Biochemical Pharmacology, Vol. 41, No. 12, pp. 1849–1854, 1991
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

COVALENT COUPLING OF DOXORUBICIN IN PROTEIN MICROSPHERES IS A MAJOR DETERMINANT OF TUMOUR DRUG DISPOSITION

JEFFREY CUMMINGS,*† NEVILLE WILLMOTT,‡ ELAINE MARLEY‡ and JOHN SMYTH*

*Imperial Cancer Research Fund Medical Oncology Unit, Western General Hospital, Edinburgh EH4 2XU; and ‡Department of Pharmacy, University of Strathclyde, Glasgow G1 1XW, U.K.

(*Received* 15 *September* 1990; *accepted* 14 *January* 1991)

Abstract—Doxorubicin is shown to be present in albumin microspheres (10–40 μm) in two forms: the native drug and a fraction of drug covalently coupled to the protein matrix probably via glutaraldehyde. Upon trypsin digestion the fraction covalently coupled is released and can be resolved from native doxorubicin by high performance liquid chromatography and quantitated either by using ^{14}C-labelled doxorubicin or by measuring the absorption of the doxorubicin chromophore at 480 nm. Albumin microspheres contained 6.9 μg/mg protein covalently bound drug versus 11.1 μg/mg native drug when 1% glutaraldehyde was used in microsphere preparation. The covalently bound fraction increased significantly with 2% glutaraldehyde. Albumin/polyaspartic acid microspheres lacked a covalently bound fraction when prepared under the same conditions as pure albumin micospheres (35 μg/mg native drug, 1% glutaraldehyde) but transferrin microspheres contained similar amounts of bound and native albumin. *In vivo*, albumin microspheres altered the disposition of doxorubicin in a rat mammary carcinoma (Sp107) compared to albumin/polyaspartic acid microspheres by reducing the rate of parent drug elimination from the tumour and by reducing its biotransformation to 7-deoxyaglycone metabolites. These data indicate that covalent coupling is a key component in the way doxorubicin is handled in tumours after administration of protein microspheres.

Intratumoural (i.t. §) administration of doxorubicin loaded albumin microspheres to a rat mammary carcinoma (Sp107) results in a 5-fold increase in antitumour activity compared to the drug free in solution administered in an identical fashion [1]. The increased activity is associated with alterations in tumour drug disposition where the microspheres produce: (a) elevated and steady state parent drug concentrations and (b) a large stimulation of anaerobic quinone reduction to 7-deoxyaglycone metabolites. Either of these two mechanisms could account for the enhanced activity of the microspheres. Recent studies with a close structural analogue of the drug, 4′-deoxydoxorubicin, also encapsulated in microspheres, has shown that increased antitumour activity does not necessarily follow stimulation of anaerobic quinone reduction [2] and suggests that with doxorubicin parent drug pharmacokinetics rather than drug metabolism is the more important determinant of antitumour activity. In this paper we present a possible explanation for elevated doxorubicin concentrations after administration of albumin microspheres, namely covalent coupling of the drug to the protein matrix of the microspheres.

MATERIALS AND METHODS

Preparation and digestion of microspheres. Microspheres were prepared by stabilisation through cross-linking of protein by glutaraldehyde of a water in oil emulsion and is described in detail elsewhere [3, 4]. The proteins and polyamino acid used as microsphere matrix materials were bovine serum albumin, human transferrin and poly-L-aspartic acid (20–30 kDa) and were from the Sigma Chemical Co. (Poole, U.K.). The drug investigated was doxorubicin and was from Farmitalia (Milan, Italy). [^{14}C]Doxorubicin was from Amersham International (Amersham, U.K.) and was used at a specific activity of 15 nmol of drug = 5000 dpm. The mixed protein microsphere system of albumin and polyaspartic acid was prepared as for the pure albumin system [4] except that 200 mg of albumin plus 25 mg polyaspartic acid were used. Microsphere diameter of individual preparations varied from 15–40 μm (50% weight average) by laser diffraction measurements (Malvern 2600C Particle Sizer, Malvern, U.K.) and the weight of the particles (protein plus drug content) was determined gravimetrically. A known weight of microspheres was digested in 0.4% (w/v) trypsin by incubation overnight at 37°. This procedure resulted in complete digestion of intact microspherical structure, as evidenced by microscopic examination, for all microsphere systems studied.

Determination of doxorubicin covalently coupled to the protein matrix of microspheres. To determine the fraction of doxorubicin covalently coupled to the protein matrix, microspheres were first digested with trypsin (as above) and then 100 μL were subjected to HPLC using previously published methods which resolve the drug from its five major metabolites and from chemical degradation products [5, 6]. Selective detection of all doxorubicin related species present in digests was achieved by connecting the liquid

† All correspondence and proofs should be sent to Dr Jeffrey Cummings at the above address.

§ Abbreviations used: dpm, disintegrations per minute; HPLC, high performance liquid chromatography; i.t., intra tumoural.

chromatograph firstly to a fluorescence detector set at 480 nm excitation and 560 nm emission and secondly to a diode array detector programmed to monitor chromatographs at 480 and 233 nm simultaneously and to record a UV-visible absorption spectrum from 200–600 nm of all chromatographic peaks greater than 0.1 mAU in height. Additionally, sequential 1 mL/min fractions were collected during chromatographic runs of microsphere digests incorporating [^{14}C]doxorubicin using a Gilson 201 collector (Gilson, Villiers-le-Bel, France). The fractions were added to 10 mL of scintillation fluid (Hionic-fluor, Canberra-Packard, Pangbourne, U.K.) and counted in a Packard Tri Carb model 460 CD counter (Canberra-Packard). Chromatographic peaks due to doxorubicin covalent coupling were quantitated as doxorubicin equivalents using absorption at 480 nm.

Determination of doxorubicin disposition in the Sp107 *tumour.* Two determinations were performed on each tumour: (1) the free native drug and its metabolites were measured by HPLC and (2) the total drug content of the tumour including the fraction of drug covalently coupled to the microsphere matrix was measured by counting the radioactivity of [^{14}C]doxorubicin as described below. For both determinations tumours were homogenized as follows. After thawing tumours were suspended in distilled water (20% w/v) then disaggregated using a Silverson blender (Silverson Machines Ltd. Waterside, U.K.) and finally homogenized using a Potter type homogenizer (Braun Megulsen, F.R.G.). Homogenates were then treated with 33% (w/v) silver nitrate (0.2 mL per 1 mL homogenate). Free doxorubicin and metabolites were determined by HPLC (as above) after homogenates were extracted with chloroform: propan-2-ol (2:1, v/v) as previously described in detail [7]. Total drug content was determined by adding 2 mL of soluene (Canberra-Packard) to 1 mL homogenate and incubating at 37° overnight. After incubation samples were thoroughly mixed with 15 mL of scintillation fluid and counted twice with a 24 hr interval between to control for spurious counts due to chemi- and photoluminescence.

Animal studies. Rats (Nottingham Wistar) bearing a solid-sub-cutaneously growing mammary carcinoma (Sp107) 1–4 g in weight were treated with 70 μg of [^{14}C]doxorubicin (specific activity as above) incorporated in either pure albumin or albumin/polyaspartic acid microspheres by direct i.t. injection. At 5 min, 1 day, 2 days and 3 days after treatment 3–4 animals were killed, their tumours were collected and immediately frozen to −60° with solid CO_2. Tumours were analysed for total drug content and native drug and metabolite profiles as described above within 1–2 months.

RESULTS AND DISCUSSION

Identification and quantitation of the covalently bound fraction of doxorubicin in protein microspheres

Upon trypsin digestion of drug containing microspheres additional chromatographic peaks were resolved from native doxorubicin by HPLC which were not present in control digestions of non-drug containing microspheres. These chromatographic peaks are more hydrophilic than native doxorubicin and are illustrated in Fig. 1 for trypsin digested albumin microspheres. Although they had different retention times from native drug (2.7 min and 3.2 min versus 4.3 min for doxorubicin, Fig. 1) they retained the characteristic absorption of doxorubicin (Fig. 2), were fluorescent (Fig. 1) and retained the ^{14}C-label (Table 1). We believe these peaks represent the fraction of drug that was originally covalently bound to the microsphere matrix and therefore not amenable to analysis until matrix digestion and separation by HPLC. Whilst these peaks fluoresced their intensity relative to native doxorubicin was quenched by a factor of 4.5 for the first main peak (2.7 min, Fig. 1) and by a factor of 2 for the second main peak (3.2 min, Fig. 1). Not all microspherical systems studied contained covalently coupled drug. With albumin/polyaspartic acid particles no significant new peaks were detectable in digests by HPLC apart from native doxorubicin and this is illustrated in Fig. 3. The amount of ^{14}C-label that migrated with these drug/microsphere peaks during HPLC correlated well with their peak heights measured by absorption at 480 nm (compare Fig. 1 and Fig. 2 with Table 1) suggesting that whilst their fluorescence is quenched, visible absorption is not quenched to any significant degree. Approximately 5–10% of the total radioactivity loaded on to the HPLC column eluted with a similar retention time to the peaks due to covalent

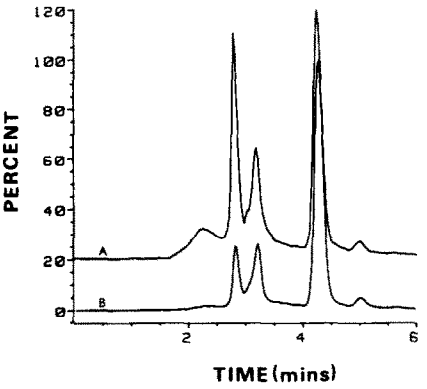

Fig. 1. Detection of doxorubicin/microsphere covalent complexes by HPLC in albumin particles. Microspheres prepared with doxorubicin were digested with trypsin (0.4% w/v) overnight and 100 μL of the digest were injected onto the HPLC column. Upper chromatogram (A) was monitored for visible absorption at 480 nm and lower chromatogram (B) was monitored for fluorescence at 480 nm excitation and 560 nm emission. The two chromatograms have been overlayed and normalized such that the native doxorubicin peak (4.3 min) represents 100%. Two other main chromatographic peaks were detected which were not present in control digestions where doxorubicin was mixed with non-drug containing albumin microspheres and treated with trypsin. These peaks had retention times of 2.7 min and 3.2 min and represent drug/microsphere covalent complexes. (See Results and Discussion.)

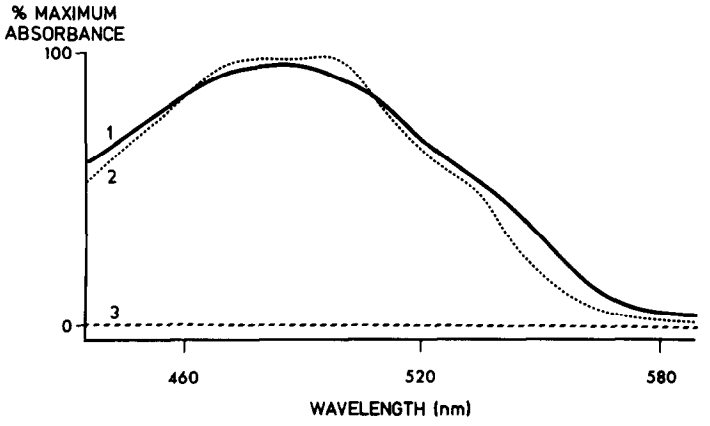

Fig. 2. Absorption spectra of the chromatographic peaks liberated from albumin microspheres after digestion with trypsin (0.4% w/v). Spectra of the three main chromatographic peaks detected in Fig. 1 were recorded during HPLC using a diode array detector. Spectrum 1 (solid) is from the two main peaks related to covalent coupling (retention time 2.7 min and 3.2 min, Fig. 1) and was identical for both. Spectrum 2 (dotted line) is from native doxorubicin (retention time 4.3 min, Fig. 1). Spectrum 3 (broken line) is from a control digestion of non-drug containing pre-formed microspheres mixed with native doxorubicin (not shown) and was taken at 2.7 min and 3.2 min to show that the doxorubicin chromophore was only present at this time when microspheres were prepared in the presence of the drug.

Table 1. HPLC radioactivity profiles of [^{14}C]doxorubicin containing protein microspheres after digestion with trypsin (0.4% w/v)

Microsphere type	dpm* Albumin	Albumin/polyaspartic acid
Chromatographic peak		
†Doxorubicin/microsphere covalent complexes	332 ± 42	120 ± 28
Doxorubicin	297 ± 17	886 ± 139
Total in digest before HPLC	962 ± 16	1268 ± 23
% Recovery of loaded radioactivity from the column	65 ± 6	79 ± 7

Radioactivity migrating with each chromatographic peak was determined by collecting sequential 1 mL fractions during HPLC and counting those in a liquid scintillation counter. Counts were only associated with two main fractions: one corresponding to native doxorubicin and one corresponding to a series of early eluting peaks associated with doxorubicin covalent coupling to the protein matrix of microspheres.

* Mean value ± SD, four replicate determinations.

† For examples of these early eluting peaks see Fig. 1 for albumin and Fig. 3 for albumin polyaspartic acid.

coupling even when these peaks were not present as evidenced by a lack of absorption at 480 nm (compare Tables 1 and 2). This may reflect the 95% radiochemical purity of [^{14}C]doxorubicin quoted by the manufacturers and/or may be due to release of label from native doxorubicin by drug chemical decomposition during microsphere digestion. Additionally, the percentage recovery of loaded radioactivity from the HPLC column was significantly less for albumin microspheres compared to albumin/polyaspartic acid microspheres ($P < 0.01$, Student's t-test). This may be explained by larger doxorubicin/protein matrix fragments becoming retained on the top of the HPLC column. Based on quantitation at 480 nm, the amount of covalently bound versus free in all the microspherical systems studied is reported in Table 2.

Perhaps the strongest evidence that the early eluting peaks in Fig. 1 are doxorubicin/microsphere covalent complexes is the fact that their visible absorption spectrum from 440–600 nm is almost identical to that of native doxorubicin. Chemical degradation products of doxorubicin, which might possibly be present in microspherical systems, are

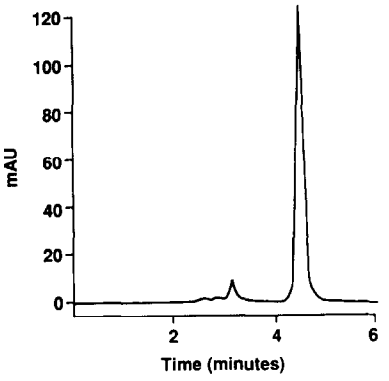

Fig. 3. Lack of detection of doxorubicin/microsphere covalent complexes by HPLC in albumin/polyaspartic acid particles. Microspheres prepared with doxorubicin were digested with trypsin (0.4% w/v) overnight and 100 μL were subjected to HPLC analysis. The chromatogram was monitored at 480 nm and only one main peak was evident, native doxorubicin.

without both fluorescence and visible absorption [6] and apart from doxorubicinol which has a retention time of 3.9 min by our HPLC method, all the major known metabolites of the drug are lipophilic aglycone species which elute with a longer retention time than doxorubicin [8, 9]. Also, chemical forms of doxorubicin with slight alterations in the structure of the benzanthraquinone chromophore have UV-visible absorption spectra completely different from the native drug [6, 10]. However when doxorubicin binds to proteins covalently its UV-visible absorption spectrum does not change significantly [11].

The exact chemical nature of the covalent complexes are not known at present but their formation appears to be related to the presence of glutaraldehyde. At the higher concentration of glutaraldehyde used as a cross-linker during microsphere production (2%), a greater amount of doxorubicin bound covalently to the protein matrix ($P < 0.01$, Student's t-test, Table 2). Doxorubicin is known to react with aldehyde groups of glutaraldehyde by a Schiff's base condensation through the amino group of its sugar moiety to form a stable imino complex [12]. Such a reaction would result in no change in the structure of the doxorubicin chromophore and little change in its UV-visible absorption spectrum, which is exactly what we have found. The lack of covalent coupling in microspherical systems incorporating polyaspartic acid may be due to the negative charge of the polyamino acid competing successfully with glutaraldehyde for the positively charged amino group of the sugar moiety of doxorubicin to form a reversible ionic association. Similar ionic interactions for doxorubicin with microspheres have been reported with polyglutamic acid [13] and Aminex 50 W-X4 ion exchange resin [14].

Tumour drug disposition of microspheres containing and lacking a covalently bound fraction of doxorubicin

To investigate whether covalent coupling alters *in vivo* parameters of microspheres, we studied tumour drug disposition after i.t. administration of 70 μg of doxorubicin incorporated within either pure albumin microspheres which contain high levels of covalently bound drug or albumin/polyaspartic acid microspheres which contain negligible levels of covalently bound drug. Two determinations were performed on each tumour specimen: free native drug and its metabolites were measured by HPLC and total drug content (including the fraction covalently bound to the protein matrix of microspheres) was measured by counting labelled [^{14}C]doxorubicin. In Fig. 4 the tumour concentration/time profiles for native doxorubicin and its 7-deoxyaglycone metabolites are superimposed on the profiles for total drug content. For albumin/polyaspartic acid microspheres, the fall in concentration of total drug paralleled closely the fall in concentration of free, native drug and was similar to the tumour concentration/time profiles of doxorubicin free in solution [2]. Taking into account the high levels of 7-deoxyaglycone metabolites formed, there was no significant difference between native and total drug. In contrast, with pure albumin microspheres, whilst the concentrations of free, native doxorubicin fell quickly, significantly higher

Table 2. Quantitation of the covalently bound and free native fraction of doxorubicin in protein microspheres

Microscopy	Doxorubicin μg/mg protein*					
Glutareldehyde content	Albumin		Albumin/polyaspartic acid		Transferrin	
	Bound†	Native	Bound	Native	Bound	Native
1%	6.9 ± 1.9 (N = 6)	11.1 ± 1.7	0 (N = 12)	32.0 ± 5.4	5.7 ± 0.4 (N = 4)	9.7 ± 3.5
2%	12.2 ± 0.5 (N = 4)	9.6 ± 0.3	—	—	—	—

The bound fraction was determined by measuring the peak height at 480 nm of a series of drug-complexes which were liberated upon digestion with trypsin and resolved from the native drug by HPLC.

* Mean ± SD.

† Results expressed as doxorubicin equivalents.

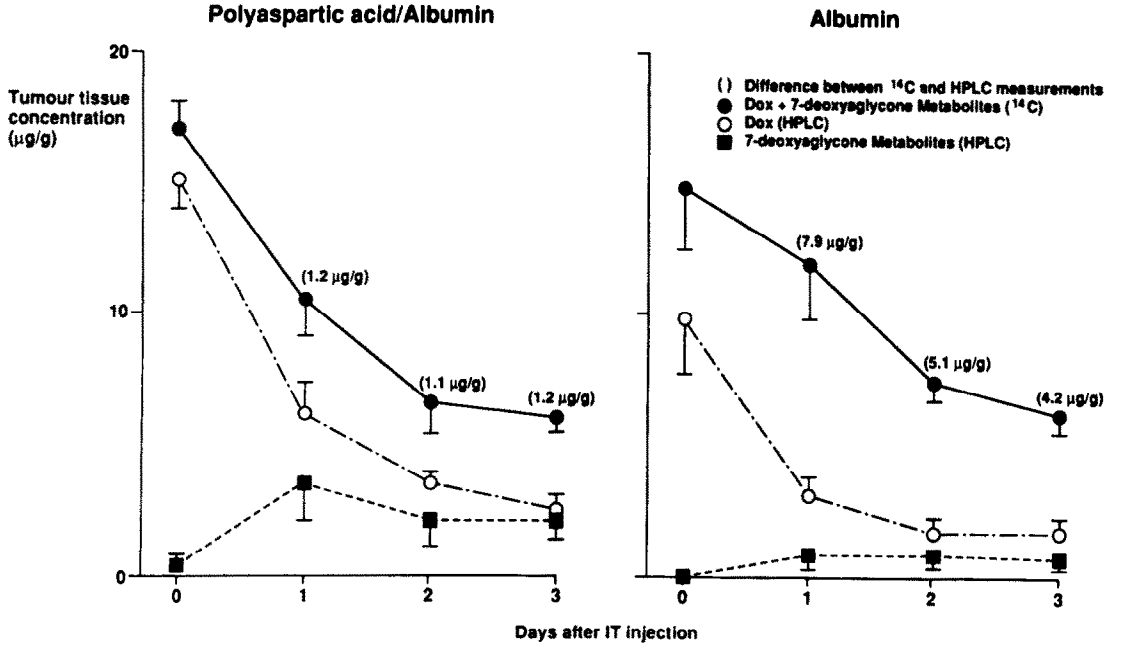

Fig. 4. Tumour concentration/time profiles of doxorubicin and its 7-deoxyaglycone metabolites after i.t. administration of either albumin microspheres or albumin/polyaspartic acid microspheres. Total drug content including particle bound was determined by solubilising a sample of tumour homogenate and counting the radioactivity of the [^{14}C]doxorubicin (solid line). The free native drug (hatched line) and 7-deoxyaglycone metabolites (broken line) were determined by HPLC after extracting the same tumour homogenates with an organic solvent mixture (chloroform:propan-2-ol, 2:1). The difference between the total drug content (^{14}C measurements) and native drug plus 7-deoxyaglycone metabolites (HPLC measurements) is contained in brackets. Each value represents the mean ± SEM from the 3–4 separate tumours.

concentrations of total (including covalently bound) were maintained over the 72 hr study period. However, significantly lower concentrations of 7-deoxyaglycones were produced (AUC_{0-72hr}: albumin, 2.03 μg/g × hr; albumin/polyaspartic acid, 7.12 μg/g × hr). It appears that the covalently coupled fraction plays a dual role in modulating doxorubicin disposition in the tumour: (1) it slows down elimination of the parent drug and (2) reduces the rate of its biotransformation to 7-deoxyaglycones.

Albumin/polyaspartic acid microspheres are no more effective than doxorubicin free in solution as an antitumour agent in our model after i.t. administration (100 μg doxorubicin in solution produced 6.4 days growth delay; 121 μg doxorubicin in albumin/polyaspartic acid microspheres produced 4 days growth delay). In the same model pure albumin microspheres containing only 85 μg native doxorubicin were 3–5 times more effective (21 days growth delay). From these data it is clear that covalently coupled drug is contributing significantly to antitumour activity in albumin microspheres.

Why covalently coupled doxorubicin retains antitumour activity is unclear. It is possible that as microspheres are biodegraded, drug/microsphere complexes are released which are then taken up into cells to produce an effect. If this is the case, then the immobilized fraction of the drug may be seen as acting as a slow release depot. A similar argument has been proposed for doxorubicin covalently bound to synthetic co-polymers [15]. It is also possible that immobilized drug does not even need to be released to be active. When doxorubicin is covalently bound to polyglutaraldehyde microspheres and does not enter cells, it retains full cytotoxicity and even has the ability to overcome drug resistance [12]. Also, when doxorubicin is covalently coupled to the surface of sepharose beads the drug remains cytotoxic without entering cells [16]. Whatever the reason, the fact that doxorubicin can be incorporated into protein microspheres in an immobilized form which retains antitumour activity will further improve the therapeutic advantage of this drug delivery system.

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