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RELATIONSHIP BETWEEN REDUCTIVE DRUG METABOLISM IN TUMOUR TISSUE OF ANTHRACYCLINES IN MICROSPHERICAL FORM AND ANTI-TUMOUR ACTIVITY

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Abstract—Increased activity against a rat solid tumour of doxorubicin incorporated into protein microspheres and administered intratumourally was associated with both increased duration of exposure of tumour tissue to native drug and anaerobic bioreduction of doxorubicin to 7-deoxyaglycones, indicating formation of reactive drug intermediates within tumour tissue. To investigate which of these aspects of drug disposition determined activity we have compared the *in vivo* fate (clearance from and metabolism by tumour tissue) of doxorubicin in microspherical form with the analogue 4′-deoxydoxorubicin and related this to the tumour growth delay recorded for these drugs. Within the dose range 42 to 55 μg, growth delay (14–18 days) of doxorubicin in microspherical form was markedly superior to drug in solution, whereas growth delay of 4′-deoxydoxorubicin in microspherical form (4.3–7.2 days) was not greater than drug in solution. Metabolism to 7-deoxyaglycones by tumour tissue was not a prominent feature of either drug when administered in solution. However, in microspherical form both drugs were extensively metabolized (peak concentrations: 3.6 μg/g doxorubicin 7-deoxyaglycone; 2.5 μg/g 4′-deoxydoxorubicin 7-deoxyaglycone). Native drug concentrations in tumour tissue were similar after administration in microspherical form at 48 hr (doxorubicin 3.8 μg/g; 4′-deoxydoxorubicin 3.7 μg/g) and 72 hr (doxorubicin 2.4 μg/g; 4′-deoxydoxorubicin 2.7 μg/g). At both time points, following administration in microspherical form, tumour tissue concentrations of doxorubicin were significantly greater than when drug was administered in solution, whereas no significant differences were observed for 4′-deoxydoxorubicin. The results are inconsistent with the process of anaerobic bioreduction of doxorubicin to 7-deoxyaglycones being an important component of its anti-tumour activity in microspherical form and point to the importance of increased duration of exposure to native drug.

Protein microspheres of mean diameter 10–50 μm are designed to (a) localize cancer therapeutic agents via embolization in capillary beds of desired organs; (b) increase duration of exposure through sustained release of incorporated agent; and (c) biodegrade to permit multiple dosing. Targetting of microspheres to solid tumour deposits in the target organ can be achieved using the vasoactive agent angiotensin II [1, 2].

Once embolized, a number of factors will influence subsequent performance of incorporated agents; in particular, sustained release from microsphere matrix, resulting in extended duration of exposure, would be expected to alter both the drug's fate (clearance and metabolism) in tumour tissue and thereby its activity. To examine this an animal model was developed involving intratumoural administration of cancer chemotherapeutic agents either in solution or in microspherical form. It was observed that doxorubicin in microspherical form was considerably more active than a comparable dose of native drug in solution and that this was associated with increased duration of exposure of tumour tissue to the drug. Unexpectedly, it was also found that doxorubicin in microspherical form was metabolized by tumour tissue via anaerobic bioreduction to 7-deoxyaglycones [3].

For quinones such as doxorubicin, metabolism via anaerobic bioreduction could theoretically result in increased or decreased drug activity. It is clearly important to find which is the case. Therefore, as part of our programme on the optimization of delivery of cancer therapeutic agents we have incorporated 4′-deoxydoxorubicin, a closely related analogue of doxorubicin, into protein microspheres to examine the relationship in this system between drug potency and fate (clearance and metabolism) in tumour tissue.

MATERIALS AND METHODS

Animal model. Inbred rats of the WAB/Not strain and the syngeneic, undifferentiated mammary carcinoma (Sp107) were used [4]. For the growth delay studies, SC tumours of approximately 1 g, arising after inoculation of 4×10^4 viable tumour cells, were injected intratumourally with drug (doxorubicin or 4′-deoxydoxorubicin) in microspherical form or a comparable dose of drug in solution. Injection volume was 0.3 or 0.4 mL. At intervals, tumours were measured with calipers and a weight in grams derived [3]. Growth delay is defined here as the mean time in days for treated tumours to reach 12 g minus mean time in days for untreated tumours to reach 12 g.

Anthracycline analysis. For measurement of tumour tissue concentrations of doxorubicin, 4′-deoxydoxorubicin and 7-deoxyaglycone metabolites SC tumours of approximately 2 g were injected intra-

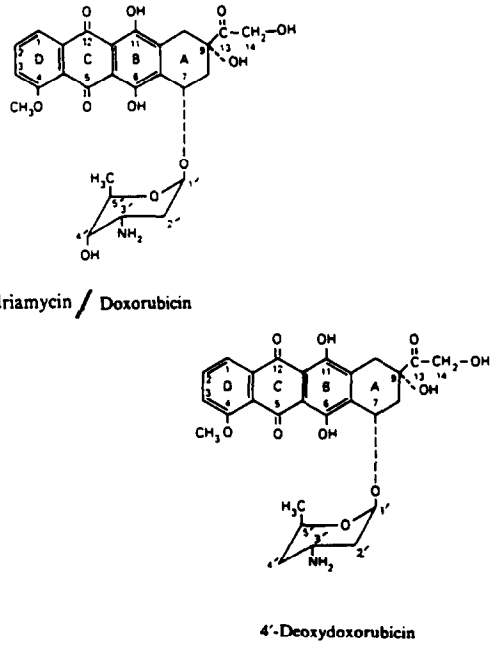

Fig. 1. Chemical structures of doxorubicin and 4'-deoxydoxorubicin. Note that aglycones of both drugs are identical.

tumourally with either doxorubicin or 4'-deoxydoxorubicin (see Fig. 1 for structures) in microspherical form or a comparable dose of drug in solution. Injection volume was 0.4 or 0.5 mL. At intervals, animals were killed, tumours excised and immediately frozen on solid CO_2 until analysis. Drug and metabolite concentrations were estimated (following homogenization of solid tumours, treatment with $AgNO_3$ to free intercalated drug and extraction with chloroform/isopropanol) by isocratic, reversed-phase HPLC with fluorescence detection using daunorubicin as internal standard [5]. Extraction efficiency was generally between 40 and 80%. Note that the aglycones of doxorubicin and 4'-deoxydoxorubicin are identical (Fig. 1). Tissue concentrations of 7-deoxyaglycones were computed assuming a molar fluorescence twice that of doxorubicin [6].

Microsphere preparation. Drug-loaded microspheres were prepared by stabilization with glutaraldehyde of a water in oil emulsion containing albumin and drug in the aqueous phase [7]. Following washing with petroleum ether, isopropanol and aqueous buffers, microspheres were ready for use. Drug content was estimated by HPLC analysis as above following digestion and solubilization of protein microspheres in trypsin. In these studies, mean diameter of microspheres was 20–50 μm (50% weight average) and native drug content 5–9 μg/mg.

Statistics. For comparisons of native drug and metabolite concentrations in tumour tissue, the two-tailed Student's *t*-test was used. In these cases, the mean and 95% confidence interval (CI) are quoted.

RESULTS

Anti-tumour activity of anthracyclines

In previous work we have shown that doxorubicin incorporated into protein microspheres has superior antitumour activity to a comparable dose of drug in solution [3]. The results in Fig. 2 demonstrate that this is a reproducible finding; thus, in this experiment 55 μg of native doxorubicin in microspherical form exhibited superior activity to a higher dose (125 μg) of drug in solution. In marked contrast, 42 μg of 4'-deoxydoxorubicin in microspherical form was not superior to a comparable dose of drug in solution (Fig. 3). In this model, when administered in solution, 4'-deoxydoxorubicin (growth delay 13.6 days; 100 μg drug) was more active than doxorubicin (growth delay 12.8 days; 125 μg), as expected from animal [8] and *in vitro* cytotoxicity [9] studies (see also Table 1 for a comparison of activities of doxorubicin and 4'-deoxydoxorubicin in solution).

Results from repeat experiments with albumin microspheres containing doxorubicin or 4'-deoxydoxorubicin are shown in Table 2. Microspherical systems prepared with high ratios of cross-linking agent (glutaraldehyde) to matrix material (albumin) appeared somewhat more active in this model. However, the most important point to note is the marked lack of activity of 4'-deoxydoxorubicin in microspherical form.

Clearance and metabolism of anthracyclines in tumour tissue

In order to correlate the fate (clearance and metabolism) of doxorubicin and 4'-deoxydoxorubicin (50–60 μg dose) in tumour tissue, when administered in microspherical form or in solution, with subsequent drug activity animals were killed at 1 min, 24 hr, 48 hr and 72 hr after injection and the amount of native drug and 7-deoxyaglycone metabolites measured by HPLC. The analysis is simplified by the fact that, whilst doxorubicin and 4'-deoxydoxorubicin have different retention times, their aglycones are identical (Fig. 1).

We have shown previously [3] that by 48 hr after injection there is an increase in doxorubicin levels in tumour tissue when administered in microspherical form. This was also the case in the larger series reported here (Fig. 4) where by 48 hr there was a greater than two-fold increase in mean native doxorubicin concentration after administration in microspherical form (microspherical form 3.8 μg/g; solution 1.7 μg/g; $P < 0.005$), that was maintained at 72 hr (microspherical form 2.4 μg/g; solution 1.1 μg/g; $P < 0.005$). It should be noted that this occurred despite the extensive metabolism of doxorubicin at 48 hr (*vide infra*).

On the contrary, Fig. 5 shows, in a series of roughly equivalent size, that tumour tissue concentrations of 4'-deoxydoxorubicin were not significantly different ($P > 0.05$) at any of the time points studied whether administered in microspherical form or in solution. Thus, at 48 hr mean tumour tissue concentrations of 3.7 μg/g (microspherical form) and 3.3 μg/g (solution) were found and at 72 hr the values were 2.7 μg/g (microspherical form) and 2.1 μg/g (solution).

Anaerobic bioreduction of anthracyclines to 7-deoxyaglycones in tumour tissue was not a prominent

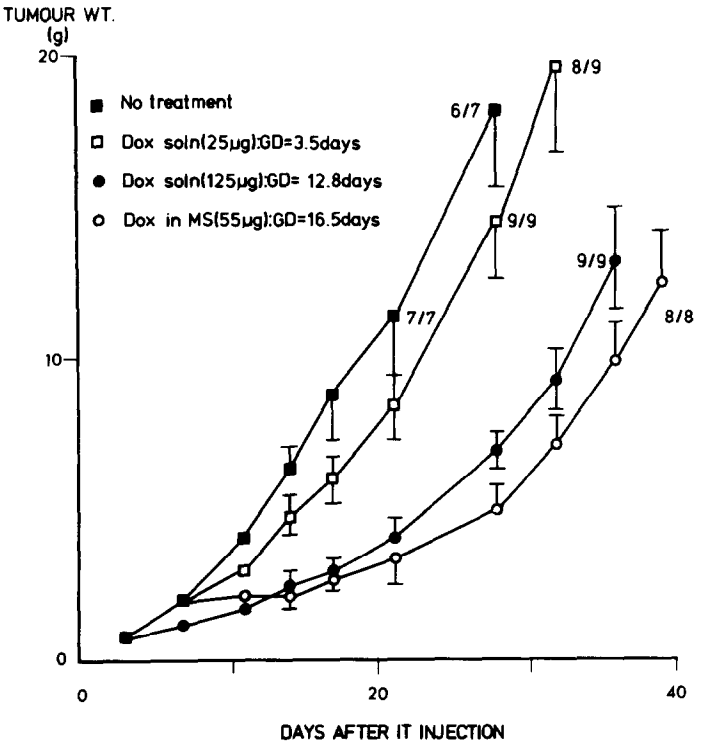

Fig. 2. Anti-tumour activity of doxorubicin-loaded microspheres. Rats bearing SC tumours of approx. 1 g were randomized into four groups and treated as shown in the key. Microspheres were suspended or drugs dissolved in phosphate buffered saline + 0.5% Tween. At intervals tumours were measured and a weight in grams calculated. Error bars represent mean ± SE. Animals were killed when the mean tumour diameter was >4 cm. Numbers on curves are animals remaining/animals per group. GD, growth delay.

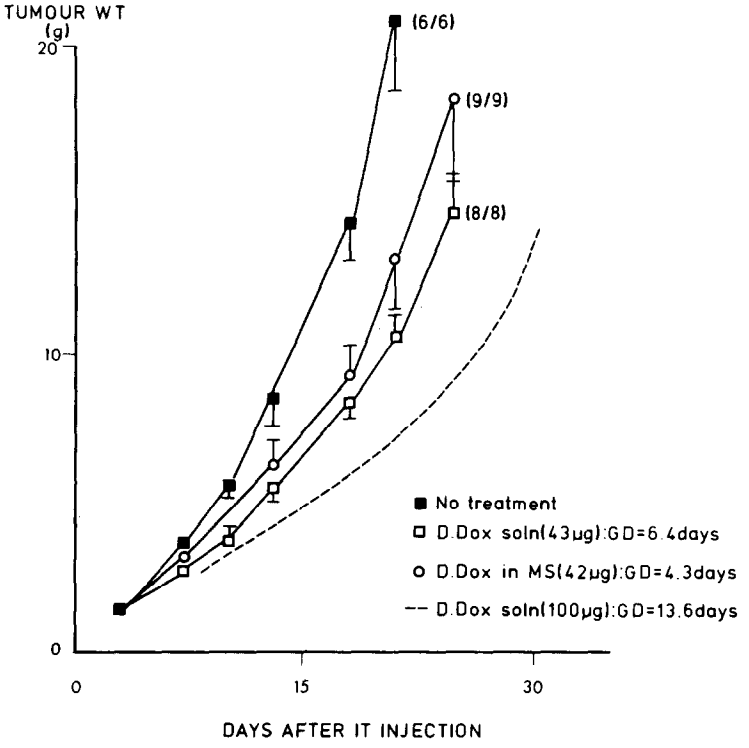

Fig. 3. Anti-tumour activity of 4′-deoxydoxorubicin-loaded microspheres. Conditions as for Fig. 2. Dotted line is data from a separate experiment and illustrates activity of a high dose of 4′-deoxydoxorubicin in solution in this model.

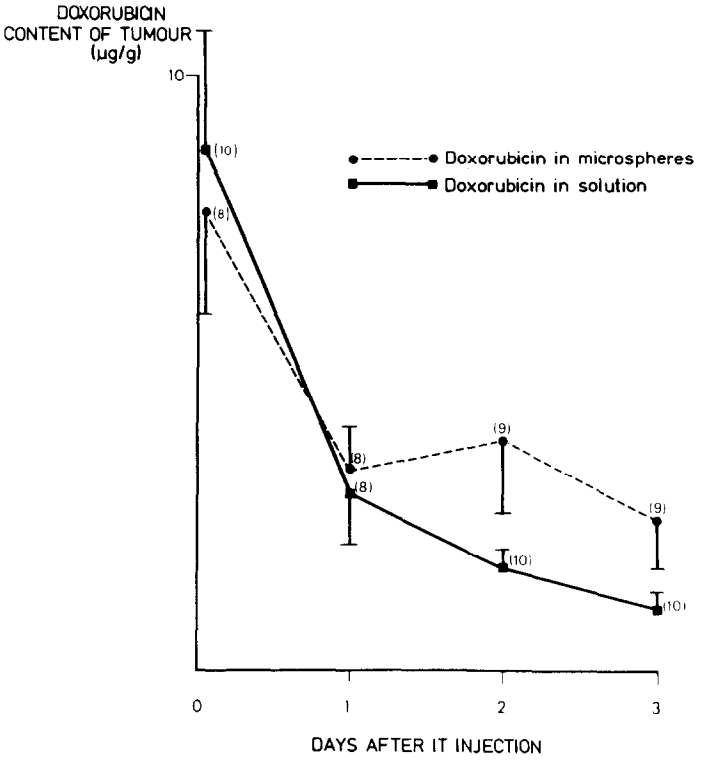

Fig. 4. Concentration–time profile of doxorubicin in tumour tissue. Drug in solution or in microspherical form (50–60 μg in phosphate buffered saline +0.5% Tween) was injected intratumourally into SC growths. At intervals rats were killed, tumours excised and weighed, then the content of parent drug and metabolites determined by HPLC. Error bars represent 95% confidence interval and number of animals examined is adjacent to the symbol. Significant differences were recorded ($P < 0.05$ by Student's two-tailed t-test) between doxorubicin concentrations when administered in microspherical form and in solution at 2 and 3 days.

feature of drugs administered in solution. Indeed, for doxorubicin virtually no metabolism occurred via this route. When administered in microspherical form no metabolism was observed immediately after injection; however, tumour tissue extensively metabolized both drugs to 7-deoxyaglycones at later time points (Table 3). Although values for 7-deoxyaglycone metabolite concentrations are available for all time points, only peak values are shown in Table 2. Thus, both drugs were metabolized to 7-deoxyaglycones to a significantly greater extent than drug in solution ($P < 0.001$, doxorubicin; $P < 0.005$, 4′-deoxydoxorubicin). Peak metabolite concentration in tumour issue following administration of doxorubicin in microspherical form (3.6 μg/g) was not significantly different to 4′-deoxydoxorubicin (2.5 μg/g). Thus, quantitatively, the two drugs are indistinguishable as regards the extent of metabolism via anaerobic bioreduction to 7-deoxyaglycones. These data should be compared with the tumour growth delay values in Table 1.

DISCUSSION

We have previously shown that direct intratumoural injection of doxorubicin in microspherical form markedly altered subsequent drug fate when compared to drug in solution administered in the same way [3]. In particular, it was observed that doxorubicin concentrations in tumour tissue were maintained for longer and metabolism of the drug to 7-deoxyaglycones occurred. Either or both of these factors could account for the activity of doxorubicin in microspherical form seen in this animal model. In order to investigate which aspect of the fate of doxorubicin in tumour tissue was most closely associated with subsequent drug activity we have used an analogue, 4′-deoxydoxorubicin (Fig. 1), and examined both its fate in tumour tissue when administered in microspherical form and also its activity in our model system.

Protein-based microspherical systems are designed to embolize in target organs following administration via the arterial system [1, 2]; therefore, there are limitations to the animal model used in this study that employs direct intratumoural injection. This is because of difficulties (paradoxically not present in humans) in modelling the targetting and therapeutic properties of microspheres when administered via the hepatic artery to experimental animals with liver tumour deposits. These are essentially due to differences of scale. Thus, in humans, the diameter of

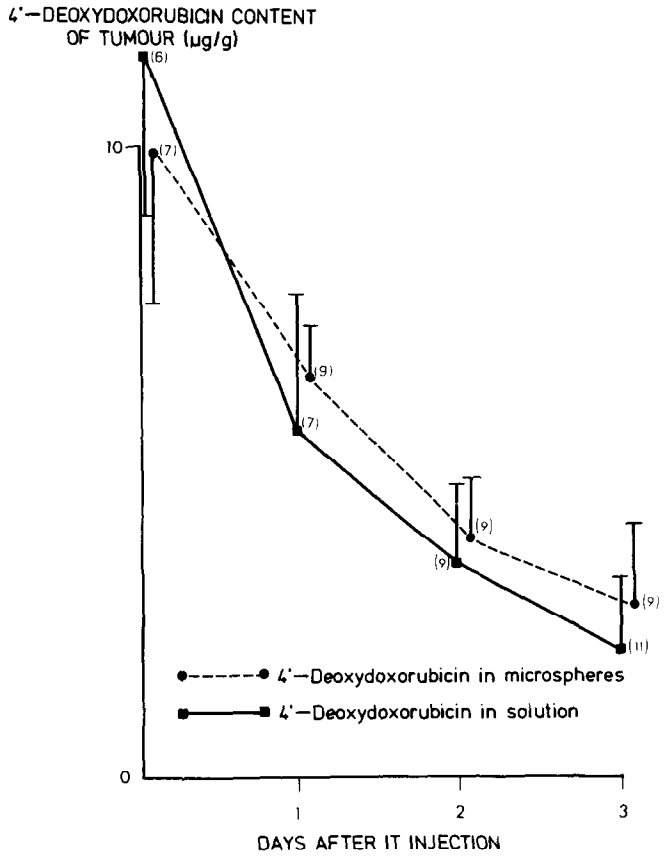

Fig. 5. Concentration–time profile of 4′-deoxydoxorubicin in tumour tissue. Conditions as in Fig. 3. No significant differences ($P < 0.05$ by Student's two-tailed t-test) were observed between 4′-deoxydoxorubicin concentrations when administered in microspherical form and in solution.

Table 1. Comparison of activities of doxorubicin and 4′-deoxydoxorubicin in microspherical form and in solution

	Dox	4′-Deoxydox
Microspherical form	GD = 18.6 days [85 μg]	GD = 4.3 days [42 μg]
Solution	GD = 6.5 days [100 μg]	GD = 6.4 days [43 μg]

GD, growth delay recorded when tumours reached 12 g.
Value in square brackets is amount of drug to achieve stated growth delay.

the hepatic artery is adequate for cannulation, and hepatic blood flow (approx. 300 mL/min) is not markedly perturbed by administration of microspheres. In small animals, injection of even a small volume of material into the hepatic artery causes a major perturbation of haemodynamics. Therefore, the current study is restricted to considering sustained release aspects of microspheres and drug activity. As regards these topics, the model has val idity because it clearly shows that 4′-deoxydoxorubicin is more active than doxorubicin in conventional form (i.e. solution), as would be

Table 2. Activity of doxorubicin and 4′-deoxydoxorubicin in microspherical form

Glutaraldehyde (mg) used per 100 mg albumin	Growth delay (days)	
	Doxorubicin	4-Deoxydoxorubicin
5	18.6 [85 μg] 14.0 [52 μg]	4.3 [42 μg]
8	16.5 [55 μg]	NT
10	18.0 [43 μg]	7.4 [52 μg]

Values in square brackets are amount of native drug necessary to achieve stated growth delay.
NT, not tested.

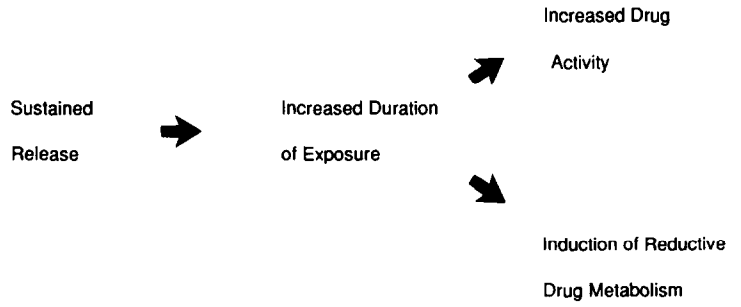

Fig. 6. Proposed relationship between fate of doxorubicin in microspherical form in tumour tissue, reductive drug metabolism and resultant activity.

Table 3. Comparison of 7-deoxyaglycone metabolite concentrations in tumour tissue following administration of doxorubicin and 4′-deoxydoxorubicin in microspherical form and in solution

	Dox 7-deoxyaglycone (48 hr)	4′-Deoxydox 7-deoxyaglycone (24 hr)
Microspherical form	Mean = 3.6 μg/g 95% CI 1.5–5.7* N = 9	Mean = 2.5 μg/g 95% CI 1.3–3.7* N = 9
Solution	Mean = 0.03 μg/g 95% CI 0–0.06 N = 10	Mean = 0.4 μg/g 95% CI 0.1–0.7 N = 7

CI, confidence interval.

* Metabolite concentrations following administration of both drugs in microspherical form significantly higher than solution ($P < 0.001$ for doxorubicin; $P < 0.005$ for 4′-deoxydoxorubicin). No significant difference between metabolite concentrations following administration of doxorubicin and 4′-deoxydoxorubicin in microspherical form.

Note that 48 hr point for dox and 24 hr point for 4′-deoxydox refer to peak concentration times.

expected from the 1.5–2-fold differential in activity accepted for these drugs against solid tumours (cited in Ref. 10).

Our original work recording the increased activity of doxorubicin in microspherical form suggested that anaerobic bioreduction of the drug was an important component [3], and there is indeed a theoretical justification for this [11]. However, the fact that 4′-deoxydoxorubicin was less active than doxorubicin in microspherical form, and yet both drugs were extensively metabolized to 7-deoxyaglycones, is inconsistent with this hypothesis. Thus, if drug-derived reactive species (semiquinone free radical, quinone methide) were formed in tumour tissue following administration of doxorubicin or 4′-deoxydoxorubicin in microspherical form, as would be predicted from the formation of 7-deoxy-aglycones, then they appear to be essentially inactive. Interestingly, it has been reported that anthracycline quinone methides showed little electrophilic character as regards covalent adduct formation with model nucleophiles, under conditions where, for example, mitomycin C was markedly active [12].

Whilst considerable evidence exists for the importance of quinone methide-like intermediates, obtained by bioreductive activation, in the biological activity of mitomycin C (quoted in Refs 13 and 14), there is little evidence of a similar role for reductive drug metabolism in the anti-tumour activity of doxorubicin. As pointed out above, this may be due to the inately weak electrophilic character of the intermediate generated under anaerobic conditions (and/or instability of the adduct formed with cellular nucleophiles e.g. DNA). In addition, it is probable that an electrophile generated from doxorubicin would form only a monoaduct [12], in contrast to mitomycin C, which can form a bis adduct [13]. Studies on the interaction of doxorubicin and mitomycin C with DNA should be useful here.

A comparison of Figs 4 and 5 shows that by 48 hr there was a greater than two-fold increase in native doxorubicin concentration in tumour tissue when administered in microspherical form (despite extensive drug metabolism at this point) and this was still apparent at 72 hr. On the contrary, tumour tissue concentrations of 4′-deoxydoxorubicin were similar whatever the mode of delivery. Thus, increased duration of exposure to native drug appears a more likely explanation of the increased activity of doxoru-

microspheres drugs that are known to require data in Table 1 showing that systems with slower rates of drug release [15] (i.e. high ratio of cross-linking agent glutaraldehyde/albumin) tended to show the longest growth delay.

Tissue concentrations of the native, unmetabolized forms of these drugs administered in solution can explain their relative activities in this model, which is consistent with other observations. Thus, drug concentrations in tumour tissue were highest with 4′-deoxydoxorubicin, the more active analogue when administered in solution, at 48 and 72 hr (Figs 4 and 5). In other models involving 3-dimensional arrays of tumour cells, increased uptake of 4′-deoxydoxorubicin by the tumour was reflected in increased activity relative to doxorubicin when drugs were administered in solution [8, 16]. This makes even more striking the marked activity of doxorubicin in microspherical form relative to 4′-deoxydoxorubicin formulated in the same way when it is considered that tumour tissue concentrations were similar at 48 and 72 hr. Clearly, whilst total tumour tissue concentrations of these drugs administered in conventional form (i.e. solution) give an indication of subsequent activity, this is not the case for drugs in microspherical form. Here, variation of rate of drug release and duration of exposure reveal wide differences in drug activity. Interestingly, it has been reported that for equivalent drug concentrations within the cell, doxorubicin is in fact more active than 4′-deoxydoxorubicin [17].

Our working hypothesis regarding the causal chain of events following intratumoural administration of doxorubicin-loaded protein microspheres is shown in Fig. 6. In this scheme both increased activity and reductive metabolism of the drug via anaerobic bioreduction are seen as a consequence of slow rate of drug release from microspheres and increased duration of exposure of tumour to doxorubicin, rather than anaerobic bioreduction of doxorubicin being causally related to increased drug activity in microspherical form. Interestingly, it has recently been suggested that the kinetics (i.e. the rate) of mitomycin C reduction determines the metabolic fate of the drug [18]. Furthermore, a relation between duration of hypoxic exposure and reductive drug metabolism [19] has been recorded for 2-nitroimidazoles, which may be analogous to our observations. In this regard, it is of interest that compared to drug in solution, reductive metabolism of doxorubicin in microspherical form is stimulated 100-fold (Table 3) accompanied by increased duration of exposure to native drug (Fig. 4), whereas reductive metabolism of 4′-deoxydoxorubicin was stimulated six-fold (Table 3), possibly due to no significantly increased exposure of tumour to drug in microspherical form. As yet it is not known whether microspheres cause hypoxia in this model, or there is some interaction between increased duration of exposure to drug and pre-existing hypoxia that results in reductive drug metabolism.

In the system described it appears that increased duration of exposure to drug enhances activity whereas reductive drug metabolism does not. A logical progression for this work is to incorporate into bicin in microspherical form. This is consistent with reductive metabolism for maximum cytotoxic activity.

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