Biochemical Pharmacology, Vol. 44, No. 11, pp. 2165–2174, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

THE CONSEQUENCES OF DOXORUBICIN QUINONE REDUCTION *IN VIVO* IN TUMOUR TISSUE

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(*Received* 26 *May* 1992; *accepted* 28 *August* 1992)

Abstract—A clear role for quinone reduction in the mechanism of action of doxorubicin has still to be established. There are three possible outcomes of this form of doxorubicin metabolism: (1) drug free radical formation, redox cycling and generation of reactive oxygen species (ROS) resulting in lipid peroxidation and DNA damage; (2) covalent binding of reactive drug intermediates to DNA; and (3) formation of an inactive 7-deoxyaglycone metabolite. In this work, the occurrence of each of these pathways has been studied *in vivo* in a subcutaneously growing rat mammary carcinoma (Sp 107). Doxorubicin was administered by direct intratumoural injection either as the free drug or incorporated in albumin microspheres (10–40 μm diameter). There was no evidence of an increase in lipid peroxidation over background after either treatment at any time point studied. In fact, doxorubicin administration resulted in a statistically significant reduction in lipid peroxidation at the later time points studied compared to control (no drug treatment), e.g. 24 hr: control, 21.7 ± 2.8 SD nmol malondialdehyde/g tissue; free doxorubicin (70 μg drug), 14.5 ± 4.0 SD nmol/g ($P < 0.01$ Student's *t*-test) and doxorubicin microspheres (70 μg drug), 17.4 ± 1.1 nmol/g ($P < 0.05$). Covalent binding to DNA was measured by a ^{32}P-post-labelling technique. Low levels of four putative drug–DNA adducts were detected; however, there were no qualitative or quantitative differences in profiles between free drug and microspheres. High 7-deoxyaglycone metabolite concentrations comparable to the parent drug itself were detected after administration of microspheres (3.0 μg/g ± 1.7 SD at 24 hr and 3.1 μg/g ± 1.1 SD at 48 hr). In contrast, these metabolites were present at levels close to the limit of detection of our HPLC assay after free drug (0.04 μg/g ± 0.03 SD at 24 hr and 0.02 μg/g ± 0.03 SD at 48 hr). Thus, 7-deoxyaglycone metabolite formation can occur in tumour tissue (indicating active drug quinone reduction) without concomitant increases in the level of lipid peroxidation or the levels of drug–DNA adducts. In conclusion, the main biological consequence of doxorubicin quinone reduction *in vivo* in tumour tissue would appear to be drug inactivation to a 7-deoxyaglycone metabolite rather than drug activation to DNA reactive species or ROS.

Despite over 20 years of investigation the mechanism of the antitumour action of doxorubicin still remains to be established. Four possible candidates have emerged based largely on *in vitro* studies: (a) DNA intercalation and stabilization of a drug–nucleic acid–topoisomerase II ternary complex referred to as the cleavable complex [1]; (b) enzyme catalysed and iron-mediated free radical formation resulting in lipid peroxidation and DNA damage probably mediated via the hydroxyl radical ($OH^{\cdot}$) [2]; (c) covalent binding to DNA by reactive drug species [3]; and (d) interaction with the phospholipid bilayer of the plasma cell membrane producing an over-stimulation of signal transduction pathways [4]. The evidence for and against each of these has been reviewed recently [5]. Two of the above mechanisms, free radicals (b) and covalent binding (c), are dependent on prior activation of the drug by metabolism to produce a semi-quinone drug free radical. Although, the processes of doxorubicin quinone reduction are complex and controversial there are three generally accepted end points and these are illustrated in Fig. 1. However, whether these actually occur *in vivo* is still debatable. Pathway 1 operates under aerobic conditions: once the semi-quinone is formed it immediately redox cycles with molecular oxygen generating a cascade of damaging reactive oxygen species (ROS‖) [6]. Under anaerobic conditions the semi-quinone free radical rearranges chemically by eliminating the daunosamine sugar group to produce a series of reactive aglycone intermediates which are proposed to bind to DNA covalently (pathway 2) [7]. The third alternative is that instead of rearranging to DNA reactive species the free radical degrades directly to an inactive 7-deoxyaglycone drug metabolite (pathway 3) [8]. Whilst the latter is considered a pathway of drug inactivation, the first two are considered pathways of activation and hence are implicated in the drug's mechanism of action.

After incorporation of doxorubicin into albumin microspheres followed by direct intratumoural (i.t.) injection to the subcutaneously growing rat mammary carcinoma (Sp 107) a marked stimulation (up to 100-fold) in 7-deoxyaglycone metabolite formation

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‖ Abbreviations: ROS, reactive oxygen species; i.t., intratumoural; MDA, malondialdehyde; TBA, thiobarbituric acid; t_R, retention time.

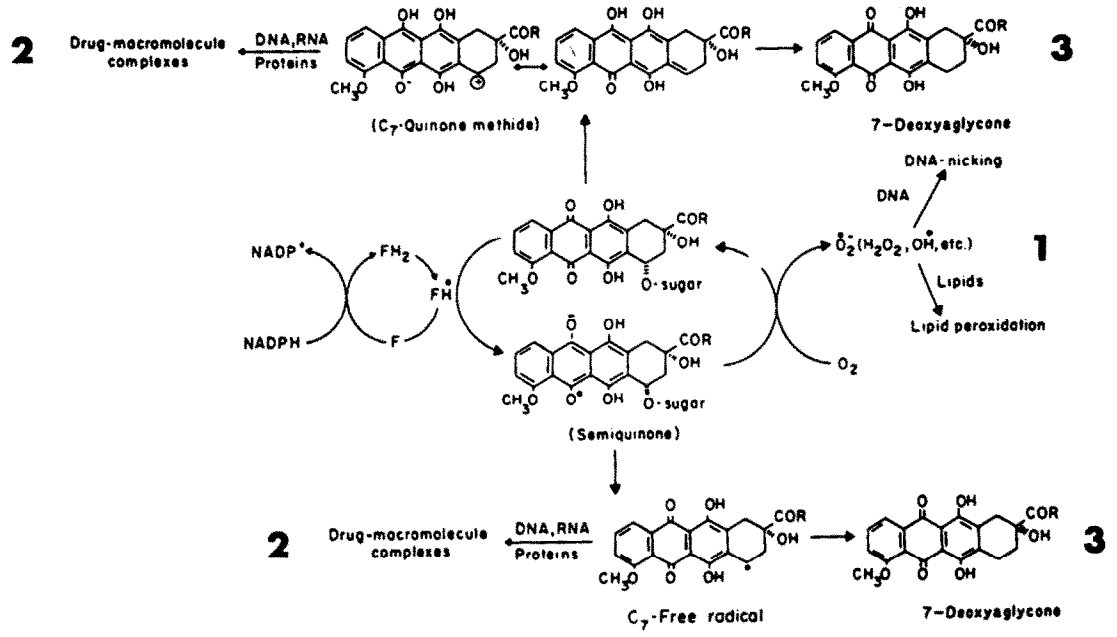

Fig. 1. The three possible outcomes of doxorubicin quinone reduction.

(pathway 3, Fig. 1) occurs in the tumour itself as compared to free drug [9]. In this paper we have extended this preliminary observation by looking simultaneously for the three possible outcomes of doxorubicin quinone reduction in the Sp 107 mammary carcinoma after i.t. drug administrations. Of particular interest was to examine whether the enhanced 7-deoxyaglycone metabolite formation induced by the albumin microspheres was also coupled to increases in lipid peroxidation or drug–DNA modification. From this point of view the Sp 107 mammary carcinoma and the microspheres have been used as a model to study the consequences of doxorubicin quinone reduction *in vivo* in tumour tissue. The i.t. injection was originally chosen to replicate, as closely as is technically feasible in a small animal, local arterial injection of microspheres (and their subsequent chemoembolization in the tumour-bearing target organ) which is their normal route of administration in larger animal models and in man [10, 11]. The i.t. injection has been validated previously as reflecting accurately the comparative chemosensitivity of the Sp 107 to a series of anthracyclines and for demonstrating a linear dose–response curve with doxorubicin [12].

MATERIALS AND METHODS

Materials. Spermidine, zinc chloride, sodium succinate, calcium chloride, sodium acetate, magnesium chloride, dithiothreitol, single-stranded DNA from calf thymus, bovine serum albumin, 2′-deoxyadenosine 3′-monophosphate, 2′-deoxycytidine 3′-monophosphate, 2′-deoxyguanosine 3′-monophosphate, thymidine 3′-monophosphate, spleen phosphodiesterase, micrococcal nuclease, nuclease P1, thiobarbituric acid (TBA) and glutaraldehyde were all from the Sigma Chemical Co. (Poole, U.K.). Polynucleotide kinase (mutant form without 3′-phosphatase activity) and polynucleotide kinase (wild type with 3′-phosphatase activity) were from BCL (Mannheim, Germany). [γ-^{32}P]ATP tetra (triethylammonium) salt specific activity 3000 Ci/nmol was from the New England Nuclear division of Dupont (Stevenage, U.K.). Pure doxorubicin hydrochloride salt was from Farmitalia Carlo Erba Ltd (Milan, Italy), doxorubicin 7-deoxyaglycone, doxorubicinol 7-deoxyaglycone and other metabolite standards were as described previously [13], and daunorubicin was from May and Baker (Dagenham, U.K.). Cytochrome P450 reductase was purified by affinity chromatography [14] and malondialdehyde (MDA) tetramethyl acetal was synthesized in house. Albumin microspheres incorporating doxorubicin were prepared by stabilization through cross-linking of protein by glutaraldehyde of a water-in-oil emulsion as described fully elsewhere [15]. Microsphere diameter of individual preparations varied from 15 to 40 μm, 50% weight average by laser diffraction measurements and the content of doxorubicin was 1–3% (w/w). Since drug loading efficiency of microspheres varied from preparation to preparation drug content could not be predicted in advance and had to be determined by HPLC retrospectively after a sample had been digested with trypsin. All solvents/buffers for HPLC were HPLC reagent grade and all other chemicals, reagents and buffers were of the highest grade available commercially. Water was deionized and bidistilled in a quartz glass still.

Animal model and in vivo *drug treatments.* The animal model consisted of inbred rats of the WAB/

NOT strain and the syngeneic, undifferentiated mammary carcinoma (Sp 107), a tumour that originally arose spontaneously in a female rat [16]. Tumours were transplanted s.c. into the flank of animals. For all measurements (doxorubicin and 7-deoxyaglycone metabolite concentrations, lipid peroxidation levels and ^{32}P-post-labelling studies) tumours were allowed to grow to 2.5 g before injection of drug as described in Results. At different time intervals (see Results) animals were killed, and tumours and livers excised and immediately frozen on solid CO_2 prior to analysis.

Drug analysis. Doxorubicin and 7-deoxyaglycone metabolite concentrations were determined by isocratic, reversed-phase HPLC with fluorescence detection using daunorubicin as internal standard [13]. Prior to HPLC, tumours or livers were homogenized in 3 vol. (w/v) phosphate-buffered saline, treated with 33% (w/v) silver nitrate (0.2 mL/mL of homogenate) and extracted with 5 vol. (v/v) chloroform–propan-2-ol (2:1) [17].

Lipid peroxidation. Peroxidation of lipids was measured by detection of MDA through reaction with TBA essentially according to the method of Sunderman *et al.* [18] except that samples were finally subjected to HPLC to reduce background interference from other common biological molecules which can react with TBA [19].

Once thawed whole tumours were immediately homogenized in 10 mL cold potassium chloride (KCl, 0.154 M). Aliquots (0.25–1 mL) were then added to 3 mL of cold orthophosphoric acid and made up to a total volume of 4 mL with cold KCl. Elapsed time from beginning of homogenization to acidification never exceeded 15 min. To this solution was added 1 mL of TBA (30 mM) and samples were then incubated at 100° for 45 min. After cooling samples were extracted with 4 mL butanol for 1 min. The butanol layer was separated and stored at −20° prior to HPLC. HPLC was performed as follows. The stationary phase was Lichrosorb RP-18 (Merck, Darmstadt, Germany) prepacked in a 25 cm long, 4 mm internal diameter stainless steel column, and the mobile phase was 1 M orthophosphoric acid: water:propan-2-ol:acetonitrile (5:345:50:100). Elution was isocratic at a flow rate of 1 mL/min and detection was by fluorescence at 532 nm excitation and 553 nm emission. Quantitation was by reference to a standard curve of known MDA tetramethyl acetal concentrations which were put through the assay from the point of acidification with phosphoric acid and was linear over the range 0–10 nmol.

^{32}P-Post-labelling studies. DNA for post-labelling was extracted from tumours by the method of Marmur [20] with the inclusion of proteinase K to assist in the separation of DNA from protein. A post-labelling procedure was developed from methods described already [21–23]. Briefly, the procedure requires four separate enzymic incubations which were all carried out at 37°. DNA (10 μg) at a concentration of 1.66 μg/μL was first digested in a total volume of 12 μL with micrococcal nuclease (4.2 U) and spleen phosphodiesterase (40 mU) at pH 6.0. The sample was then incubated with nuclease P_1 (1.6 U) to concentrate modified nucleotides by removing the 3′-phosphate group from unmodified nucleotides only. The third step was the labelling stage using polynucleotide kinase (mutant form) (10 U) in order to transfer γ-^{32}P-phosphate from ATP to available 3′-monophosphates. Finally, the sample was incubated with polynucleotide kinase (wild type) (5 U) to remove the 3′-monophosphate from the bisphosphates produced, in order to facilitate HPLC.

HPLC of the modified labelled nucleotides was performed as follows. The stationary phase was a 25 cm by 4.6 mm diameter stainless column packed with 7 μm Adsorbosphere Nucleotide-Nucleoside (Alltech, Carnforth, U.K.). Gradient elution was employed at a flow rate of 2 mL/min, at ambient room temperature. Buffer A was 60 mM ammonium dihydrogen phosphate and 5 mM tetrabutylammonium phosphate, pH 5.0; buffer B was 5 mM tetrabutylammonium phosphate in methanol. The starting proportion of the gradient at time zero was 0% B (100% A) which was increased linearly to 36% B over 28 min, and samples were injected every 38 min. Prior to HPLC samples were centrifuged at 4500 *g* for 1 hr through regenerated cellulose membranes which had a 10,000 nominal M_r limit (Amicon, Stonehouse, U.K.), diluted and 100 μL was injected onto the column. ^{32}P-Labelled chromatrographic peaks were detected by a continuous on-line radioactivity monitor connected to an IBM computer for data storage and evaluation.

RESULTS

Lipid peroxidation, doxorubicin disposition and metabolism to 7-deoxyaglycones

Table 1 shows the values for lipid peroxidation measured in the liver and Sp 107 tumour after control treatments and intravenous (tail vein injection) doxorubicin administration. Table 2 shows the corresponding concentrations of doxorubicin and 7-deoxyaglycone metabolites measured in the liver and Sp 107 tumour after the same drug exposure. Halothane anaesthesia, which has been reported to cause liver damage possibly by lipid peroxidation of endoplasmic reticulum [24] was used for all drug administrations but not for control saline injections. Under our conditions of light anaesthesia, halothane had no effect on endogenous levels either in the liver or tumour (data not shown). Nickel chloride was used as a positive control to stimulate lipid peroxidation. At a dose of 0.15 mmol it increased the concentration of TBA-reactive material in the liver 2-fold (Table 1), which is in accordance with previously published data [18]. No increase was recorded in the tumour. An i.v. injection of 5 mg/kg doxorubicin is the maximum tolerated dose which can be administered to the rat model and inhibits tumour growth (2–3 days growth delay). Intravenous doxorubicin at 5 mg/kg did not stimulate lipid peroxidation over control values either in the tumour or the liver despite production of significant concentrations of 7-deoxyaglycone metabolites in the latter (Table 2). 7-Deoxyaglycones were not detected in the Sp 107 tumour and this is consistent with data generated with another s.c. rat tumour, the MC40A [25]. Studies with anaerobic rat liver microsomes have shown that 7-deoxyaglycones are

Table 1. Levels of lipid peroxidation in liver and the Sp 107 tumour after intravenous administration of doxorubicin

Time (hr)	Control saline		Nickel chloride (0.15 mmol)		Doxorubicin (5 mg/kg)	
	Liver (nmol MDA/g)	Tumour (nmol MDA/g)	Liver (nmol MDA/g)	Tumour (nmol MDA/g)	Liver (nmol MDA/g)	Tumour (nmol MDA/g)
1	61.9 ± 2.5	22.4 ± 1.1	—	—	60.6 ± 1.8	23.4 ± 5.1
24	77.3 ± 12.6	20.9 ± 4.1	146.1 ± 12.1†	15.4 ± 2.1	58.6 ± 6.2	16.8 ± 2.3*

Rats bearing approximately 2.5 g tumours were dosed as above. At 1 and 24 hr livers and tumours were collected and assayed for lipid peroxidation levels as described in Materials and Methods.

Each value represents the mean ± SD for three to five animals per time point.

* $P < 0.05$, Student's t-test compared to an overall control arrived at by taking the mean value for control saline at 1 and 24 hr (21.7 ± 2.8 SD).

† $P < 0.01$, Student's t-test compared to 24 hr control saline value.

Table 2. Doxorubicin and 7-deoxyaglycone metabolite concentrations in the liver and Sp 107 tumour after an intravenous dose of 5 mg/kg drug

Time (hr)	Liver		Tumour	
	Parent drug (μg/g)	7-Aglycones (μg/g)	Parent drug (μg/g)	7-Aglycones (μg/g)
1.0	9.2 ± 0.7	1.7 ± 0.2	1.9 ± 0.1	<0.01
24	1.3 ± 0.5	0.1 ± 0.01	1.1 ± 0.1	<0.01

Doxorubicin and 7-deoxyaglycone metabolites (7-aglycones) were measured by HPLC as described in Materials and Methods.

Each value represents the mean ± SD for three to five separate determinations per time point.

Table 3. Levels of lipid peroxidation in the Sp 107 rat mammary carcinoma after intratumoural administration of different doxorubicin treatments

Time (hr)	Doxorubicin (70 μg) (nmol MDA/g)	Doxorubicin + empty microspheres (70 μg) (nmol MDA/g)	Doxorubicin incorporated in microspheres (70 μg) (nmol MDA/g)
0.1	19.8 ± 2.7	18.5 ± 4.8	21.4 ± 1.8
24	14.5 ± 4.0*	11.8 ± 4.7*	17.4 ± 1.1*†
48	16.1 ± 1.8*	12.3 ± 5.3*	22.8 ± 12.2

Rats bearing approximately 2.5 g tumours were dosed as above. At 0.1, 24 and 48 hr tumours were collected and assayed for lipid peroxidation levels as described in Materials and Methods.

Each value represents the mean ± SD for three to five animals per time point.

* $P < 0.01$, Student's t-test compared to an overall control arrived by taking the mean value for control saline at 1 and 24 hr (21.7 ± 2.8 SD).

† $P < 0.05$, Student's t-test compared to value at 0.1 hr.

formed by a linear sequential pathway where doxorubicin is first converted to doxorubicin 7-deoxyaglycone and then to doxorubicinol 7-deoxyaglycone [26, 27]. Subsequently, this pathway has been shown to operate in whole animals and man [17], and in the Sp 107 tumour [9]. For clarity, all data presented on 7-deoxyaglycones in Tables 2 and 4 are the combined values for the two metabolites.

Levels of lipid peroxidation measured in the Sp 107 tumour after i.t. injection of doxorubicin are contained in Table 3 and the corresponding values for doxorubicin parent drug and 7-deoxyaglycone metabolite concentrations are to be found in Table 4. Intratumoural administration of free doxorubicin at a similar dose level to that used in this present work produces a growth delay in the Sp 107 tumour of 6.5 days [9]. This effect is achieved at

Table 4. Doxorubicin and 7-deoxyaglycone metabolite concentrations in the Sp 107 tumour after different intratumoural drug treatments

Time (hr)	Doxorubicin (70 μg) Parent drug (μg/g)	Doxorubicin (70 μg) 7-Aglycones (μg/g)	Doxorubicin + empty microspheres (70 μg) Parent drug (μg/g)	Doxorubicin + empty microspheres (70 μg) 7-Aglycones (μg/g)	Doxorubicin incorporated in microspheres (70 μg) Parent drug (μg/g)	Doxorubicin incorporated in microspheres (70 μg) 7-Aglycones (μg/g)
0.1	7.5 ± 2.3	<0.01	10.3 ± 3.4	<0.01	8.2 ± 1.4	<0.01
24	4.3 ± 2.4	0.04 ± 0.03	4.9 ± 2.2	<0.01	3.6 ± 0.5	3.0 ± 1.7
48	2.6 ± 1.0	0.02 ± 0.03	4.1 ± 2.4	<0.01	3.9 ± 1.3	3.1 ± 1.1

Doxorubicin and 7-deoxyaglycone metabolites (7-aglycones) were measured by HPLC as described in Materials and Methods.

Each value represents the mean ± SD for three to five separate determinations per time point.

approximately 4-fold higher parent drug concentrations in the tumour compared to i.v. administration of 5 mg/kg but without any appreciable formation of 7-deoxyaglycones (compare Tables 2 and 4). Three treatments were given to animals by i.t. injection: free drug, free drug admixed with non-drug containing preformed microspheres (empty microspheres) and drug incorporated in microspheres. Each achieved similar levels of the parent drug at the three time points studied (Table 4) and each produced a similar tumour growth delay (free doxorubicin, 6.5 days; doxorubicin plus empty microspheres, 5.5 days; doxorubicin incorporated in microspheres 7.4 days) after equivalent doses [28]. Only the drug-loaded microspheres stimulated the production of 7-deoxyaglycones (155-fold increase in concentration at 48 hr compared to drug free in solution) and to a level significantly higher than the liver after i.v. drug ($P < 0.01$ at 48 hr, $P < 0.05$ at 24 hr, Student's *t*-test). However, with all three treatments no evidence was detected of an increase in TBA-reactive material. In contrast, there was actually a marked decrease in endogenous levels of lipid peroxidation in the tumour after all doxorubicin treatments (i.t. and i.v.), both with time and compared to an overall control value arrived at by taking the mean of the 1 and 24 hr control saline injection data presented in Table 1 (21.65 ± 2.8 SD). This effect reached statistical significance in several cases (see Tables 1 and 3).

DNA covalent binding by ^{32}P-post-labelling

Post-labelling experiments were performed in two separate groups of animals only after i.t. drug treatments and at 48 hr, the time of maximum production of 7-deoxyaglycones in the tumour after administration of microspheres [9]. In the first group ($N = 5$) the dose of doxorubicin was 158 μg for solution and 185 μg for drug-loaded microspheres; the control received physiological saline. In the

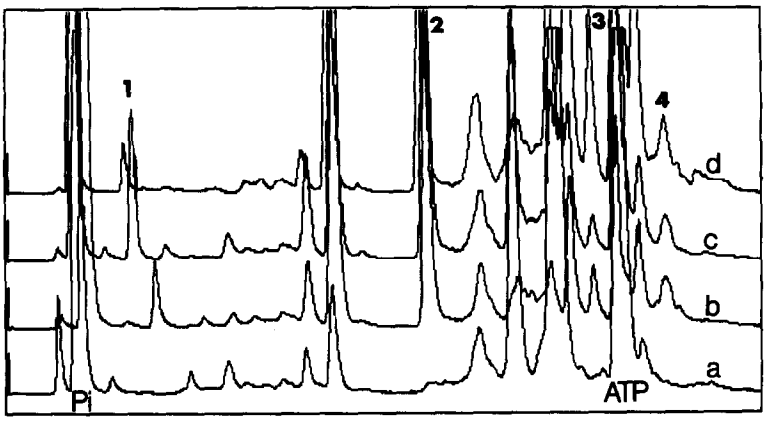

Fig. 2. Detection of doxorubicin–DNA covalent adducts *in vivo* by ^{32}P-post-labelling utilizing HPLC. DNA (10 μg) extracted from the Sp 107 rat mammary carcinoma was post-labelled as described in Materials and Methods. Chromatogram (a) control tumour injected i.t. with 0.4 mL saline; (b) tumour injected i.t. with 0.4 mL of doxorubicin (185 μg) incorporated in albumin microspheres (10–40 μm diameter); (c) separate tumour injected with same dose of drug-loaded microspheres and (d) tumour injected i.t. with 0.4 mL of free doxorubicin (158 μg). Tumours were collected 48 hr after drug treatments, at the time of maximum production of 7-deoxyaglycone drug metabolites. Four putative adduct peaks (1–4) are identified which were absent in control tumours. Time frame (*x*-axis) is 28 min; *y*-axis is radioactivity.

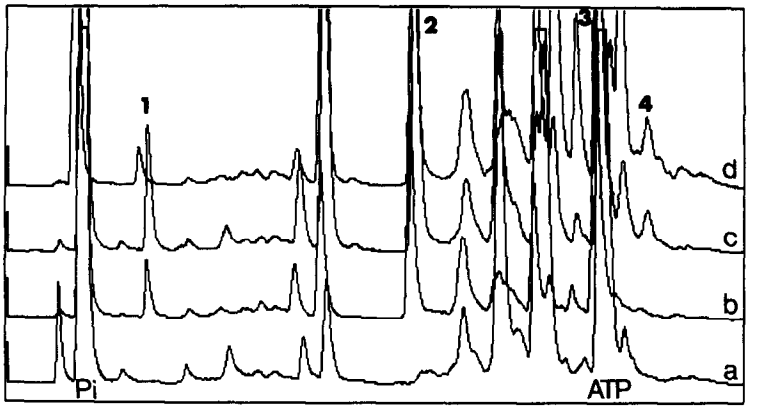

Fig. 3. Detection of doxorubicin–DNA covalent adducts *in vivo* by ^{32}P-post-labelling utilizing HPLC. Repeat but separate analyses of the tumour specimens shown in Fig. 2 in order to illustrate the reproducibility of the post-labelling method employed. Experimental procedures and labelling of chromatograms and axes as in Fig. 2.

second group (N = 9) the dose of doxorubicin was 160 μg for solution and drug-loaded microspheres. Figure 2 shows typical HPLC chromatograms from the first study where tumour DNA specimens: a control (chromatogram a), two microsphere-treated (chromatograms b and c) and one free drug-treated (chromatogram d), have been overlayed. Four peaks are identified in drug-treated specimens which were absent in the control: peak 1 with a retention time (t_R) of 4.7 min; peak 2, 15.3 min; peak 3, 21.4 min and peak 4, 24.3 min. ATP had a t_R of 22.6 min and is indicated along with ^{32}P-P$_i$ (2.7 min). Retention times for labelled DNA nucleobase 5′-monophosphate standards are: dC, 4.0 min; dG, 11.3 min; T, 13.3 min and dA, 19.7 min. Peak 4 (t_R 24.3 min) eluted later than ATP and the four monophosphate standards and may possibly be related to incomplete digestion of DNA. Several repeat labelling and chromatographic analysis of DNA samples extracted from the same tumours consistently produced evidence of these four putative nucleotide–doxorubicin adducts with good reproducibility. This is illustrated in Fig. 3 which represents a replicate analysis of the four specimens shown in Fig. 2. Almost identical adduct profiles were recorded with the possible exception of adduct peak 4 in chromatogram b which was reduced in height. No marked quantitative or qualitative differences in profiles were evident between free drug and microspheres. Typical chromatograms from the second study are shown in Fig. 4 where: chromatogram a is a control; chromatogram b, microspheres and chromatogram c, free drug. Evidence for specific doxorubicin–DNA adducts in drug-treated tumours compared to controls is less striking.

To further characterize doxorubicin–DNA adducts by ^{32}P-post-labelling two additional studies were performed *in vitro*. In the first, 50 or 100 μM doxorubicin were incubated with purified cytochrome P450 reductase in the presence of single-stranded DNA. In the second, 100 μM doxorubicin was incubated with 1 mM formaldehyde (HCHO) in the presence of single-stranded DNA. HCHO has been shown recently to cross-link daunorubicin to DNA hexamers by forming a covalent methylene bridge between the N3 position of daunosamine (on the drug) and the N2 of guanine or 2-aminoadenine [29]. In the cytochrome P450 reductase studies some evidence of formation of adduct peak 1 was detected but no other modification was present (data not shown). HCHO did not appear to cross-link doxorubicin as evidenced by a lack of modified nucleotides (Fig. 5).

DISCUSSION

In this work, the occurrence of the three proposed consequences of doxorubicin quinone reduction (see Fig. 1) have been studied *in vivo* in tumour tissue. Pathway 1, ROS generation, was measured indirectly as lipid peroxidation by HPLC. Using this methodology, a pronounced decrease in endogenous levels was detected in the Sp 107 tumour at the later time points studied (24 and 48 hr) after all forms of doxorubicin treatment from i.v. free drug to i.t. drug-loaded protein microspheres (maximal effect approaching a 100% reduction compared to control). In contrast, no effect was evident in the liver after i.v. drug. ROS generation and subsequent peroxidation of membrane phospholipids result as a byproduct of aerobically metabolizing cells [30]. The different dose schedules of doxorubicin used in this study all produced a significant antitumour response [28] and consequently are likely to influence the number of metabolically active cells present in the total tumour population. This may explain the reduction observed. In a sister paper [31], we have also shown in the same rat/tumour model that i.t. doxorubicin markedly reduces the activity of three major quinone reductase enzymes (DT-diaphorase, NADPH cytochrome P450 reductase and NADH cytochrome b_5 reductase) by at least 2-fold. It is less likely that doxorubicin is lowering endogenous lipid

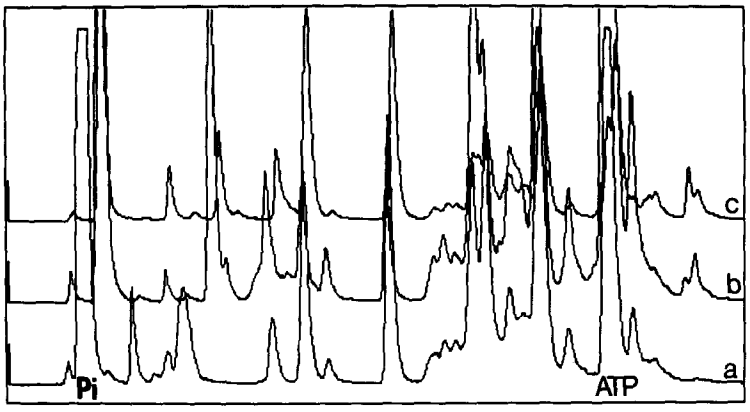

Fig. 4. Detection of doxorubicin–DNA covalent adducts *in vivo* by ^{32}P-post-labelling utilizing HPLC. Experimental procedures as in Fig. 2 except that the analysis was performed on a new set of tumours from a different group of animals. Tumours were collected at 48 hr. Chromatogram (a) control tumour injected i.t. with 0.4 mL saline; (b) tumour injected i.t. with doxorubicin (160 μg) incorporated in albumin microspheres (10–40 μm diameter) and (c) tumour injected i.t. with doxorubicin (160 μg) free in solution. In this study putative adduct peaks are less evident. Axes as in Fig. 2.

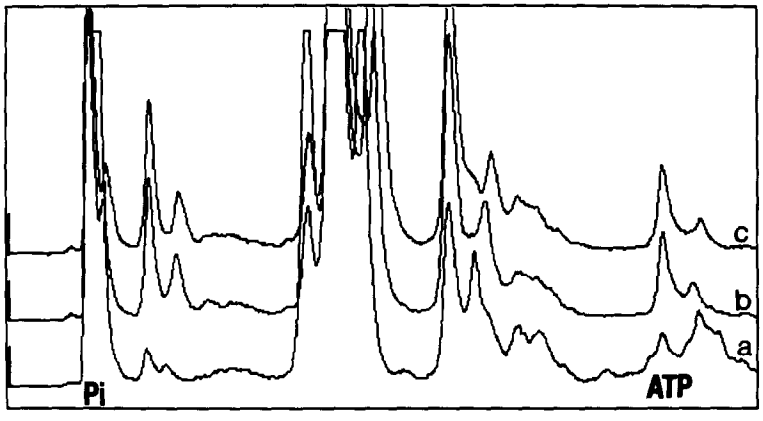

Fig. 5. Detection of doxorubicin–DNA covalent adducts by ^{32}P-post-labelling utilizing HPLC. Single-stranded calf thymus DNA (67.6 μg) was incubated with 100 μM doxorubicin and 1 mM formaldehyde in a total volume of 2 mL for 3 hr at 37°. At the end of the incubation the sample was placed on an Amicon 30 micro-concentrator and washed three times with 2 mL H_2O, and then washed repeatedly with 60% ethanol until the absorbance at 490 nm (doxorubicin) remained steady. For post-labelling the first two enzyme incubations (see Materials and Methods) took place on the filter membrane and 36 μL was taken for labelling. Chromatogram (a) calf thymus DNA incubated with 1 mM formaldehyde minus doxorubicin, control; (b) calf thymus DNA, 1 mM formaldehyde, 100 μM doxorubicin; (c) separate labelling of calf thymus DNA, formaldehyde and doxorubicin. No drug adduct peaks evident. Time frame (x-axis) is 22 min.

peroxidation levels by acting as a free radical scavanger since the drug can spontaneously generate oxygen-derived reactive species in its own right [32]. A lack of an increase in lipid peroxidation in both the liver and tumour after i.v. doxorubicin (and all i.t. treatments) corroborates the findings of several whole animal studies published in the past [33–35].

Of major interest was the observation that i.t. doxorubicin-loaded microspheres did not produce an elevation in lipid peroxidation in the tumour when they stimulated 7-deoxyaglycone metabolite formation by up to 155-fold. To protect against the toxic effects of ROS cells have evolved a large number of antioxidant defense mechanisms ranging from detoxification enzymes such as superoxide dismutase to small molecule free radical scavangers like vitamin E. The net result of a drug-induced ROS insult in a particular tissue will depend upon both its capacity to generate the reactive species and its ability to dispose of them once formed [30]. Tumour cells tend to have a lower content of radical detoxifying enzymes and free radical scavangers than

normal tissues [36–38] and should therefore be more sensitive to the deleterious effects of ROS. Since there was no increase in lipid peroxidation, it is concluded that active quinone reduction of doxorubicin in the Sp 107 tumour *in vivo* does not result in a significant burst of ROS. This conclusion is corroborated by our recent findings which show that the Sp 107 tumour has only a limited capability to support doxorubicin quinone reduction (30–40-fold less activity than rat liver microsomes [31]).

At least three different routes have been postulated by which doxorubicin can bind to DNA covalently: (1) aerobic quinone reduction and direct addition of the semi-quinone free radical [39], although this is unlikely due to the high instability of the radical, its limited ability to diffuse through the cell and its preferential reactivity with molecular oxygen [40]; (2) chelation of iron followed by irreversible binding of the ternary complex to DNA [41]; and (3) anaerobic quinone reduction to a quinone methide aglycone or a C-7-centred radical aglycone [42]. Low levels of doxorubicin covalent binding to DNA (pmol/100 μg nucleic acid) have been demonstrated *in vitro* by several different groups using a number of different activating systems: chemical reducing agents, microsomes, isolated nucleii and cells in culture [43]. In all these studies non-physiological drug concentrations (up to 1 mM) were a pre-requisite to measure covalent binding and drug associated with intact DNA was quantitiated by indirect methods (spectrophotometry or radio-actively labelled drug) where extensive sample preparation procedures had to be applied to remove non-covalently bound intercalated drug. Since individual adducts were never, or could never, be identified a question mark remains against the validity of these observations [44].

We have attempted to measure doxorubicin covalent binding to DNA *in vivo* in tumour tissue after administration of a therapeutic dose of drug and identify individual adducts by ^{32}P-post-labelling. In the first group of animals studied, low levels (approximately one adduct per 10^8 nucleotides) of four putative adducts were detected. The major peak had a retention time close to but later than a labelled deoxyguanosine 5'-monophosphate stand-ard and may be related to a dG monofunctional adduct. From *in vitro* studies, doxorubicin has been proposed to form DNA adducts with the highest frequency to dG [45]. No quantitative or qualitative increases in DNA modification levels were observed after i.t. injection of doxorubicin-loaded albumin microspheres when the formation of 7-deoxy-aglycones was stimulated by 155-fold. We believe this indicates that adduct formation was probably not dependent on quinone reduction in the first place but could be due to complexation of iron. A recent *in vitro* study, using quantitation of stable transcriptional blockage sites to measure DNA covalent adducts of doxorubicin, has reported a dependency on the presence of iron in incubations [41]. In the second group of animals studied evidence of DNA modification was less striking.

Specific doxorubicin–DNA adducts have never been purified and characterized chemically and, therefore, their ability to survive the post-labelling procedure is unknown. Loss of adducts can often result from incomplete digestion of DNA and adduct instability [46]. From *in vitro* studies, anthracyclines cross-linked to DNA have been shown to be both temperature sensitive (100°) and alkaline (0.03 N NaOH) labile [47]. Nevertheless, the post-labelling technique used in this work does not involve procedures anywhere near as severe as those above and positive detection of specific adducts was achieved. Thus, active quinone reduction of doxorubicin *in vivo* in tumour tissue would also seem not to result in covalent binding to DNA but rather solely the formation of a 7-deoxyaglycone metabolite.

7-Deoxyaglycone metabolites are preferentially and almost exclusively formed under anaerobic conditions by a process that was originally referred to as reductive glycosidic cleavage [8]. The chemical pathways of reductive deglycoslation are now much better understood, although still debated. It has been proposed that the semi-quinone free radical degrades directly to the 7-deoxyaglycone via a C7-centred aglycone radical based on ESR detection of an immobilized signal consistent with a non-water soluble aglycone species [48]. Others have argued convincingly, on chemical groups, that the C-7 radical is not adequately reactive to alkylate DNA [49]. The majority of evidence favours anaerobic bioreductive deglycosylation proceeding through a fully reduced hydroquinone, formed either by two electron quinone reduction or as is more likely by disproportionation of the semi-quinone free radical after one electron reduction, and producing a quinone methide aglycone as intermediate (see Fig. 1) [5]. The quinone methide has a half-life of several seconds which is long enough for diffusion through the cell and alkylation of DNA [50]. However, it has only a limited capability to react with sulphydryl groups of proteins and is probably not sufficiently reactive to bind to DNA, but preferentially abstracts a solvent proton to form the 7-deoxyaglycone metabolite [51]. Therefore, anaerobic bioreduction primarily results in drug metabolism to a 7-deoxyaglycone without the evolution of DNA covalent binding species. This conclusion would appear to hold *in vivo*, from data presented in this work. In their own right 7-deoxyaglycone metabolites are inactive against tumour cells and lose the ability to bind to DNA [52]. Their formation should be considered a pathway of drug inactivation.

At present it is unknown how the microspheres are stimulating doxorubicin quinone reduction. It has been reported previously that there is a delay of 16–24 hr before high metabolite levels appear [9] which is in marked contrast to their rapid formation within minutes by anaerobic rat liver microsomes [27] and in normal tissues (liver and heart) after i.p. drug administration to mice [17]. This long time lag mitigates against the i.t. administration of microspheres inducing hypoxia by mechanically restricting blood flow which would be excepted to occur immediately after injection. Another possibility is that the high steady state drug levels and continuous drug exposure achieved by the micropheres in the tumour [9] may be resulting in enzyme induction. We rule this out because our recent results

show that i.t. doxorubicin (either free drug or microspherically bound) actually reduces the activity of all the major quinone reductases enzyme present in the Sp 107 tumour [31]. A delay of 16–24 hr is, however, consistent with the time at which the microspheres begin to be biodegraded accompanied by an inflammatory response involving phagocytic cells [15]. Either this could be inducing hypoxia, or the activated macrophages are themselves metabolizing the drug. Activated phagocytic cells display increased plasma membrane NADPH cytochrome P450 reductase which catalyses efficiently doxorubicin quinone reduction [9]. Further studies are required to understand this phenomenon more fully.

In conclusion, the main consequence of doxorubicin quinone reduction *in vivo* is now shown to be drug inactivation to a 7-deoxyaglycone metabolite without either ROS generation or the evolution of DNA binding species. This occurs at a dose level of doxorubicin which produces a significant delay in tumour growth. These data raise doubts over a role for quinone reduction in the antitumour activity of doxorubicin *in vivo*.

Acknowledgements—The authors would like to thank Eirian Wynne Jones for her technical assistance. Part of this work was supported by the Cancer Research Campaign.

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