fractions L and P, rich in lysosomal markers and devoid of DNA, suggests the association of part of the drug with the lysosomes rather than with mitochondria. After isopycnic centrifugation of HS fraction from hearts of rats injected with DOX or DOX–DNA a very close overlapping of equilibration profiles of the drug and DNA is observed (Fig. 4). It appears also that a much smaller proportion of the drug can be associated with *N*-acetyl-β-glucosaminidase or cytochrome oxidase. The results obtained after differential centrifugation (Fig. 6) do not allow us to assign more precisely this small part of fluorescent material either to lysosomes or to mitochondria.

These results indicate that DNR and DOX accumulate in the heart by permeation and become associated to a greater extent with the nuclei probably by binding to the nuclear DNA. In addition, however, part of the accumulated DNR can be tentatively attributed to lysosomes and a much smaller part of DOX either to lysosomes or mitochondria. This is in good agreement with previous data [39, 43] which indicate that in cultured fibroblasts, on one hand, free DNR and DOX saturate first the nuclei and are then accumulated inside lysosomes, and on the other hand, that DNR is accumulated to a greater extent than DOX. The similar accumulation levels of DNR and DOX in the heart could be explained if the lysosomal compartment is much smaller for the cardiac cells than for fibroblasts.

When rats are perfused with DNR–DNA or DOX–DNA, the accumulation levels of the drugs are lower, and DOX is accumulated to a lesser degree than DNR.

In order to explain these two differences two mechanisms can be suggested. As proposed by Trouet *et al.* [16, 17, 19] DNA complexes could be taken up by cardiac cells through endocytosis; DNA be digested in lysosomes and the drug released and trapped by nuclei. The low levels found in the heart after administration of complexes could, in this way, result from the low endocytic rate of cardiac cells.

An alternative mechanism is based on recent data obtained *in vivo* [17] or *in vitro* [44] indicating that DNR–DNA and, to a much lesser extent, DOX–DNA are partially dissociated in the blood stream or in serum. The uptake of the anthracyclines by the heart would in this case be due to the presence of the small amounts of free DNR and free DOX.

Although the results obtained after acute administration of the drugs cannot be directly related to their cardiotoxicity after long term treatment, we can try to correlate the subcellular localization of the drugs with their toxicity.

An association of the anthracyclines with mitochondria seems unlikely since for DOX there is very little analogy between the distributions of the drug and cytochrome *c* oxidase after isopycnic centrifugation. The presence of anthracyclines in the M fraction, on the other hand, could be related to the presence of DNA in this fraction, the amount of which cannot be accounted for by the mitochondrial DNA, By analogy to the cardiomyopathy induced by chloroquine [45, 46], a drug which in many cell types is concentrated in lysosomes [47] and induces vacuolization, the accumulation of DNR and DOX inside lysosomes could explain their toxicity. However, our results which suggest that DNR has a greater degree of accumulation in lysosomes than DOX are in contradiction with such an hypothesis.

We have to remeber that experimental [17] and clinical data [48] indicate that doxorubicin is more toxic than daunorubicin. The association of DNR and DOX to nuclei could explain the impairment of DNA and protein synthesis and the cardiotoxicity of both drugs. To attempt to reduce their cardiotoxicity without impairing their activity towards tumor cells, the rational approach is to prevent their uptake by heart muscle cells. In this paper we have shown that by complexing DNR and DOX to DNA we achieve such a reduction. Those results are in agreement with those obtained in mice [17]. On the other hand, we have shown recently that the DNR–DNA but mainly the DOX–DNA complexes are less cardiotoxic in the chronic test on rabbits [49] and also less cardiotoxic in clinical trials conducted on more than 700 patients [50].

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