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Oxygen-Dependent Effect of Microsomes on the Binding of Doxorubicin to Rat Hepatic Nuclear DNA

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SUMMARY

Doxorubicin is metabolically activated by microsomal NADPH-dependent cytochrome P_{450} reductase as well as by intact nuclei forming semiquinone free radicals, which reoxidize to doxorubicin in the presence of oxygen. Nuclear activated doxorubicin became bound to DNA in a time-dependent fashion. The addition of microsomal protein to intact nuclei increased the amount of doxorubicin bound to DNA under aerobic conditions. In contrast, inclusion of microsomes virtually abolished DNA binding under anaerobic conditions. Disruption of the nuclear membrane by sonification increased the amount of drug bound to DNA, indicating that the nuclear envelope serves as a partial barrier to the diffusion of microsomal DNA-directed intermediates. The data indicate that under aerobic conditions metabolites produced by microsomes either traverse the nuclear membrane and bind to DNA or act indirectly by disrupting the nuclear membrane. In contrast, inhibition of DNA binding by doxorubicin under anaerobic conditions suggests either that the microsomal metabolites do not diffuse across the nuclear membrane or that the metabolites are not capable of binding to DNA. The decreased diffusibility of the anaerobic metabolites may represent either the generation of metabolites having lower diffusion constants or the formation of highly reactive intermediates which preferentially bind in the immediate vicinity to the site of generation on the microsomal surface. In conclusion, it appears that, under aerobic conditions, metabolic activation of doxorubicin on the surface of the endoplasmic reticulum can contribute to the amount of the drug which becomes closely associated with nuclear DNA. Consequently, the microsomal drug-metabolizing system may be important in affecting the therapeutic or cytotoxic properties of the drug.

Doxorubicin is an anthracycline antibiotic with potent antineoplastic properties (1). Its clinical use, however, is limited by a cumulative cardiotoxicity resulting in congestive failure (2). Investigations into the biochemical mechanism of doxorubicin action have revealed a variety of explanations which may account for the observed therapeutic as well as toxic effects of the drug. Doxorubicin has been shown to intercalate with DNA (3), cause DNA strand scission (4, 5), and bind to DNA (6), thereby altering the DNA template and inhibiting DNA replication and RNA and protein synthesis. Doxorubicin also enhances lipid peroxidation (7), depletes tissue reducing equivalents (8) and disrupts cellular membrane integrity (9).

Metabolic activation of doxorubicin has been implicated in several of the biological actions of the drug. The reaction, univalent reduction to form the semiquinone free radical, is catalyzed by NADPH-dependent cytochrome P_{450} reductase (10). In the presence of oxygen, the semiquinone radical rapidly reoxidizes to the parent compound at the expense of oxygen radical formation (11, 12). In contrast, under anaerobic conditions, the semiquinone is diverted into alternate pathways resulting in the formation of numerous metabolites including the C_7-quinone radical, the 7-deoxyaglycone, and the C_7-quinone methide. The C_7-radical and C_7-quinone methide have been implicated as reactive intermediates that bind to DNA (13). The metabolism of doxorubicin by microsomes is well documented; however, the role of microsomes in generating intermediates which bind to DNA of intact nuclei is currently under investigation (13, 14).

Recently, Bachur *et al.* (14) demonstrated that isolated rat liver nuclei contain the enzymes necessary for the activation of doxorubicin to free radical intermediates. Accordingly, it was postulated that nuclei may be capable of activating the drug to DNA-directed metabolites independent of microsomal involvement. The generation of intermediates in close proximity to DNA could facilitate the genotoxic effects of the drug. Sinha *et al.* (13) provided evidence which suggests that nuclear activation contributes significantly to the binding of doxorubicin to DNA and that this process is enhanced under anaerobic conditions.

The present investigation was designed to further characterize the metabolic activation of doxorubicin to DNA-binding

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ABBREVIATIONS: G6P, glucose 6-phosphate; G6PD, glucose-6-phosphate dehydrogenase; SDS, sodium dodecyl sulfate; ASA, *p*-aminosalicylic acid.

intermediates by intact nuclei, and to assess the contribution of microsomal protein to the generation of DNA-directed metabolites. Since anaerobic metabolites have been implicated in the alkylation of DNA (13), we also investigated the modulating effect of oxygen on the binding of doxorubicin to nuclear DNA in the absence and presence of microsomes.

Materials and Methods

Doxorubicin hydrochloride (Adriamycin) was purchased from Adria Laboratories (Columbus, OH). The solvents were analytical grade (Fisher Scientific Co., Fairlawn, NJ). NADPH, G6P, G6PD, SDS, ASA, and proteinase K (type XI, from *Tritirachium album*) were obtained from Sigma Chemical Co. (St. Louis, MO).

Microsomes and intact nuclei were isolated from livers of male Sprague-Dawley rats weighing 170–200 g. Food and water were provided *ad libitum*. The animals were decapitated and the liver was removed to cold isotonic NaCl. A portion of the liver was minced in 8 volumes of 2.4 M sucrose, 3 mM $CaCl_2$ and nuclei were isolated as described by Bresnick *et al.* (15) and Wallace (16). The liver was homogenized in a glass-Teflon pestle and filtered through two layers of surgical gauze. After centrifugation for 60 min at 40,000 × *g* and 4°, the nuclear pellets were washed twice with 1.0 M sucrose, 1 mM $CaCl_2$ and resuspended in cold 50 mM Tris-HCl (pH 7.4), 150 mM KCl at 1/10 the original volume by weight (ml/g). The preparations were routinely examined under a light microscope to ensure that the nuclear preparation did not deteriorate, lyse, or aggregate during the incubation. Electron microscopic inspection of the nuclear preparations revealed occasional small fragments resembling endoplasmic reticulum; however, extensive washing of the preparations did not significantly diminish metabolic activity (16). This was taken to indicate that the fragments were in close association with the nuclei, perhaps functionally integrated into the nuclear membrane. Microsomes were isolated from the remaining portion of liver according to the method of Omura and Sato (17). The final microsomal pellets were resuspended in cold 50 mM Tris-HCl (pH 7.4), 150 mM KCl at 1/10 the original volume by weight (ml/g).

The standard 5.0-ml incubation mixture contained 0.75–1.0 mg/ml nuclear protein, 2.0 mg/ml G6P, and 5 units/ml G6PD. All reagents were dissolved in 50 mM Tris-HCl (pH 7.4), 150 mM KCl. The mixture was allowed to equilibrate at 37° either in air or under a stream of argon gas for 10 min before adding 1.0 mM doxorubicin and 1 mg/ml NADPH to initiate the reaction. When the reactions were performed with continuous mixing in room air, oxygen tension, as recorded with an oxygen electrode, remained within 80% of that of an air-saturated solution (16). In contrast, the concentration of dissolved oxygen was maintained at less than 10% of that of air-saturated solutions by incubating the mixtures under a steady stream of argon gas.

The incubation was stopped by making the mixture 1% SDS/6% ASA, followed immediately by the addition of an equal volume of phenol/chloroform (1:1). The interval between lysing the nuclei and adding phenol/chloroform was minimized to avoid continued interaction of exposed DNA with unreacted drug. The samples were shaken vigorously for 20 min and centrifuged at 10,000 × *g* for 45 min at 4°. The upper aqueous phase and interface were retained for further processing and the phenol/chloroform phase was discarded. The interface was resuspended in 5 ml of 50 mM Tris-HCl (pH 7.4), 150 mM KCl and digested with 1 mg/ml proteinase K at 37° for 30 min, then extracted with phenol/chloroform. The proteinase K concentration was sufficient to digest virtually all of the interface, providing high and consistent DNA recovery (greater than 80%). Ten ml of absolute ethanol were added to the combined aqueous extracts to precipitate DNA, which was then wound onto a glass rod, washed with 70% ethanol, and dissolved in 2.0 ml of 50 mM Tris-HCl (pH 7.4), 150 mM KCl. Five ml of absolute ethanol were added to reprecipitate DNA. The extracts were then centrifuged at 6,000 × *g* for 10 min at 4° and the DNA pellets were sequentially washed with ethanol, ethanol/ether (1:1), and, finally, ether. The ether was evaporated and the dried DNA pellets were redissolved in 2 ml of 50 mM Tris-HCl (pH 7.4), 150 mM KCl.

An aliquot of the DNA extract (0.75 ml) was combined with 2.0 N $HClO_4$ (0.25 ml) and incubated at 70° for 20 min. The final DNA acid hydrolysates were scanned spectrophotometrically to determine the concentration of doxorubicin extracted with the DNA. Careful analyses of the spectral data revealed that the peak absorbance at 490 nm was a representative measure of the concentration of drug in the DNA extract. The molar extinction coefficient for the drug in 0.5 N $HClO_4$ was determined to be 9564 $(M \cdot cm)^{-1}$. DNA concentrations were measured by the diphenylamine reaction (18). Binding was expressed as nmol of doxorubicin/mg of DNA. Protein content in the extracts, nuclei, and microsomes was measured by the ninhydrin reaction as described by Lowry *et al.* (19). Less than 1% of the added protein was extracted with the DNA. The Student's *t* test was used for statistical analysis of the data and a probability of $p < 0.05$ was selected as the criterion for significance.

Results

In a separate set of experiments, addition of trichloroacetic acid precipitated contaminating protein without altering the absorbance of the extracts at 490 nm, suggesting that an insignificant amount of doxorubicin was bound to protein in the final DNA extracts. Furthermore, ribonuclease digestion of the extracts failed to alter the absorbance and did not affect the DNA measurement. Thus, traces of protein or RNA extracted with DNA did not appear to affect the reported drug/DNA binding.

Preliminary data demonstrated a dose-dependent increase in the binding of doxorubicin to nuclear DNA (data not presented). Maximum binding was attained at 0.75 mM doxorubicin. Fig. 1 illustrates a time-dependent 8-fold increase in the binding of doxorubicin to DNA for nuclei incubated in room air for up to 6 hr. The recovery of DNA from the reaction mixtures did not change with time of incubation and averaged 86%. Nuclei were examined under a light microscope at various intervals and no significant loss of membrane integrity or

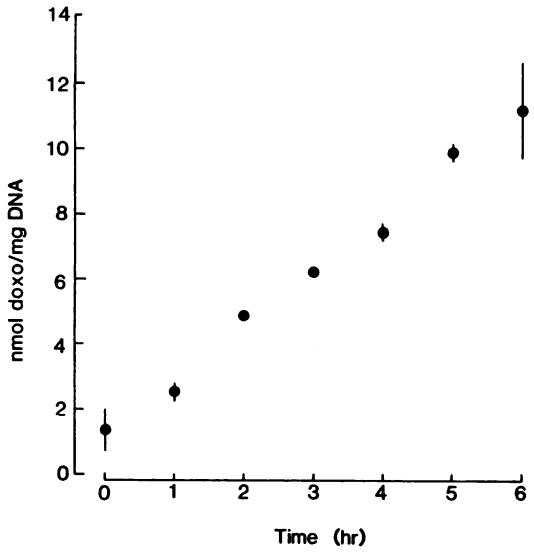

Fig. 1. Time-dependent binding of doxorubicin to DNA of intact nuclei. Aerobic incubations contained 1.0 mM doxorubicin (*doxo*), 0.75–1.0 mg/ml nuclear protein, 2.0 mg/ml G6P, 5 units/ml G6PD, and 1.0 mg/ml NADPH in 50 mM Tris-HCl (pH 7.4), 150 mM KCl. Each point represents the mean ± standard deviation ($n = 2$). Points without error bars indicate that the standard deviation was less than the radius of the symbol.

aggregation was observed. The time between lysing the nuclei with SDS/ASA and adding phenol/chloroform may allow for the interaction of exposed DNA by unreacted doxorubicin and account for the binding observed at the start of the reaction.

There was also a time-dependent 12-fold increase in the binding of doxorubicin to DNA for nuclei incubated in the presence of microsomes (2.6–2.9 mg of protein/ml) in room air for 2 hr (Fig. 2). The amount of nuclei and microsomes added to the incubation mixture was proportionate to that recovered from liver on a per gram basis. The rate of binding decreased progressively over the 2 hr. DNA recovery did not change over the course of the incubation and averaged 82%. Comparing the amount of drug bound to DNA for nuclei incubated alone for 1 hr, 2.49 ± 0.16 (Fig. 1), to that for nuclei incubated for an hour with microsomes, 16.25 ± 3.68 (Fig. 2), indicates that, under aerobic conditions, addition of microsomal protein enhanced the binding of drug to nuclear DNA by 6-fold.

A microsomal protein-dependent increase in the binding of doxorubicin to DNA is illustrated in Fig. 3. At microsomal protein concentrations above 2.5–3.0 mg/ml the binding of drug to nuclear DNA was 4- to 5-fold greater than that of nuclei incubated alone for 1 hr in room air. DNA recovery did not change with increasing microsomal protein addition and averaged 91.3%.

The amount of drug bound to DNA of intact nuclei incubated alone either in room air or argon gas for 4 hr was not significantly different (Table 1). Addition of microsomes under aerobic conditions significantly increased the binding of doxorubicin to DNA by 3-fold at 4 hr (Table 1). In contrast, the addition of microsomes to anaerobic incubations inhibited 79% of the binding of doxorubicin to nuclear DNA. In the presence of microsomes under anaerobic conditions, the binding of drug to DNA at 1 and 4 hr (Table 1) was not significantly different from that observed at the start of the reaction, suggesting that, under anaerobic conditions, DNA binding is abolished by microsomal addition. The quantity of drug bound to DNA in the presence of microsomes was significantly greater for sonically disrupted than for intact nuclei under both anaerobic and aerobic conditions, indicating that the intact nuclear envelope

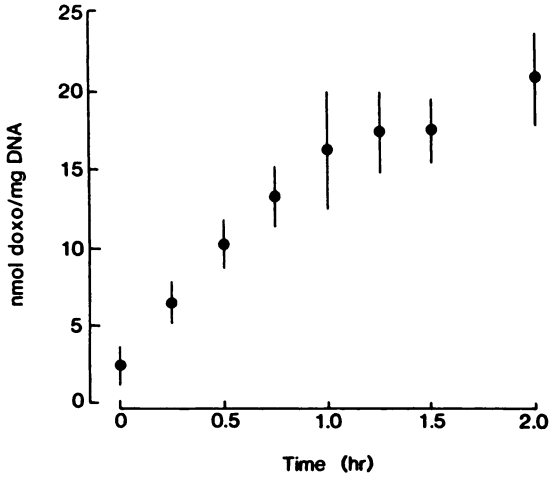

Fig. 2. Time-dependent effect of microsomal protein on the binding of doxorubicin to nuclear DNA. Microsomal protein (2.6–2.9 mg/ml) was added to aerobic incubations containing 1.0 mM doxorubicin (*doxo*), 0.75–1.0 mg/ml nuclear protein, 2.0 mg/ml G6P, 5 units/ml G6PD, and 1.0 mg/ml NADPH in 50 mM Tris-HCl (pH 7.4), 150 mM KCl. Binding is expressed as the mean ± standard error of three experiments.

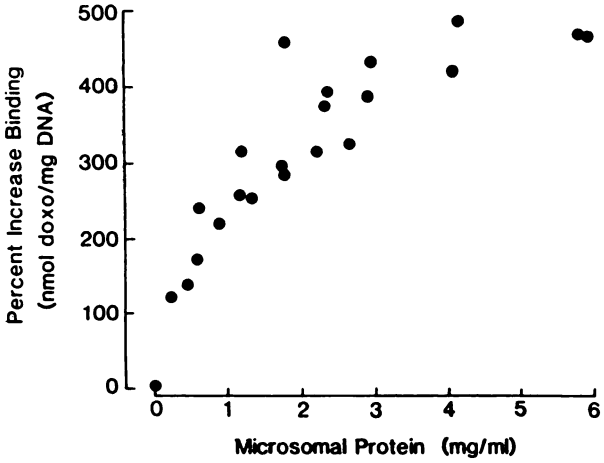

Fig. 3. Effect of increasing microsomal protein on DNA binding by doxorubicin. Increasing amounts of microsomal protein (0–6 mg/ml) were added to incubations containing 1.0 mM doxorubicin (*doxo*), 0.75–1.0 mg/ml nuclear protein, 2.0 mg/ml G6P, 5 units/ml G6PD, and 1.0 mg/ml NADPH in 50 mM Tris-HCl (pH 7.4), 150 mM KCl. Samples were incubated for 1 hr in room air. The individual values are expressed as per cent increase with respect to the binding observed when nuclei were incubated alone.

provides at least a partial barrier to the diffusion of microsomal metabolites of doxorubicin. Finally, the binding of doxorubicin to nuclear DNA, in the presence or absence of microsomal protein, was significantly, but not completely, inhibited by omitting NADPH (Table 1).

Discussion

The results indicate that doxorubicin is metabolically activated by both nuclei and microsomes to intermediates which bind to DNA. The binding of the drug to DNA was both dose and time dependent and was inhibited by omitting NADPH. The less than complete inhibition of drug binding in the absence of exogenous NADPH may reflect either the presence of residual endogenous NADPH in the tissue samples or that a portion of the measured binding occurred via NADPH-independent reactions such as the nonenzymatic association of the drug with DNA to form a rather stable complex (20). A final observation was that addition of purified bovine serum albumin to reaction mixtures containing isolated nuclei failed to alter the binding of doxorubicin to DNA under aerobic conditions (data not presented). These results suggest that the stimulatory effect of microsomes on the binding of doxorubicin to nuclear DNA was due to more than an allosteric effect of the protein itself.

Following metabolic activation, the fate of the semiquinone radical is dependent on oxygen tension (13, 14). Under aerobic conditions a cyclic process predominates wherein the semiquinone is reoxidized to doxorubicin, at the expense of liberating oxygen radicals. Under anaerobic conditions, however, the semiquinone is diverted into alternate pathways leading to numerous aglycoside metabolites. Sinha *et al.* (13) propose that anaerobic intermediates are responsible for binding to DNA, whereas Scheulen *et al.* (21) suggest that aerobic metabolites mediate the binding of doxorubicin to microsomal proteins.

The results presented herein contrast with those reported by Sinha *et al.* (13). These investigators observed a 43% decrease in the binding of doxorubicin to nuclear DNA when oxygen was added to an anaerobic incubation. However, our data

TABLE 1

Effect of microsomes on the oxygen-dependent alkylation of nuclear DNA by doxorubicin

	Time of incubation[a]	
	1 Hr	4 Hr
Intact nuclei[b]		
Air	3.11 (1)	6.87 ± 1.13 (3)
Argon	3.21 (1)	6.43 ± 0.25 (3)
Intact nuclei plus microsomes[b]		
Air	12.61 ± 1.48 (3)	22.14 ± 3.07 (3)[c]
Argon	1.77 ± 0.13 (3)[d]	1.37 ± 0.29 (3)[e,f]
Sonified nuclei plus microsomes[b]		
Air	16.46 ± 0.83 (3)[d]	
Argon	2.55 ± 0.09 (3)[g,h]	
Intact nuclei in air[i]		
(+)-NADPH		7.05 ± 0.49 (7)
(−)-NADPH		5.20 ± 0.32 (2)[j]
Intact nuclei plus microsomes in air[i]		
(+)-NADPH	14.44 ± 1.44 (8)	
(−)-NADPH	7.08 ± 0.74 (3)[j]	

[a] Doxorubicin/DNA binding ratios (nmol of doxorubicin/mg of DNA) are expressed as mean ± standard error. The number of replications are in parentheses.
[b] Significance determined by the Student's t test ($p < 0.05$).
[c] Significantly different from intact nuclei in air at 4 hr.
[d] Significantly different from intact nuclei plus microsomes in air at 1 hr.
[e] Significantly different from intact nuclei in argon at 4 hr.
[f] Significantly different from intact nuclei plus microsomes in air at 4 hr.
[g] Significantly different from intact nuclei plus microsomes in argon at 1 hr.
[h] Significantly different from sonified nuclei plus microsomes in air at 1 hr.
[i] Significance determined by unpaired Student's t test ($p < 0.05$).
[j] Significantly different from that observed in the presence of NADPH.

indicate no significant difference in the amount of doxorubicin bound to DNA when comparing nuclei incubated in argon to nuclei incubated in an aerated mixture (Table 1). Sinha *et al.* (13) also reported that the addition of microsomes to nuclei incubated under anaerobic conditions increased the binding of doxorubicin to nuclear DNA by 30%. Based on this finding, it was concluded that the semiquinone and/or anaerobic intermediates are sufficiently stable to diffuse across the nuclear membrane and bind to DNA. In contrast to these observations, our data demonstrate that, under anaerobic conditions, the addition of microsomal protein dramatically inhibits the binding of doxorubicin to DNA of intact nuclei (Table 1). Furthermore, the amount of doxorubicin bound to DNA under anaerobic conditions in the presence of microsomes was less than that observed for nuclei incubated alone for 4 hr and was not significantly different from that recorded at the start of the reaction (Table 1). The apparent discrepancy between our results and those reported by Sinha *et al.* (13) may be attributed to differences in either the conditions of the incubation or the method for extracting DNA. For example, Sinha *et al.* (13) employed a lower concentration of microsomal protein relative to that of nuclei. Metabolic activation of doxorubicin results in the binding of metabolites to microsomal proteins in the immediate vicinity of their generation (16, 20, 22). Consequently, the availability of freely diffusible metabolites for interacting with DNA is diminished. Accordingly, under the conditions employed by Sinha *et al.* (13), insufficient microsomes may be present to effectively compete for binding of reactive intermediates formed anaerobically, thereby preserving a greater fraction of the microsomal metabolites available to the nuclear DNA.

Three possible explanations for the dramatic inhibition of doxorubicin binding to DNA by microsomes added under an atmosphere of argon gas are: 1) the intermediates formed by microsomes under anaerobic conditions may not be capable of binding to DNA; 2) the anaerobic metabolites may not diffuse across the nuclear membrane; and/or 3) the reactive intermediates may preferentially bind to microsomes in close proximity to the site of generation. For example, the lower water solubility of aglycone metabolites of doxorubicin formed anaerobically may impede their diffusion in the aqueous reaction medium. Each mechanism would effectively reduce the availability of intermediates to bind to nuclear DNA.

The third possibility, sequestration by binding, is supported by the results of Scheulen *et al.* (21) who demonstrated that metabolically activated doxorubicin binds to microsomal protein. Wallace (16) also provided evidence for the binding of doxorubicin metabolites to microsomes. Under anaerobic conditions, the total amount of extractable doxorubicin and doxorubicin metabolites, principally aglycones, decreased progressively, whereas the degree of nonextractable drug associated with the pellets increased with time. In contrast, under aerobic conditions, the extraction of drug and metabolites and the binding of doxorubicin to microsomal pellets were not significantly altered (16). These data suggest that the observed inhibition of DNA binding by microsomes under anaerobic conditions may be due to the competitive binding of aglycone metabolites to microsomal protein with the consequence of limiting the availability of the free drug.

The enhanced binding of doxorubicin to nuclear DNA resulting from the addition of microsomes under aerobic conditions may reflect one or more of several possible interactions. The reoxidation of the semiquinone radical in the presence of oxygen may spare the drug from binding to microsomal protein (16), thereby conserving the concentration of doxorubicin available to bind to nuclear DNA. Alternatively, the oxgyen radical-dependent stimulation of lipid peroxidation associated with the aerobic metabolism of doxorubicin by microsomes (7, 23) may result in the formation of unstable hydroxyalkenals capable of disrupting either the integrity of the nuclear membrane or the

tertiary structure of DNA. Either effect would feasibly increase the accessibility of doxorubicin and its metabolites to interact with nuclear DNA (24–26). In support of this possibility, Roders *et al.* (24) and Benedetti *et al.* (25) demonstrated that alkenal products of microsomal lipid peroxidation catalyze the lysis of erythrocyte membranes. Although no disruption of the nuclear membrane was visualized microscopically, it is possible that sufficient damage had occurred to increase the membrane permeability and/or accessibility of nuclear DNA to binding by the drug.

In conclusion, although the nuclear envelope appears to serve as a partial barrier to passive diffusion, metabolic activation of doxorubicin by microsomes contributes to the extent of binding of the drug to nuclear DNA under aerobic conditions. This may reflect either the generation of DNA-directed metabolites capable of traversing the nuclear membrane or the destruction of the nuclear envelope either by reactive metabolites of the drug or products of peroxidized membranes leading to an increase in the partitioning of the drug to the nuclear contents. In contrast, inhibition of doxorubicin binding to DNA following the addition of microsomes to anaerobic reactions most likely reflects the preferential binding of highly reactive metabolites in the immediate vicinity of their formation on the surface of microsomal membranes. Regardless of the mechanisms, the data suggest that metabolic activation of doxorubicin by enzyme systems associated with the endoplasmic reticulum may be important in determining the extent of binding of the drug to nuclear DNA and, consequently, may modulate the therapeutic and/or cytotoxic effects of the drug.

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