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Doxorubicin-loaded Casein Microspheres: Protean Nature of Drug Incorporation

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Abstract—We have studied incorporation of [^{14}C]doxorubicin within protease-sensitive casein microspheres both by ^{14}C-activity, measuring total drug, and HPLC, measuring free drug only. It was found that total drug content (27·7 μg mg^{-1}) exceeded free drug content (3·2 μg mg^{-1}) suggesting that the major portion of doxorubicin was incorporated via a covalent linkage to matrix protein. In-vivo drug disposition and activity studies suggested that this fraction of doxorubicin was the major species within tumour tissue (total vs free: 5 min, 14·3 μg g^{-1} vs 0·7 μg g^{-1}; 24 h, 11·7 μg g^{-1} vs 1.1 μg g^{-1}; 48 h, 11·2 μg g^{-1} vs 1·2 μg g^{-1}; 72 h, 10·0 μg g^{-1} vs 0·8 μg g^{-1}), did not exhibit a 'burst' effect, was slowly cleared (30% loss over 3 days), and was equiactive (growth delay = 12 days) compared with drug in solution (growth delay = 10 days). This work clearly implicates in-vivo microsphere matrix biodegradation in drug release and subsequent disposition and activity.

Protein microspheres incorporating the cytotoxic agent doxorubicin have been prepared, extensively characterized as regards drug-loading and release rate (Willmott et al 1985, 1988; Chen et al 1987, 1988; Willmott & Harrison 1988) and are currently undergoing assessment following regional administration in patients with localized solid tumours accessible to arterial catheterization. In this therapeutic approach, if microspheres are prepared of appropriate size, it is possible to embolize selectively solid tumours in target organs with little exposure of non-target organs (Goldberg et al 1991a, b; Willmott et al 1991). The clinical utility of this and other approaches has recently been reviewed (Gupta 1990).

During these studies it became apparent that doxorubicin was incorporated within protein microspheres in both a freely diffusible, extractable form and also in an immobilized, non-extractable form covalently bound to protein matrix. Following digestion and solubilization of drug-loaded microspheres with trypsin, the two forms of doxorubicin could be distinguished chromatographically. In addition, incorporated drug in each form could be quantitated using [^{14}C]doxorubicin to obtain the total doxorubicin incorporation and HPLC to obtain the amount incorporated in the free form (Cummings et al 1991).

To investigate the activity and fate within tumour tissue of free and covalently bound doxorubicin, an animal model involving direct intratumoural injection of drug-loaded microspheres was employed. We use this animal system as a model of post-embolization events (e.g. drug diffusion, clearance and metabolism, and particle biodegradation) that determine anti-tumour activity in-vivo. It has been recently demonstrated that doxorubicin in protein microspherical form is active in animal models both after intratumoural administration (Willmott et al 1990) and on embolization in solid tumour deposits after administration via the hepatic artery (Goldberg et al 1992).

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Materials and Methods

Preparation and characterization of doxorubicin-loaded casein microspheres

Drug-loaded microspheres were prepared by stabilization with glutaraldehyde of the aqueous phase of a water/oil emulsion containing protein (200 mg) and doxorubicin (10 mg). The basic process (Willmott et al 1985) and the modifications necessary with the lipophilic protein casein (Chen et al 1987; Willmott et al 1989) are described elsewhere. To incorporate [^{14}C]doxorubicin, a known activity between 2×10^6 and 6×10^6 d min^{-1} (sp. act. $0{\cdot}3 \times 10^6$ d min^{-1} μg^{-1}) in methanol was evaporated to dryness. Ten mg of unlabelled doxorubicin and 200 mg casein were added and the standard procedure for microsphere preparation followed. For storage, microspheres were lyophilized (Willmott & Harrison 1988).

Doxorubicin-loaded microspheres were digested and solubilized in 0·4% trypsin and characterized as regards drug content both chromatographically and by ^{14}C-activity. In the first technique a sample of trypsin digest was extracted with chloroform/isopropanol (2/1) using daunorubicin as internal standard; the solvent was evaporated and the drug reconstituted in methanol for HPLC analysis using fluorescence detection (Willmott et al 1985, 1990). Because of the extraction step and chromatographic separation this technique is both sensitive and specific for doxorubicin, no other doxorubicin-derived peaks being present. From samples with added drug, drug recoveries are typically 90%.

Drug loading was also assessed by ^{14}C-activity. Samples of trypsin digests of microspheres incorporating [^{14}C]doxorubicin were counted in a Packard Tri-Carb 460CD using Emulsifier Safe (Canberra Packard) as scintillant. By reference to quench curves, constructed using ^{14}C-activity quenched with carbon tetrachloride, it was found that counting efficiency was > 90% for microsphere digests.

Microsphere diameter and susceptibility to digestion by the protease trypsin were both obtained using laser diffraction measurements (Malvern 2600C Particle Sizer). In the latter case the variation of volume concentration (derived from particle size and amount of light transmitted) of microspheres suspended at 37°C in 0·4% trypsin in phosphate buffered saline (pH 7·2) containing 0·05% sodium azide, was plotted against time.

Analysis of doxorubicin in tumour tissue

SC growing solid tumours (Sp107 mammary carcinoma (Kamel et al 1989)) were injected intratumourally with [^{14}C]doxorubicin in microspherical form. Rats were killed, tumours excised, snap frozen on solid CO_2 and stored at −20°C. For analysis, tumours were thawed and suspended in 10 mL phosphate buffered saline: tumour tissue was disaggregated and homogenized using a Silverson Mixer fitted with a tubular head, and a Potter S homogeniser. Analysis of the homogenate (1 mL) with respect to total drug (^{14}C-activity) was performed by solubilizing in 2 mL Soluene (Canberra-Packard) at 37°C overnight in scintillation vials. After addition of 15 mL scintillant (Emulsifier Safe), samples were counted. Counting efficiency was always >70%.

Free doxorubicin concentration in solid tumour tissue was determined as follows: 1 mL of tumour homogenate plus daunorubicin as internal standard, was treated with 33% (w/v) silver nitrate (0·2 mL) to free intercalated drug (Cummings et al 1986). Following extraction into chloroform/isopropanol, extracts were evaporated to dryness and reconstituted in methanol for HPLC as described earlier.

Assessment of drug activity in microspherical form

SC growing Sp107 tumours of approximately 1 g were injected intratumourally with 100 μg of doxorubicin either in microspherical form or in solution. Tumours were measured with callipers and a weight in grams derived (Willmott et al 1990).

Results

Characteristics of microspheres

Doxorubicin-loaded casein microspheres were characterized as regards particle size, drug content and degradation by trypsin, both in the freshly prepared and lyophilized state (Table 1). In this system lyophilization had little effect on characteristics that are of importance for in-vivo performance following embolization. In a previous, less comprehensive, study that examined particle size and free drug content of doxorubicin-loaded albumin microspheres, similar results were obtained (Willmott & Harrison 1988).

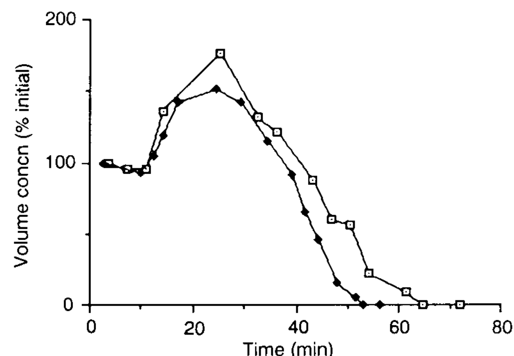

FIG. 1. In-vitro degradation profiles of doxorubicin-loaded casein microspheres. Using a Malvern 2600C Particle Sizer, volume concentration of a microsphere suspension was plotted against time. Time courses for microsphere degradation are shown to illustrate latent period, swelling and solubilization (see also Table 1). Enzyme reaction conditions: 0·4% trypsin in phosphate buffered saline (pH 7·2) made 0·05% in sodium azide; temp. 37°C. Pre-freeze-dried, ◆; post-freeze dried, □.

The time course of microsphere degradation was complex (Fig. 1), consisting of a latent period followed by swelling of particles and finally particle solubilization. To introduce a quantitative element into these profiles for comparative purposes we have defined two parameters (see footnote c to Table 1 for definitions): T_L, which reflects the length of the latent period before swelling occurs, and T50, which reflects the rate of particle solubilization. From Fig. 1 it is clear that casein microspheres are totally digested to water soluble material and that lyophilization has no effect on this process.

In freshly prepared microspheres, whilst total drug content is 27·7 μg mg^{-1}, the content of native, chromatographically pure doxorubicin is only 3·2 μg mg^{-1} (11·6% of total). Because the HPLC traces show no evidence of doxorubicin hydrolysis or breakdown products we interpret this to mean that the bulk of doxorubicin incorporated within casein microspheres is covalently bound to the protein matrix.

Tumour tissue clearance of doxorubicin administered in microspherical form

To assess drug disposition within solid tumour tissue, [^{14}C]doxorubicin-loaded casein microspheres were injected intratumourally, animals killed and tumours individually processed for analysis. The same tumour homogenate was

Table 1. Characteristics of lyophilized doxorubicin-loaded casein microspheres.

	Size (μm)[a]	Drug content[b] Free (μg mg^{-1})	Drug content[b] Total (μg mg^{-1})	Degradation by trypsin[c] T_L (min)	Degradation by trypsin[c] T50 (min)
Pre-lyophilization (freshly prepared)	61	3·2	27·7	14·4, 12·3	44·1, 44·1
Post-lyophilization	58	3·6	26·3	14·4, 17·2	54·0, 51·5

[a]Fifty% weight average diameter determined using Malvern 2600C Particle Sizer. [b]Free drug content estimated by HPLC; total drug content estimated using [^{14}C]doxorubicin. [c]Measured using Malvern 2600C Particle Sizer. T_L defined as time taken for microspheres to swell by 15% of initial value; T50 defined as time taken to reduce volume concentration by 50% of initial value. Results of repeat experiments quoted.

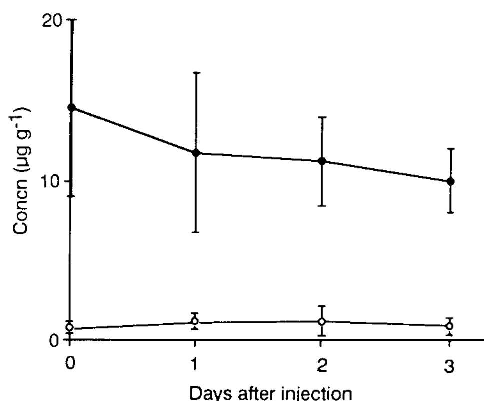

FIG. 2. Doxorubicin disposition in solid tumour tissue. [14C]Doxorubicin-loaded microspheres were administered intratumourally and at intervals tissue concentrations of drug assessed by ^{14}C-activity and by HPLC. Four rats per group. Error bars represent mean and 95% confidence interval. Total doxorubicin (^{14}C), ●; native doxorubicin (HPLC), ○.

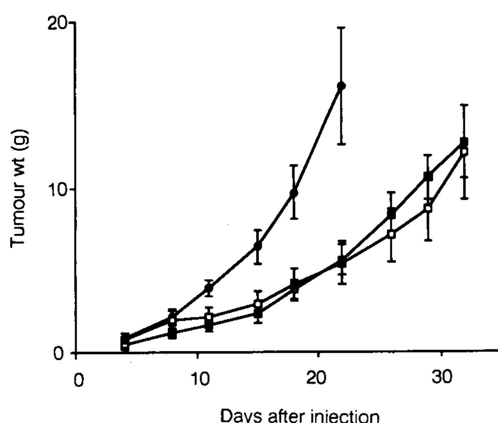

FIG. 3. Anti-tumour activity of doxorubicin-loaded casein microspheres. Subcutaneous tumours (~1 g) were injected either with doxorubicin in microspheres or in solution and subsequent tumour growth monitored by calliper measurements, 6–8 rats per group. Error bars represent mean±s.e. No treatment, ●, doxorubicin in solution (100 µg), ■; doxorubicin in microspheres (100 µg), □.

used for measurements of ^{14}C-activity and free doxorubicin concentration (Fig. 2). At all time points studied, free doxorubicin concentration was significantly ($P<0{\cdot}05$, Student's t-test) lower than total doxorubicin concentration. Presumably, the major doxorubicin species present in solid tumour tissue is covalently bound to protein matrix and not detectable by HPLC which involves an extraction step into organic solvent. Metabolism, either hydrolytic to 7-hydroxyaglycones or reductive to 7-deoxyaglycones, was not a prominent feature of this analysis.

To demonstrate that the difference in concentrations as measured by total radioactivity and HPLC was not due to inadequate extraction of doxorubicin from tumour homogenate, blank tumour homogenate to which had been added [^{14}C]doxorubicin was processed. It was found that doxorubicin concentration by HPLC analysis (free doxorubicin) accounted for 84–92% (n=3) of the ^{14}C-activity (total doxorubicin) present. Moreover, when [^{14}C]doxorubicin in solution was administered intratumourally, drug concentrations in tumour tissue were not significantly different whether assessed by ^{14}C-activity or by HPLC (data not shown).

Anti-tumour activity of doxorubicin-loaded casein microspheres

Our previous data (Chen et al 1987) on activity of this microspherical system showed an apparent superiority over doxorubicin in solution. However, this work was performed before we became aware that free doxorubicin was not the only form in which the drug was incorporated. Thus, this original work compared doxorubicin in solution with a comparable amount of free drug (i.e. based on HPLC analysis) in the microspheres. Here we compare doxorubicin in solution with a comparable amount of total drug (i.e. based on ^{14}C-activity) in the microspheres.

Fig. 3 shows the tumour growth delay curves. From day 11 onwards, tumours treated either with drug in solution or in microspheres were significantly ($P<0{\cdot}05$) smaller than untreated tumours. To achieve a tumour weight of 10 g, tumour growth was delayed for drug in solution by 10 days and for drug in microspheres by 12 days. At no time point were the two treatment groups significantly different.

Discussion

We consider that the major findings from this work are the detection and quantitation of a doxorubicin-derived species within microspheres which is only made biologically available on degradation of the carrier matrix and which retains anti-tumour activity. We have shown elsewhere that when [^{14}C]doxorubicin-loaded microspheres are digested and solubilized in trypsin two doxorubicin-derived entities, separable chromatographically, are present in the unextracted digest. These are free doxorubicin and a rapidly eluting material that possesses the distinctive doxorubicin chromophore. Moreover, both entities contained ^{14}C-activity (Cummings et al 1991). In extracts of microsphere digests considered in the present work, only doxorubicin was seen. We interpret these results to mean that a proportion of doxorubicin is covalently bound to protein matrix during manufacture, probably via a glutaraldehyde bridge between amine groups of drug and protein. When microspheres are solubilized in trypsin this form of doxorubicin is not extracted into chloroform/isopropanol, whereas the free drug is.

It is instructive to compare drug disposition in tumour tissue after injection in microspheres with that after injection in solution. Our previous work has shown that the initial clearance (over 24 h) of doxorubicin from tumour tissue is rapid and occurs at a similar rate both for drug in solution and in microspheres (Willmott et al 1990; Cummings et al 1991), probably due to a burst effect in the latter case. By contrast the clearance of total doxorubicin (Fig. 2) is relatively constant over 72 h: indeed by 72 h total drug concentration within tumour tissue is still 70% of the initial value.

Immediately after injection, we may assume that most of the doxorubicin is present in complexed form incorporated within microspheres that are too large (60 µm) for cellular ingestion; that is, microspheres and incorporated doxorubicin are situated extracellularly. However, at subsequent time

points it is not known whether complexed doxorubicin in tumour tissue remains within microspheres or whether the particles have been digested and solubilized and doxorubicin (bound to protein fragments) has been taken up by tumour cells or cells of host origin residing in the solid tumour, such as macrophages. It has been reported that doxorubicin bound to non-biodegradable microspheres incapable of entering cells retains activity (Tritton & Yee 1982). Dual isotope experiments, in which protein matrix and incorporated drug are separately radiolabelled, are planned to elucidate how close is the association between microsphere integrity and drug retention in tumour tissue.

Although the concentration-time profile of total doxorubicin in this study (Fig. 2) is markedly different from that of drug in solution (Willmott et al 1990) there was no significant difference in anti-tumour activity (Fig. 3). Thus, the sustained release and slow clearance of doxorubicin from casein microspheres did not improve activity. Nevertheless, an important conclusion, based on the low levels of free doxorubicin in tumour tissue, is that anti-tumour activity is mediated predominantly by doxorubicin covalently bound to the microsphere matrix.

Others have reported the activity of mitomycin C (Hashida et al 1983) and doxorubicin (Pratesi et al 1985; Seymour et al 1990) covalently bound to synthetic macromolecules by biologically labile linkages. In both systems it is considered that the regeneration of free drug is responsible for anti-tumour activity. In this respect, the microspherical system studied here appears different because doxorubicin in the free form was always present in tumour tissue at low concentration (Fig. 2) relative to the total doxorubicin present. This data and our chromatographic data (Cummings et al 1991) suggest that doxorubicin bound to a protein fragment, produced on digestion of microsphere matrix, retains activity. Whether cellular (Storm et al 1988) or soluble extracellular (e.g. Fig. 1, Table 1) proteases are involved in this mode of drug release is not known.

These results point to the importance of rate of biodegradation of protein microsphere matrix for release of doxorubicin from this system. Consequently, we have developed an in-vitro assay (Fig. 1, Table 1) to estimate susceptibility of microspheres to protein digestion and examine the effect of factors such as lyophilization on degradation rate. We are currently developing the system involving trypsin and other protease enzymes with the aim of yielding predictive information in-vivo.

In view of the results on drug disposition and activity reported here, and the targeting potential of this carrier system in regional cancer therapy, the covalent binding of drugs to the carrier matrix may be a useful feature to incorporate into microsphere design.

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