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Correlation between tumour drug disposition and the antitumour activity of doxorubicin-loaded microspheres: implications for the drugs' *in vivo* mechanism of action

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Abstract—Doxorubicin (DOX) has been incorporated into five different formulations of protein microspheres, each altering tumour drug disposition in a characteristic manner. There was no correlation between the stimulation in anaerobic quinone bioreduction over the levels produced by free DOX and tumour growth delay against the Sp 107 rat mammary carcinoma. A strong correlation ($r^2 = 0.948$, $P < 0.01$, two-tailed t statistic) was observed between a slower decline in parent drug levels and antitumour activity. These data support the view that free radical processes are not involved in the mechanism of action of DOX and suggest that the optimum way to deliver the drug to the Sp 107 tumour is through sustained release of lower concentrations (approximately 2 μM) from a large extracellular pool.

Protein microspheres can actively alter both drug disposition parameters and the therapeutic properties of incorporated doxorubicin (DOX*) once they have reached the tumour. In an initial report, intra-tumoural (i.t.) injection of DOX-loaded albumin microspheres to the rat mammary carcinoma Sp 107 was shown to increase antitumour activity 5-fold compared to equivalent administration of free drug [1]. Increased efficacy was associated with tumour parent drug concentrations (not including complexed drug, see below) which did not change significantly over 72 hr, whereas with free DOX parent drug concentrations fell 5-fold over the same period to much lower levels. Surprisingly the microspheres stimulated DOX anaerobic quinone reduction (AQR) to 7-deoxyaglycone metabolites by a maximum factor of 155-fold at 48 hr, from almost negligible levels after free DOX (0.02 μg/g tissue) to concentrations comparable to the parent drug itself after microspheres (3.1 μg/g tissue) [1, 2]. This phenomenon only became apparent after a 16–24 hr latent period and active drug metabolism could still be recorded 1 week after administration of microspheres. Enhancement of AQR persisted when DOX was substituted with a closely related (anthracycline) structural analogue, 4′-deoxydoxorubicin [3]. Only when DOX was incorporated in microspheres did the stimulation of bioreduction occur; when free DOX was co-administered with non-drug-containing "empty" pre-formed microspheres no drug biotransformation was detected [2].

Shortly after these initial observations, it came to light that a proportion of incorporated drug became complexed (probably covalently via a molecule of glutaraldehyde) to the protein matrix of the microspheres during particle formation and that this fraction was responsible for their sustained release properties [4, 5]. Due to its nature, covalently coupled drug was not amenable to conventional analysis by HPLC techniques [1], but its presence in the tumour could be detected by incorporating radioactively labelled [^{14}C]DOX into microspheres as a tracer [5, 6]. The amount of coupling varied depending on the protein in use and the amount of glutaraldehyde employed as a cross-linker, and in turn this modulated the extent of drug metabolism observed in the tumour. In this report, DOX has been formulated into a number of different protein microsphere systems so that the relationship between various aspects of tumour drug disposition and antitumour activity could be examined.

* Abbreviations: DOX, doxorubicin; i.t., intra-tumoural; AQR, anaerobic quinone reduction.

Materials and Methods

Preparation of DOX-loaded protein microspheres. Drug-loaded microspheres were prepared by stabilization with glutaraldehyde (3.5–7 mg/100 mg of protein) of the aqueous phase of a water/oil emulsion containing protein (200 mg; or 200 mg albumin and 25 mg polyaspartic acid in the case of the mixed protein microsphere system) and DOX (10 mg), as reported in detail previously [4, 7]. The modifications necessary with the lipophilic protein casein are described elsewhere [8]. Each different microspherical system (see Table 1) contained, essentially, the same total drug content, ranging from 2.2–3.2% (w/w). The size of the resultant particles varied from 15 to 40 μm in diameter (50% weight average).

Drug analysis techniques. To measure the degree of covalent coupling of DOX in intact microspheres two different drug determinations were performed [5]. Total drug content was measured by incorporating [^{14}C]DOX (sp. act. 3×10^5 dpm/μg) as a tracer during microsphere preparation and counting radioactivity; free drug (non-complexed) was measured by HPLC after solvent extraction with 5 vol. (2:1) of chloroform:propan-2-ol [9]. For both determinations, microspheres were completely solubilized by digestion with 0.4% (w/w) trypsin overnight [6]. The fraction covalently bound to microsphere matrix components was calculated as the difference between the concentration of total drug and free drug.

Tumour DOX disposition was determined as above: free drug and metabolites by HPLC [10] and total parent drug content by counting radioactivity due to [^{14}C]DOX [5]. 7-Deoxyaglycones were the only metabolites of DOX detected in the Sp 107 tumour.

Animal studies. The animal model used throughout consisted of inbred rats of the WAB/NOT strain and the syngeneic, undifferentiated mammary carcinoma (Sp 107), a subcutaneously growing tumour that originally arose spontaneously in a female rat [11]. All drug treatments were administered by i.t. injection in a 0.5 mL vol. of phosphate-buffered saline containing 0.5% Tween 80, at the doses shown in Table 1. Antitumour activity was assessed by determining tumour weight and is represented as a growth delay, which is defined as the difference in days between the time it takes drug-treated tumours to reach 10 g compared to controls [1].

Results

The microsphere systems studied exhibited a wide range of drug complexation to the protein matrix (0–88% of total drug content, Table 1). As a consequence, they also altered

Table 1. Relationship between covalent coupling to matrix, tumour parent drug levels and metabolic activation through quinone reduction to antitumour activity of doxorubicin encapsulated in a series of protein microspheres

Microsphere matrix	% Cross-linker (glutaraldehyde)	% Complexation*	% Fall in total parent drug levels (over 48 hr)†	Free 24 hr drug levels (μg/g)†	Fold stimulation in drug metabolism to 7-deoxyaglycones†	Antitumour activity‡ (growth delay in days)
Albumin/polyaspartic acid§	5	0	74	6.1 ± 1.1	83	4.5
Albumin	3.5	49	51		81	7.4
Albumin	5	69	55	3.1 ± 0.08	26	6.8
Albumin	7	76	33		14	12
Casein‖	5	88	22	1.1 ± 0.04	0	12
Correlation to antitumour activity (r^2)		0.717	0.948	0.892	0.674	
Significance level (P value, two-tailed t statistic)		> 0.05	< 0.01	> 0.10	> 0.05	

* Degree of doxorubicin covalent binding to microsphere matrix components (complexation) was determined by comparing free drug measured by HPLC against total drug measured by incorporating [^{14}C]DOX as a tracer.

† For all drug disposition parameters N = 3–4 separate animals (± SD) and the dose of doxorubicin administered was 70 μg in each case. Total parent drug levels were measured by counting the radioactivity of the [^{14}C]DOX tracer in tumour homogenates and adjusting for 7-deoxyaglycones. Free drug and 7-deoxyaglycones were measured by HPLC after solvent extraction of tumour homogenates. Fold stimulation in 7-deoxyaglycone formation was calculated by comparing against metabolite formation when 70 μg of free DOX was injected i.t. (0.04 μg/g ± 0.03) and was determined at 24 hr.

‡ Antitumour activity was measured in groups of seven to eight animals after i.t. injection of 100 μg of DOX encapsulated in microspheres. The antitumour activity of 100 μg of free DOX ranged from 5.6–10 days throughout these experiments.

§ Part of data derived from [5].

‖ Part of data derived from [6].

in a graded manner parent drug pharmacokinetics, drug metabolism and antitumour activity (see Table 1). Parent drug pharmacokinetics are represented by two different values: (1) % fall in total parent drug levels over 48 hr and (2) free drug levels at 24 hr. These parameters were chosen to summarize the characteristics of a full tumour DOX concentration–time profile. For examples of full pharmacokinetic profiles see Refs 5 and 6. There was no significant difference in tumour total parent drug levels (15–17 μg/g) recorded at time zero after i.t. administration of all the different types of microsphere. Significance levels and the degree of correlation between drug disposition parameters and antitumour activity are also contained in Table 1.

Discussion

By regulating DOX complexation in protein microspheres it has been possible to dictate the rate at which the parent drug is cleared from the tumour and the level of metabolic activation (AQR) that occurs, and determine the relative importance of each of these processes. The results presented in Table 1 show clearly that there is no correlation between increased DOX quinone reduction to 7-deoxyglycone metabolites and antitumour efficacy. In fact, a negative relationship was observed, although this did not reach the level of statistical significance ($P > 0.05$, two-tailed t statistic). These data corroborate recent detailed studies which show that this is a pathway of drug inactivation [2, 12] and lend further support to the view that free radicals are not involved in the mechanism of action of DOX [13].

Antitumour activity was strongly correlated to parent drug levels ($P < 0.01$, two-tailed t statistic), as might be anticipated. Nonetheless, closer inspection of the data of Table 1 reveals an intriguing situation. Casein microspheres which were 3-fold more active compared to albumin/polyaspartic acid microspheres actually liberated five times less free drug into the tumour after 24 hr. However, because of the greater degree of covalent coupling in casein microspheres, drug levels were sustained far longer. Thus, two broadly different pharmacokinetic profiles were generated. First, represented by albumin/polyaspartic acid, was where higher levels of free DOX were presented to the tumour over a shorter period of time. The second, typified by casein, was where lower levels of free drug were maintained in the locus of the tumour for longer periods of time, and this type of drug delivery proved to be substantially more efficacious.

The above conclusion assumes that the immobilized fraction of drug residing in microspheres is acting as a reservoir for slow release of free DOX, and is without pharmacological properties in its own right. However, DOX immobilized covalently on to agarose beads of a diameter too large to be internalized by cells has been demonstrated to retain cytotoxicity through membrane interactions [14]. In addition, a recent study has shown that the presence of extracellular drug is necessary for DOX to exert its cytotoxic effect through stabilization of topoisomerase II cleavable complexes, despite the fact that levels of intracellular drug can be several orders of magnitude above IC_{50} values [15]. Therefore, drug covalently bound to the protein matrix need not necessarily be released to be effective and may be boosting antitumour activity by prolonged contact with tumour cell membranes.

In summary, the data presented in this work confirm that native DOX and not a biotransformed intermediate(s) is the active form of the drug. They also suggest that the optimum way to deliver DOX to the Sp 107 tumour for maximum effect is through sustained release of lower concentrations (approximately 2 μM) from a large extracellular pool.

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