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Covalent modification of DNA by daunorubicin

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Abstract. Daunorubicin, a clinically useful antitumor agent, induces mammary adenocarcinoma in Sprague-Dawley rats. As part of an investigation of the mechanism of tumor induction by daunorubicin, the formation of daunorubicin-DNA adducts has been investigated by 32P-postlabeling assay. Rat-liver DNA incubated with either 0.05 or 0.1 mM daunorubicin, rat-liver microsomes, and 5 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH) for 1 h contained covalent DNA adducts in addition to the endogenous adduct profile present in control DNA. With 1.5 mM cumene hydroperoxide serving as a cofactor, higher levels of these two adducts and two additional adducts were formed, all of which most likely were daunorubicin-DNA adducts. This latter treatment also resulted in an intensification of three endogenous DNA modifications over levels occurring in control DNA. Covalent DNA alterations in vivo were studied in rats treated with 20 mg/kg daunorubicin for 2 days and 200 mg/kg on the 3rd day. Daunorubicin-DNA adducts as observed in vitro could not be detected in DNA of liver or mammary epithelial cells. The levels of endogenous modifications in drug-treated rats were increased by 200% in mammary DNA and by 50% in hepatic DNA as compared with controls. It was concluded from these experiments that daunorubicin may be metabolically activated to a reactive metabolite that binds covalently to DNA. These daunorubicin-DNA adducts may not play a role in tumor induction because they were not detectable in vivo. However, the increase in levels of endogenous DNA modifications induced by daunorubicin both in vitro and in vivo is consistent with a role of this class of DNA modification in the carcinogenic process.

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Abbreviations: DNR, daunorubicin or daunomycin; CuOOH, cumene hydroperoxide; RAL, relative adduct levels as defined by Reddy and Randerath [12]

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Introduction

Daunorubicin (DNR), like many clinically useful antitumor agents, also induces tumors in laboratory animals [1]; for instance, it has been reported to cause mammary adenocarcinomas in female Sprague-Dawley rats [1–3]. The number of tumors induced by DNR increases in a dose-dependent manner [1]. The mechanistic details of tumor induction by DNR and also of its anticarcinogenic activity are unknown and are subject to intense debate. Both the carcinogenic and the therapeutic activities of this drug are thought to arise from the DNA modulating action of this substance. A widely accepted mechanism of DNR interaction with DNA is intercalation [4]. Second, DNR has been demonstrated to undergo redox cycling by single electron transfer catalyzed by enzymes of the microsomal, mitochondrial, and nuclear fractions of the cell [5–7]. This process of redox cycling results in the formation of reactive free radicals that are capable of covalently modifying DNA, proteins, and membranes [8]. For instance, significantly higher relative frequencies of single-strand breaks have been observed in rat mammary epithelial cells as compared with hepatocytes exposed to identical doses of DNR [9]. These single-strand breaks are attributed to the action of free radicals on DNA. A third mechanistic possibility for the nucleic acid-modulating activity of DNR is the direct covalent binding of the tetracyclic quinone or one of its metabolites to DNA, which is the subject of this investigation.

In a hepatocyte model, DNA binding has been shown for several morpholino derivatives of anthracycline antibi-

otics, including morpholinodaunomycin [10]. Weak DNA binding of the parent drug DNR has also been observed, but the DNA-reactive site appeared to be localized in the aminosugar moiety [10]. Moreover, DNR-DNA binding has been demonstrated in vitro by Sinha et al. [8, 11] in the presence and absence of metabolic activation without further clarification of the type of binding involved. Thus, DNR-DNA binding in vivo and the mechanistic details of this interaction in vitro and in vivo have not yet been clarified and were therefore investigated in this study. DNR-DNA adduct formation was examined by the nuclease P_1-enhanced ^{32}P-postlabeling assay described by Reddy and Randerath [12] both in vitro and in vivo in female Sprague-Dawley rats. In vitro, DNR-DNA adduct formation was studied in the absence and presence of metabolic activation by microsomes and reduced nicotinamide adenine dinucleotide phosphate (NADPH) or CuOOH. The organic hydroperoxide-dependent activation of DNR was carried out in analogy to the CuOOH-dependent increase in concentrations of the quinone metabolite and quinone-DNA adducts from diethylstilbestrol (A. Gladek and J.G. Liehr, unpublished data). For the in vivo examinations, DNA was isolated from mammary glands of DNR-treated rats and from liver, which is not a target of DNR-induced carcinogenesis.

Materials and methods

Materials. DNR and CuOOH were purchased from Sigma Chemical Co. (St. Louis, Mo.) and NADPH, from Boehringer Mannheim (Indianapolis, Ind.). Microsomes and DNA were isolated from livers of 4-month-old female Sprague-Dawley rats by methods described previously [13, 14]. Materials for the ^{32}P-postlabeling assay were obtained from sources described elsewhere [12, 13, 15].

Animal care. Female weanling Sprague-Dawley rats were purchased from Harlan Sprague-Dawley (Houston, Tex.). The animals were housed in groups of three or four per polycarbonate cage suspended on wire racks covered with filter paper. The room temperature was maintained at 72° ± 4° F. Lighting cycles comprised 12 h light (from 7 a.m. to 7 p.m.) and 12 h darkness. All animals were placed in quarantine for 48 h before initiation of the experimental protocols. USPHS guidelines for humane animal care and use were followed. Purina laboratory chow and tap water were available ad libitum.

Incubations in vitro. Rat-liver DNA (500 μg) and 1 mg of microsomal protein in a volume of 1 ml 0.01 *M* phosphate buffer (pH 7.5) were incubated with DNR (Sigma Chemical Co., St. Louis, Mo.) and NADPH (5 m*M*) or CuOOH (1.5 m*M*) for 1 h at 37° C. DNA in buffer, DNA and microsomes in buffer, and DNA incubated with microsomes and 0.05 m*M* or 0.1 m*M* DNR but without cofactor served as controls. The DNA was isolated from the reaction mixture by solvent extraction combined with enzymatic digestion of protein and RNA as described by Gupta [13]. The DNA was then examined by the nuclease P_1 version of the ^{32}P-postlabeling procedure [12]. These experiments were done in triplicate.

Treatment of animals. Female Sprague-Dawley rats aged 2 months received i.v. injections of 20 mg DNR/kg body weight on 2 consecutive days and on the 3rd day were given a dose of 200 mg/kg. A mixture of Cerubidine, a pharmaceutical preparation consisting of DNR and mannitol (Wyeth-Ayerst Laboratories, Philadelphia, Pa.), and pure DNR (Sigma Chemical Co., St. Louis, Mo.) was used for this treatment. Mannitol (62.5 mg/kg) was injected into control rats. The treatment and control groups consisted of three rats per group. The rats were euthanized at 6 h postinjection using carbon dioxide. Various organs were removed quickly and placed on dry ice. Mammary epithelial cells were isolated using a modification of the procedure of Moon et al. (16) and Wiepjes and Prop [17].

^{32}P-postlabeling analysis. The liver and mammary epithelial-cell DNA from individual animals was analyzed by the nuclease P_1 version of the ^{32}P-postlabeling procedure [12]. The chromatography of the ^{32}P-postlabeling procedure was carried out using the following solvents. Prior to two-dimensional mapping of adducts, the normal nucleotides were removed by chromatography with 2.3 *M* sodium phosphate (pH 5.75) in direction 1 (D1), followed by autoradiography. After D1, a 2.5- × 1-cm strip was cut from each lane of the chromatogram. The modified nucleotides in this strip were transferred to fresh polyethyleneimine-cellulose sheets using a magnet-transfer technique described elsewhere [12]. Each individual strip was attached to 16.5- × 12.5-cm acceptor sheets using magnets. The sheets were first predeveloped in deionized water to a height of 2 cm from the bottom and then chromatographed in D3 using 4.2 *M* lithium formate and 7.5 *M* urea (pH 3.35). For D4, a Whatman number 1 wick was attached to the top of the sheet. Chromatograms were predeveloped in 0.60 *M* sodium phosphate to 1 cm from the origin and then to 4 cm on the wick in 0.60 *M* sodium phosphate and 7 *M* urea (pH 6.4). The final separation of nucleotides was carried out by developing the chromatograms to 4 cm on the wick in the D4 direction (D5) using 1.7 *M* sodium phosphate (pH 6.0). Between chromatographic procedures, the polyethyleneimine-cellulose sheets were soaked twice in 1 l deionized water for 7 min each to remove solids. Autoradiography was done using Kodak XAR-5 film and DuPont Lightning Plus intensifying screens. Exposures were carried out at −70° C for 16.5 h.

Quantitation of adduct levels. Adducts were quantified by determining relative adduct levels (RAL) according to the procedure of Reddy and Randerath [12]. The autoradiograms were used to locate the areas of radioactivity. The spots were then excised and assayed by Cerenkov counting [12]. Each vial was counted three times for 2 min for the specific activity of the [^{32}P]-adenosine triphosphate (ATP) or three times for 5 min for the adducts. The specific activity and RAL values were calculated as described by Reddy and Randerath [12]. Student's t-test was used for analysis of differences between the RAL values of DNA adducts formed as a result of DNR treatment in vitro and in vivo. *P* values of 0.05 or less were considered statistically significant for determining the differences in the mean values of the various parameters under study.

Results

DNA adducts in vitro

The initial exploration of DNR-DNA adduct formation was carried out at two different substrate concentrations, 0.05 m*M* and 0.1 m*M* DNR in the presence of rat-liver microsomes and either NADPH or CuOOH as cofactors (Fig. 1). Two new adducts, spots D1 and D2, were observed after incubation of DNA with DNR, microsomes, and NADPH (Fig. 1B, E) as compared with the background radioactivity and endogenous adducts of control DNA (Fig. 1A). When CuOOH was used as a cofactor instead of NADPH, the concentrations of spot D2 were increased over the levels observed in incubations with NADPH (Table 1). Moreover, two additional adducts, spots D3 and D4, were detected (Fig. 1C, F). Spots D1, D2, D3, and D4 were formed upon incubation of DNA with DNR and a complete microsomal activating system and most likely represented DNA adducts of DNR metabo-

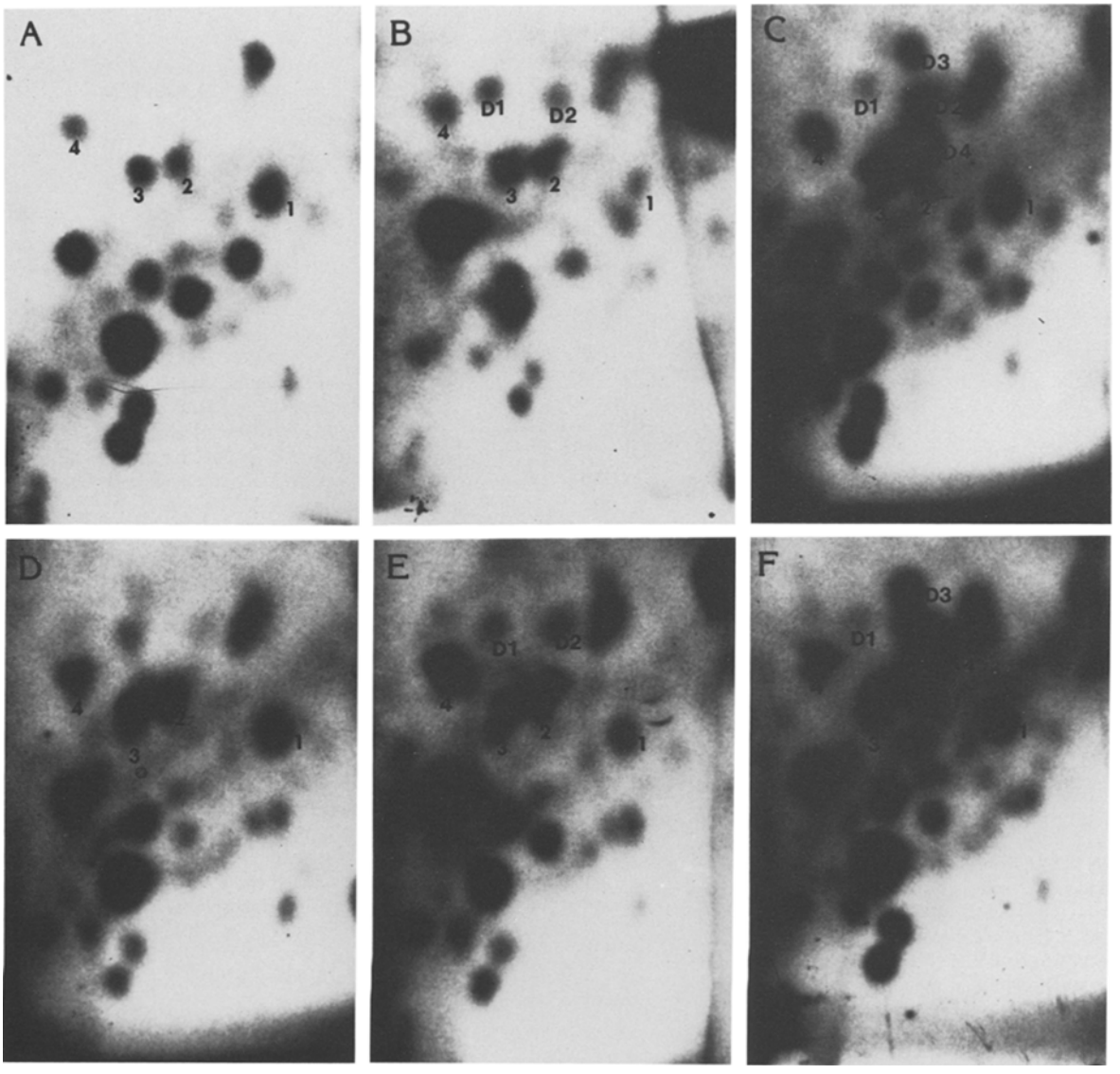

Fig. 1 A–F. ^{32}P-postlabeling analysis of the products of the incubation of rat-liver DNA and 0.05 or 0.1 mM DNR and a microsomal activating system. Pure rat-liver DNA (500 μg) and microsomal protein (1 mg) were incubated with 5 m*M* NADPH and 0.05 or 0.1 m*M* DNR (*B* and *E*, respectively) or 1.5 m*M* CuOOH and 0.05 or 0.1 m*M* DNR (*C* and *F*, respectively). Pure rat-liver DNA (*A*) and DNA incubated with 0.1 m*M* DNR and microsomes but no cofactor (*D*) served as controls. The products of the incubations were analyzed by the nuclease P_1 procedure of the ^{32}P-postlabeling assay as described previously [12]. Labeled adducts were located by autoradiography for 16.5 h at –70° C using Kodak XAR-5 film and Dupont Lightning Plus intensifying screens. Adducts are lettered *beneath* each spot except for adducts D2 and D4 (C, F), which are lettered to the *right* of each spot

lite(s). The intensity of spot D3 was increased at the higher DNR concentration ($P < 0.05$).

The endogenous adduct profile of untreated rat-liver DNA shown in Fig. 1A was comparable with that published previously [18]. The intensities of endogenous spots 1 and 2 increased when DNA was incubated with DNR, microsomes, and CuOOH (Fig. 1C, F). These increases are reflected in the RAL values of these endogenous spots (Table 2). At the elevated substrate concentration, 0.1 m*M* DNR, the DNA adduct profile was almost identical to that shown at the lower drug concentration except that spot intensities were increased further (Fig. 1, Table 2). For instance, when DNA was incubated with 0.1 m*M* DNR, microsomes, and CuOOH, the RAL values of spots 1, 2, and 3 were significantly higher than those of controls. There was no difference in adduct intensities in DNA incubated with microsomes (data not shown), DNA incubated without microsomes (Table 2), and DNA incubated with 0.1 m*M* DNR and microsomes but no cofactor (Table 2).

In summary, the incubation of DNA with DNR, microsomes, and CuOOH resulted in an intensification of several endogenous DNA adducts. Moreover, four new adducts were also detected that most likely were DNR-DNA adducts.

Effect of DNR treatment of rats on DNA adducts

The treatment of female Sprague-Dawley rats with DNR did not result in detectable levels of in vivo DNR-DNA

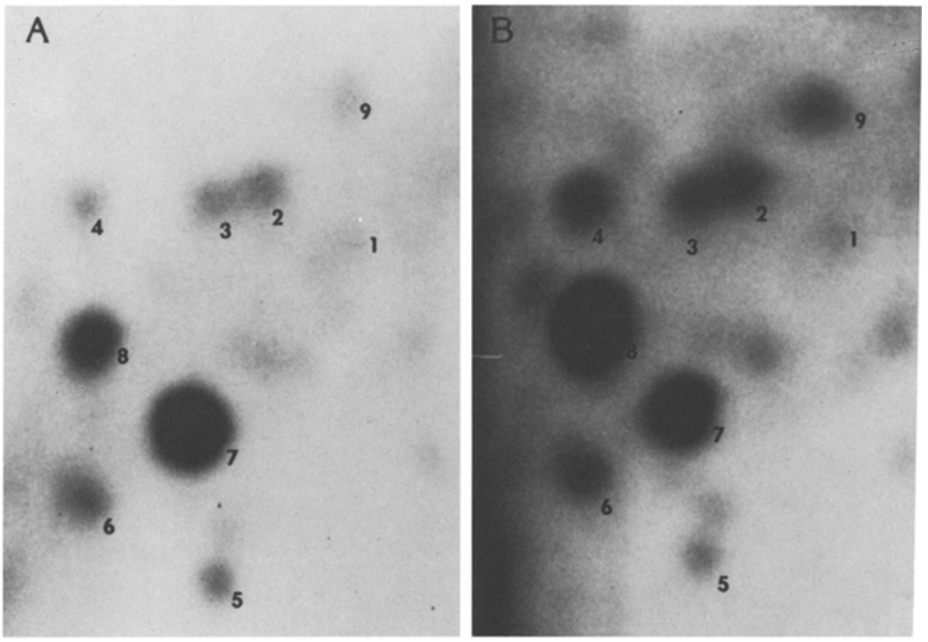

Fig. 2 A, B. ^{32}P-postlabeling analysis of liver DNA of rats treated with 20 mg/kg DNR for 2 days and 200 mg/kg on the 3rd day (**B**). Mannitol was injected into control rats (**A**). DNA was isolated and analyzed by the nuclease P_1 procedure of the ^{32}P-postlabeling assay as described previously [12]. Labeled adducts were located by autoradiography for 16.5 h at –70°C using Kodak XAR-5 film and Dupont Lightning Plus intensifying screens. Adducts 2–9 are lettered *beneath* each spot, and adduct 1 is lettered to the *right* of each spot

Table 1. RAL values of ^{32}P-labeled DNR-DNA adducts formed in vitro

Conditions	Spot number (Fig. 1)		
	D1	D2	D3
B: 0.05 m*M* DNR, NADPH	2.2±1.6	2.5±1.2	<0.1
C: 0.05 m*M* DNR, CuOOH	1.7±1.2	5.4±2.1*	3.0±0.8
E: 0.1 m*M* DNR, NADPH	2.1±2.2	2.3±1.1	<0.1
F: 0.1 m*M* DNR, CuOOH	3.4±3.7	6.1±2.0**	5.0±1.4***

The adduct spots shown in Fig. 1 were cut from the thin-layer sheets, and the radioactivity content was determined by Cerenkov counting. Counts obtained from appropriate background areas served as controls and were subtracted. The intensity of D4 was not measured, because this spot overlapped with others and could not be excised clearly. RAL × 10^9 values are means ± SD for the indicated spots as seen in Fig. 1 ($n = 3$)
* RAL value significantly higher than that in map B as determined by *t*-test ($P < 0.05$)
** RAL value significantly higher than that in map E as determined by *t*-test ($P < 0.05$)
*** RAL value significantly higher than that in map C as determined by *t*-test ($P < 0.05$)

Table 2. RAL values of ^{32}P-labeled endogenous adducts of DNA incubated with DNR in vitro

Conditions	Spot number (Fig. 1)			
	1	2	3	4
A: no microsomes	3.6±3.2	2.6±2.3	2.8±2.7	1.8±0.7
B: 0.05 m*M* DNR, NADPH	3.4±1.7	4.6±2.1	5.6±4.3	3.9±2.1
C: 0.05 m*M* DNR, CuOOH	5.6±3.3*	7.7±6.4*	6.5±5.8	2.9±2.1
D: 0.1 m*M* DNR, no cofactor	4.2±1.2	3.5±1.2	4.6±1.6	2.2±0.9
E: 0.1 m*M* DNR, NADPH	3.5±3.2	6.0±5.4	7.2±5.4	5.0±3.9
F: 0.1 m*M* DNR, CuOOH	7.9±2.3*	9.7±4.9*	6.9±1.8*	2.5±1.0

The adduct spots shown in Fig. 1 were cut from the thin-layer sheets, and the radioactivity content was determined by Cerenkov counting. Counts obtained from appropriate background areas served as controls and were subtracted. RAL × 10^9 values are means ± SD for the indicated spots ($n = 3$)
* RAL value significantly higher than that in map A as determined by *t*-test ($P < 0.05$)

adducts (spots D1–D4) as observed in vitro (Figs. 2, 3). The maps of mammary epithelial cells and also of liver, which is not a cancer target under these treatment conditions, did not show any additional DNA adducts as a result of DNR treatment. However, there were differences when intensities of endogenous adducts were compared with those of controls. There was an intensification of several endogenous adducts in hepatic DNA of the drug-treated rats (Fig. 2). The RAL values of five spots in liver DNA of treated animals were not changed as compared with controls (two-tailed *t*-test), but spots 2, 8, and 9 had significantly higher RAL values (Table 3). Also, the total values of all spots were increased by 50% over control values.

In DNA of mammary epithelial cells, the target of DNR carcinogenesis, no new adduct was seen as a result of DNR treatment of rats (Fig. 3). However, there was a significant intensification of endogenous DNA adduct spots M1, M2, and M4 in response to DNR treatment ($P < 0.05$; Table 4). The total RAL values for endogenous adducts in mammary DNA of drug-treated rats was more than triple that of control DNA.

In summary, there was a significant intensification of three of a total of four endogenous adducts in DNA of mammary epithelial cells of rats treated with DNR as compared with controls. In hepatic DNA, concentrations of five adducts remained unchanged, whereas those of three DNA

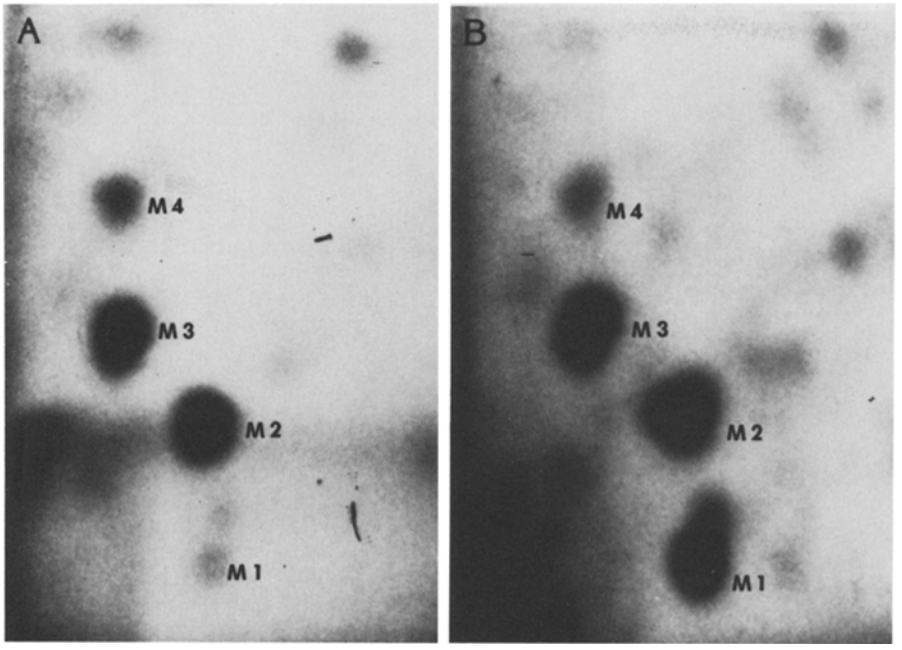

Fig. 3 A, B. ^{32}P-postlabeling of mammary DNA of rats treated with 20 mg/kg DNR for 2 days and 200 mg/kg DNR on the 3rd day (**B**). Mannitol was injected into control rats (**A**). DNA was isolated and analyzed by the nuclease P_1 procedure of the ^{32}P-postlabeling assay as described previously [12]. Labeled adducts were located by autoradiography for 16.5 h at –70° C using Kodak XAR-5 film and Dupont Lightning Plus intensifying screens. Adducts are lettered to the *right* of each spot

Table 3. RAL values of ^{32}P-labeled endogenous adducts in liver DNA of vehicle- and DNR-treated female Sprague-Dawley rats (3 rats/group)

Spot number (Fig. 3)	RAL × 10^9	
	Vehicle-treated (Fig. 3 A)	DNR-treated (Fig. 3 B)
2	1.2 ± 1.2	4.6 ± 0.6*
3	3.5 ± 1.2	4.3 ± 6.5
4	2.1 ± 1.2	3.8 ± 1.8
5	1.2 ± 0.5	0.7 ± 0.3
6	3.3 ± 0.5	4.0 ± 1.1
7	9.3 ± 5.8	6.01 ± 0.7
8	3.1 ± 2.4	17.5 ± 9.1*
9	1.9 ± 0.8	3.4 ± 0.6*
Total	26.0 ± 7.2	39.5 ± 12.9*

The adduct spots shown in Fig. 2 were cut from the thin-layer sheets, and the radioactivity content was content was determined by Cerenkov counting. Counts obtained from appropriate background areas served as controls and were subtracted. RAL × 10^9 values are means ± SD for the indicated spots shown in Fig. 3 ($n = 3$)

* RAL values significantly higher than those in vehicle-treated DNA as determined by *t*-test ($P < 0.05$)

Table 4. RAL values of ^{32}P-labeled endogenous adducts in mammary epithelial-cell DNA of vehicle- and DNR-treated female Sprague-Dawley rats (3 rats/group)

Spot number (Fig. 3)	RAL × 10^9	
	Control (Fig. 3 A)	DNR-treated (Fig. 3 B)
M1	1.2 ± 0.6	5.3 ± 3.4*
M2	4.1 ± 1.9	15.5 ± 13.8*
M3	7.1 ± 2.2	8.6 ± 5.2
M4	0.9 ± 0.4	3.8 ± 0.9*
Total	12.1 ± 4.4	37.7 ± 6.9*

The adduct spots shown in Fig. 3 were cut from the thin-layer sheets, and the radioactivity content was determined by Cerenkov counting. Counts obtained from appropriate background areas served as controls and were subtracted. RAL × 10^9 values are means ± SD for the indicated spots ($n = 3$)

* RAL values significantly higher than those of the corresponding spots in control DNA as determined by *t*-test ($P < 0.05$)

adducts increased in response to DNR treatment. Despite the treatment of Sprague-Dawley rats with very high doses of DNR, no DNR-DNA adduct could be detected in liver or mammary DNA.

Discussion

The new spots observed in ^{32}P-postlabeling maps of DNA incubated with DNR, microsomes, and cofactor (spots D1–D4) most likely represent covalent DNR-DNA adducts. Thus, direct covalent binding of DNR to DNA is demonstrated in this study as a third possible pathway of interaction of this drug with DNA in addition to intercalation and to damage by free radicals generated by redox cycling. These adducts are barely detectable in the absence of cofactor (Fig. 1D) and are therefore probably formed by a reactive metabolite of DNR. The structure of this metabolite capable of covalent DNA binding has not yet been elucidated. These DNR-DNA adducts may contribute at best marginally to carcinogenesis by the parent drug because concentrations of these adducts in vitro are very low (2–6 × 10^9 RAL) despite optimal incubation conditions. Moreover, the adducts could not be detected in vivo although the animals received very large doses of the drug.

The DNR-induced increases in concentrations of endogenous DNA modifications (I-spots), observed both in vitro and in vivo, may be biologically more significant than

the DNR-DNA adducts discussed above. Levels of endogenous adducts have previously been modulated in vivo by dietary factors [19], estrogen treatment prior to hormonal carcinogenesis [20], and other factors [21]. Our data demonstrate a modulation of endogenous adduct concentrations in vitro and in vivo, i.e., an increase in concentrations of several I-spots by incubation of DNA with DNR and a microsomal activating system and by treatment of rats with DNR. This enhancement of I-spots in vitro or in vivo has likely been mediated by free radicals generated by cytochrome P450-catalyzed redox cycling of DNR. This hypothesis is supported by the enhancement of I-spots in vitro by nickel(II) or copper(II) ions and hydrogen peroxide or in vivo by injection of nickel chloride into mice [22–24]. In that system, nickel or copper is proposed to act as a catalyst for the generation of free radicals in a Fenton-type reaction. Likewise, microsome-mediated redox cycling of DNR is known to generate free radicals [5–7], which may increase concentrations of endogenous DNA adducts in vitro or in vivo by processes similar to those known for radicals generated by metal ions and hydrogen peroxide. The structure of endogenous adducts and their origins remain to be determined in future studies.

The largest increase in the concentration of endogenous DNA adducts occurred in mammary cells, the target of DNR-induced carcinogenesis, whereas the changes in hepatic adduct levels were not as pronounced. This target-specific increase in endogenous DNA modification by DNR in vivo is consistent with a role of these DNA adducts in carcinogenesis. The endogenous adducts may have accumulated in mammary DNA by enhanced free-radical generation via redox cycling of DNR. The significantly higher relative frequencies of single-strand breaks observed in rat mammary epithelial cells as compared with hepatocytes exposed to identical doses of DNR [9] also support the proposed role of free-radical-generated DNA alterations in DNR-induced carcinogenesis. The enhancement of endogenous DNA adducts by DNR may operate in combination with other free-radical-induced changes such as strand breaks and cross-links as a possible cause of DNR-induced cancer.

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