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THE PHARMACOKINETICS OF POLYMER-BOUND ADRIAMYCIN

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Abstract—Adriamycin® (ADR) covalently bound to *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers via biodegradable (Gly-Phe-Leu-Gly) oligopeptide sequences shows antitumour activity against model tumours *in vivo*. In this study we have examined the distribution of ADR bound to such HPMA copolymers following intravenous administration to mice (ADR concentration 5 mg/kg). An established fluorimetric HPLC method was used to measure levels of free ADR in plasma and tissue samples, and a new technique was developed to quantitate levels of polymer-bound anthracycline. The high initial levels of free ADR in plasma observed following administration of free drug were absent in the case of polymer-bound ADR, and the subsequently high levels of free ADR seen in other tissues were also abolished. In contrast, the circulating half-life of HPMA copolymer–ADR was approximately 15 times longer than that of the free drug. The initial peak level of free ADR in the heart was reduced 100-fold following administration of drug–conjugate. These alterations in pharmacokinetics may account for the decreased toxicity and improved efficacy reported previously.

The antitumour antibiotic Adriamycin® (ADR§) is a small amphipathic molecule that diffuses easily through biological membranes [1]. It is used clinically to treat a variety of tumours, but efficacy is severely restricted by a range of toxic side-effects, including cumulative cardiotoxicity that can be life-threatening [2]. When administered in free form, ADR displays a ubiquitous body distribution confirmed by a recent study which showed little or no preferential accumulation of ADR in breast tumour tissue (tumour: normal tissue ratio, 1.1–1.8:1.0) [3]. Numerous attempts have been made to improve the therapeutic index of ADR by modifying its mode of delivery, including attempts to optimize kinetics of drug administration [4] or alternatively by using a variety of drug delivery systems (liposomes [5], microspheres [6], antibodies [7], poly aminoacids [8, 9] and soluble synthetic polymers [10]).

Previously we have shown that *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers can be covalently bound to ADR via oligopeptide (Gly-Phe-Leu-Gly) side chains which are cleaved intracellularly by lysosomal thiol-dependent proteinases [11]. Such conjugates, containing ADR or daunomycin (DNM), show antitumour activity *in vivo* against L1210 leukaemia [12, 13], Walker sarcoma [14] and B16 Melanoma.‖ and decreased toxicity; approximately 10-fold more ADR can be administered in conjugate form without overt signs of toxicity [13].

To understand further the mechanism of action of HPMA copolymer-ADR we have studied the pharmacokinetics of polymer conjugate and ADR released from conjugate following intravenous administration to DBA_2 mice. A technique originally described by Cummings *et al.* [15] was used to permit HPLC-fluorimetric determination of ADR in plasma and tissue samples. To determine concurrently the distribution of the polymer-bound ADR it was first necessary to liberate ADR from the polymeric carrier by an acid hydrolysis technique.

MATERIALS AND METHODS

Materials

ADR and DNM were purchased from the Sigma Chemical Co. (Poole, U.K.); all HPLC solvents were purchased from Fisons plc (Loughborough, U.K.) HPMA copolymer conjugates containing ADR (Fig. 1) were synthesized as described previously [16]. The weight average molecular weight (M_w = 24,000 daltons) and polydispersity (M_w/M_n = 1.2) of the polymeric precursor was determined by Sepharose 4B/6B gel permeation chromatography.

DBA_2 mice (male, approx. 10 weeks) were purchased from Banton and Kingman Ltd (Hull, U.K.).

Administration of ADR and polymer-bound ADR

ADR was dissolved in distilled water and added to an equal volume of double-strength phosphate

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§ Abbreviations: HPMA, *N*-(2-hydroxypropyl)methacrylamide; ADR, Adriamycin®; DNM, daunomycin; HPLC, high pressure liquid chromatography; HCl, hydrochloric acid; NaOH, sodium hydroxide; AUC, area under the curve.

‖ K. B. O'Hare, R. Duncan, P. Kopečková and J. Kopeček (1989) unpublished results.

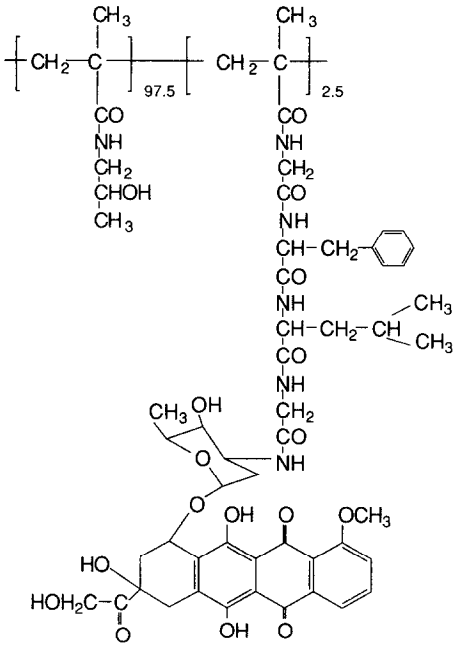

Fig. 1. Structure of the ADR–HPMA copolymer conjugate.

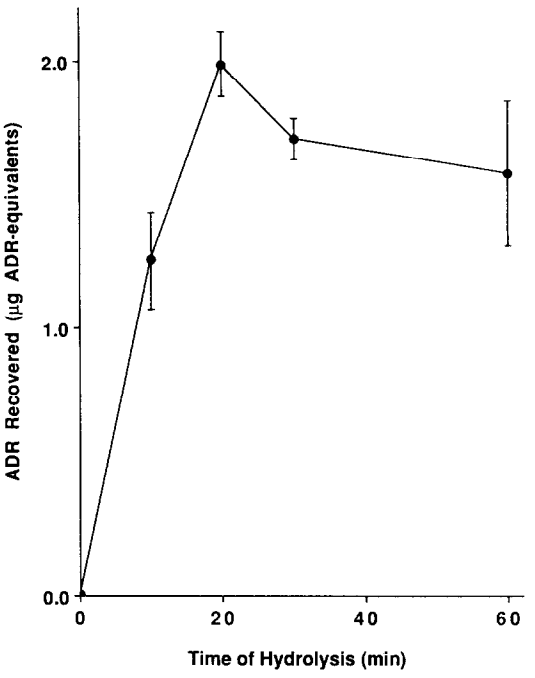

Fig. 2. Effect of duration of acid-hydrolysis of polymer-bound ADR on liberation and subsequent organic extraction of ADR-derivatives. ADR–HPMA copolymer (containing 2 μg ADR) was incubated at 85° in HCl (1 M) for various times prior to neutralization, organic extraction and subsequent quantitation as described in the text.

buffered saline (final ADR concentration 1.0 mg/mL). ADR–HPMA copolymer conjugate was dissolved in phosphate buffered saline to give the same final concentration of ADR. Mice were lightly anaesthetized and the substrates administered via the caudal vein at an ADR dose of 5 mg/kg. After various times, mice were again anaesthetized and exsanguinated. Blood samples were centrifuged (2000 *g*, 20 min) to isolate the plasma, and heart and liver were removed, washed in cold saline and immediately frozen.

Assay of ADR and polymer-ADR in plasma and tissue samples

Quantitation of free ADR. Free ADR in heart, liver and blood was estimated using the method of Cummings *et al.* [15]. Solid tissues were homogenized to known total volumes in Tris–phosphate buffer (0.2 M, pH 9.5) and appropriate-sized samples were taken (sample sizes were chosen to contain 100–500 ng ADR). These samples, and plasma samples, were diluted with the same buffer to a total volume of 1.0 mL. DNM (100 or 200 ng) was added to each tube as an internal standard, vortex-mixed, and then silver nitrate (20% v/v of a 33% w/v solution) was added. A double volume of extraction medium (chloroform:methanol 75%:25%, v/v) was then added, samples were vortex-mixed three times during a 30 min period and finally, to promote full separation of the aqueous/organic phases, were centrifuged (1000 *g*, 10 min). The upper (aqueous) layer was then carefully removed and discarded. The lower (organic) layer was removed from the silver chloride precipitate and then filtered (Millipore SR filter units, pore size 0.5 μ) to remove all particulate material. The samples were evaporated to dryness, either in a vacuum or under a stream of nitrogen and redissolved in mobile phase for HPLC analysis (see below). This protocol is hereafter referred to as "extraction procedure A".

Quantitation of polymer-bound ADR. The hydrophilicity of HPMA copolymer–ADR prevents its extraction into the organic phase. In addition, polymer-bound ADR is only weakly fluorescent. Hence, to permit accurate quantitation of polymer-bound ADR the free drug must first be released from the conjugate. ADR is linked to the copolymer by a tetrapeptide sequence attached to the sugar amine by a peptide bond (Fig. 1). This linkage is resistant to acid hydrolysis, but the glycosidic bond between the daunosamine ring and the aglycone moiety is hydrolysed relatively easily, releasing the free aglycone [17].

Preliminary investigations were conducted to design a suitable hydrolysis protocol for quantitation of polymer-ADR. Samples containing ADR (2 μg/sample) were exposed to HCl (final concentration 1.0 M) at 85° for varying lengths of time. An equal volume of Tris–phosphate buffer (pH 9.5, 1.0 M) was then added, followed by NaOH (2.0 M, volume adjusted to neutralize the acid). Subsequent extraction and processing was the same as for the free drug. The amount of fluorescence measured during HPLC depended on the duration of the hydrolysis step, and the maximum yield was seen after 20 min of hydrolysis (corresponding to release of approximately 100% of bound ADR, Fig. 2). Extended acid-treatment caused a gradual fall in the yield of fluorescence. Therefore, a hydrolysis time of 20 min

was routinely used to permit the quantitation of polymer-bound ADR. This protocol is hereafter referred to as "extraction procedure B".

HPLC chromatography

Samples (20 μL, prepared as described above) were injected into a Waters μ-Bondapak C_{18} column and eluted at a flow rate of 1.0 mL/min with a mobile phase of aqueous propan-2-ol (29%) adjusted to pH 3.2 with orthophosphoric acid. Elution was isocratic and detection fluorimetric (Milton Roy Fluoromonitor III, excitation wavelength 480 nm, emission wavelength 560 nm).

The system was calibrated with intact and aglycone forms of both ADR and DNM. In order to correct the fluorescence profiles for peaks derived from materials endogenous in the tissue samples, tissues from untreated animals, with and without added DNM as an internal standard, were routinely processed in parallel with experimental samples.

For every experimental sample the content of ADR (ng/g or ng/mL) and its metabolites was calculated by measuring the relevant peak area and calibrating against the corresponding peak area derived from the DNM internal standard. Data are presented in terms of ADR-equivalents of material extracted. For the purposes of this study the fluorescence ratio of ADR (or metabolite):DNM (or metabolite) was assumed to be 1.0.

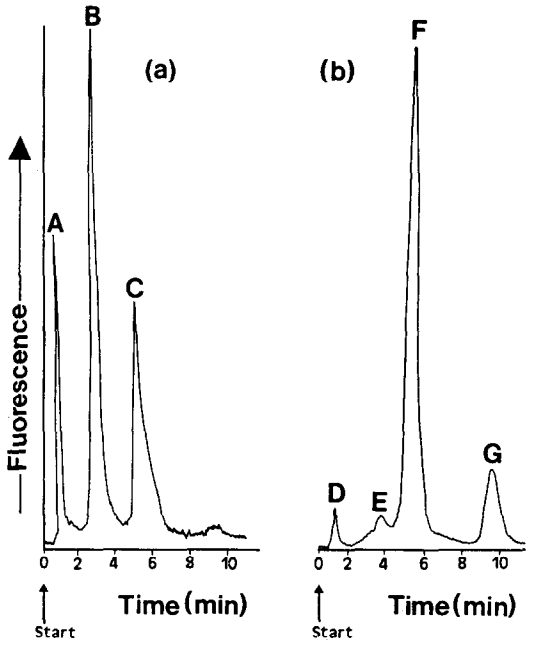

Fig. 3. Typical elution chromatograms obtained following HPLC analysis of tissues derived from mice following (10 min) intravenous administration of ADR or ADR–HPMA copolymer: (a) liver following administration of free ADR; (b) acid-hydrolysed serum following administration of ADR-HPMA copolymer conjugate. Peaks are identified A: endogenous, B: ADR, C: DNM (internal standard), D: endogenous, E: Adriamycinol, F: Adriamycinone, G: Daunamycinone (internal standard).

RESULTS

The plasma and tissue samples obtained following administration of ADR or HPMA copolymer–ADR that were subjected to simple extraction (procedure A) typically produced an elution profile which displayed two well-defined peaks corresponding to the DNM internal standard and the ADR contained in the sample (Fig. 3). Small amounts of metabolites mainly the aglycone-forms, were detected, although the parent drugs were predominant in every case. Analysis of samples extracted following acid-hydrolysis (procedure B) produced elution profiles that showed two main anthracycline-derived peaks corresponding to adriamycinone (derived from the sample) and daunamycinone (arising from the internal standard, Fig. 3b).

A complication in quantitation using the above HPLC methods was the appearance of a number of relatively hydrophilic peaks (elution time 1–2 min). Such peaks were found to be greatest for liver samples and smallest for plasma samples (Fig. 3b), and were clearly tissue-derived [18] since they were present also in tissue samples taken from untreated mice.

When free ADR was administered intravenously to mice, most drug had disappeared from the blood by the first sampling (2 min, Fig. 4a). This rapid clearance was not simply attributable to binding to plasma proteins, since the extraction technique used here is known to release protein-complexed ADR [18]. When administered in the form of polymer conjugate, there was virtually no free drug detected in the plasma at any time (the AUC for free drug in plasma during the first hour fell over 60 times as a result of conjugation), although the polymer conjugate continued to circulate at a high concentration for an extended period (the level detected in the blood fell only by 45% during the fist hour, giving an AUC increased over 70 times compared with free drug, Fig. 4b).

Although the liver contained substantial amounts of free ADR (up to 20 μg/g) following administration of drug in free form, there was a marked decrease (approximately 10-fold during the first hour) as a result of polymer-conjugation. A significant amount (up to 7 μg/g) of polymer-bound drug was detected in the liver following the administration of conjugate, but this was probably present in occluded blood (liver was not perfused prior to isolation, Fig. 5).

Heart levels of free and polymer-bound ADR followed a similar pattern to those seen in the liver, although the absolute concentrations of drug were lower (Fig. 6). The maximum level of free ADR detected in heart tissue was 12 μg/g, 15 min after administration of free drug, and the corresponding level of free drug following administration of polymer-conjugated ADR was less than 0.1 μg/g. The AUC for free ADR present in heart tissue during the first hour was consequently decreased over 70 times by conjugation of the drug. The peak maximum of free ADR seen after administration of polymer conjugate was much less than 1 μg/g, and occurred 24 hr after injection. A significant amount of polymer-conjugated ADR was detected in the heart (up

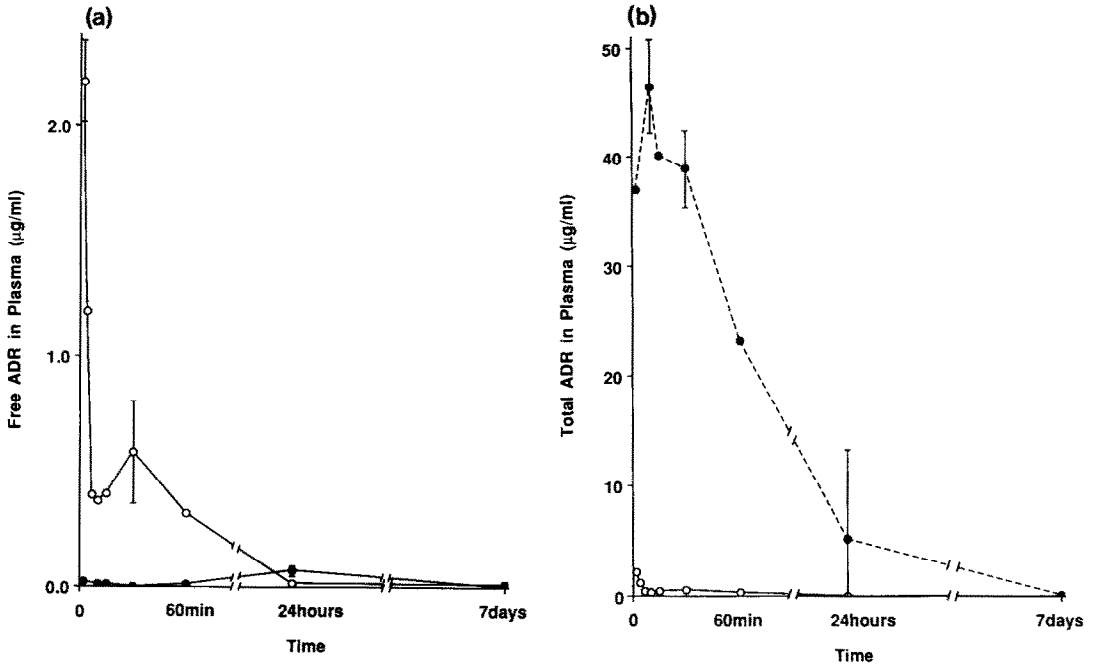

Fig. 4. Plasma clearance of ADR and polymer-bound ADR. Panel (a) shows the levels of freely-extractable ADR following the administration of (○) free ADR, (●) polymer-bound ADR. Panel (b) shows the total drug-levels observed following administration of (○) free ADR (●) polymer-bound ADR.

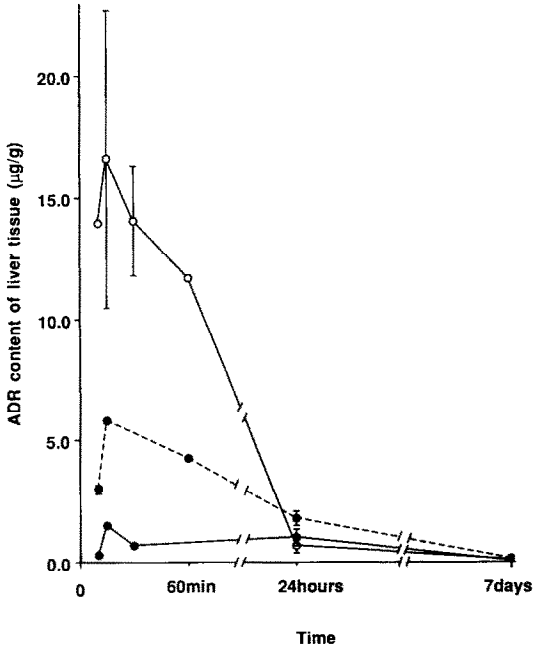

Fig. 5. Liver pharmacokinetics of ADR and polymer-bound ADR. Levels of free ADR seen in liver following administration of ADR (○—○) and polymer-bound ADR (●—●) are shown. In addition the total drug levels seen following the administration of polymer-bound ADR are shown (●– – –●).

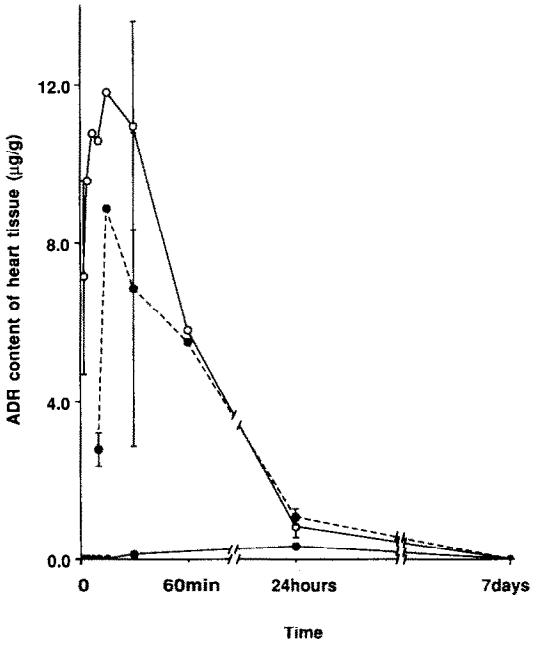

Fig. 6. Heart pharmacokinetics of ADR and polymer-bound ADR. For key see legend to Fig. 5.

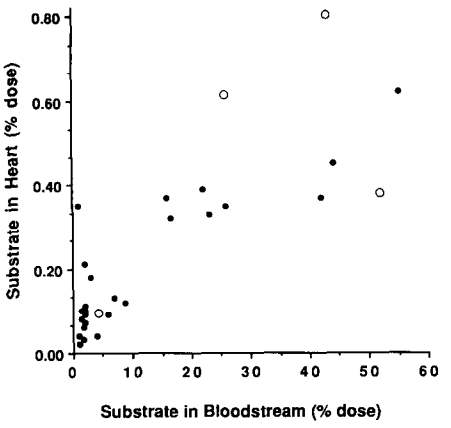

Fig. 7. Relationship between the level of HPMA copolymer–ADR measured simultaneously in heart and in blood. The amount of conjugate in blood and heart tissue was measured by either HPLC analysis of contained polymer-bound ADR (○) or by analysis of the radioactivity present (for ^{125}I-labelled copolymer conjugates (●) [13].

to 10 μg/g). Figure 7 shows the relationship between amounts of polymer-bound ADR measured in heart and those detected in blood at various times after administration of polymer conjugate. There is a clear correlation and the line passes through the origin, indicating that the levels of heart-associated polymer-bound ADR are entirely due to its presence in occluded blood. There is no evidence for penetration of the macromolecular form into heart tissue.

DISCUSSION

The pharmacokinetics of ADR were markedly altered following its conjugation to HPMA copolymer. Blood-clearance was significantly slower after conjugation, the characteristic fast initial clearance of free drug ($t_{1/2}$ = 4 min) being replaced by a prolonged high concentration of circulating polymer-bound ADR, which fell only by 45% in the first hour after injection. This rate of disappearance was consistent with renal filtration being the major route of clearance [19]. The other major change observed in the distribution of polymer-bound ADR compared with free drug was a dramatic fall (by over 99%) in the amount of free ADR measured in heart tissue. Both of these modifications result directly from changes in the ability of ADR to diffuse through membranes; the free drug rapidly leaves the circulation, entering all accessible cells and tissues (notably heart tissue), whereas the polymer-conjugated drug can leave the circulation only through interendothelial cell junctions or by uptake into pinocytic cells.

High levels of ADR in cardiac tissue are known to correlate with tissue damage and cumulative, delayed-onset cardiotoxicity [20]. Consequently, numerous attempts have been made to decrease the cardiac-levels of anthracyclines using a variety of drug-delivery systems; many of these are not actively targeted to specific cells, and operate simply by modifying the physical properties and release kinetics of the drug. Such delivery systems can be usefully distinguished into those formulations where the anthracycline is bound to a carrier molecule using a covalent linkage that is stable in the bloodstream, being cleaved only after internalization into cells (e.g. conjugates with proteins [21], polyamino acids [22] and synthetic polymers [23]), and systems based on non-covalent complexes (e.g. liposomes [24], erythrocytes [25], microparticles [26] and DNA-conjugates [27]). These latter formulations are often unsuitable substrates for pinocytosis, largely remaining dispersed in the extracellular fluid, although some do have the capacity for passive targeting and show accumulation in phagocytes or certain capillary beds. In exerting their antitumour activities, such formulations probably act mainly as depots for the sustained release of drug [28].

It is possible to obtain much greater reductions in the cardiac ADR-content using stable, covalent conjugates which are only cleaved intracellulary (e.g. 99% fall in cardiac levels of drug achieved in this study) than with sustained release formulations (e.g. liposomal preparations of ADR, which usually achieve reductions of 60% [29]–80% [28]). A recent study measuring rat cardiac output in a sensitive model for delayed-onset cardiotoxicity [30] has shown a marked reduction in the toxicity of the HPMA copolymer–conjugate of ADR, with no evidence for any decrease in cardiac output up to 20 weeks following administration of the drug (ADR was applied at 4 mg/kg, a dose that is usually lethal when it is administered to these rats in unconjugated form).

Similar HPMA copolymer–DNM conjugates used to treat Walker Sarcoma growing subcutaneously in Wistar rats were able to show both improvement in the therapeutic response for a given dose of DNM bound to HPMA compared with free DNM and also a large increase in the amount of free DNM detected in the tumour following administration of the polymer-bound form (the AUC for DNM levels in tumour tissue over the first 24 hr was increased at least four-fold) [14]. The explanation for this passive tumour targeting of the HPMA copolymer–DNM is not certain, but may be related to the prolonged circulation of the drug in the bloodstream, as described above for HPMA copolymer–ADR. Certain tumours, including Walker Sarcoma [31], are known to have high rates of pinocytosis *in vivo*, and it has been suggested that extending the circulation times of antineoplastic drugs in the circulation may elevate their relative concentrations in tumour cells [32].

If increased pinocytic capture by tumour cells is indeed the basis for enhanced efficacy of untargeted polymer-bound anthracyclines, then other non-targeted lysosomotropic delivery systems might be expected to display similarly elevated activity. DNM linked via the amine group of the daunosamine ring through a lysosomally-degradable peptide chain to albumin or directly to oxidized dextran has been shown to possess substantially increased antineoplastic activity *in vivo* against L1210 leukaemia [21] and YAC lymphoma [33] respectively. Conversely ADR linked to non-specific monoclonal antibodies via an ester bond is reported to show no such improvement, tested against various model

tumours [34]. The variations in efficacy of covalently-bound anthracycline formulations [9, 21, 32–35] may be induced by differences in the site and technique of derivatization of the anthracycline molecule, including the chemical harshness of the methods employed. In particular, it is clear from our own work that the nature of the spacer group between anthracycline and polymer carrier is crucial. The tetrapeptide linker Gly-Phe-Leu-Gly (a preferred substrate for lysosomal cathepsins) permits cleavage of the anthracycline from the carrier following internalization, promoting effective antitumour activity, whereas the non-lysosomally-degradable spacer Gly-Gly produces a conjugate molecule that is inactive *in vivo* [12, 13]. In addition, the macromolecular carrier may itself influence the activity of the conjugate (for example, pyran copolymer, which is known to have immunostimulatory activity, has been used to deliver ADR [35]). However, the majority of soluble macromolecular conjugates of ADR, based on lysosomally-degradable covalent linkages, show at least equal antitumour activity compared with the same dose of free drug, and some display much greater activity. Those soluble conjugates which do not display elevated antitumour activity tend to be based on labile bonds, such as esters, which may be broken rapidly following injection, releasing a burst of free drug into the system without involving a lysosomotropic step. Conversely, non-targeted delivery systems based on vehicles that are not suitable substrates for pinocytosis (such as liposomes and microparticles) generally do not show an increase in the specific activity of ADR [5, 24, 28, 29, 36, 37], and achieve improved efficacy only by the administration of elevated doses of the drug.

The evidence available suggests that enhanced capture of circulating drug-conjugates by pinocytic tumour cells may underlie the improved efficacy seen with soluble covalent complexes of ADR; extending the circulation-time of ADR-conjugates further may cause still greater tumour-accumulation with corresponding improved efficacy in the model systems described. Whether this principle can be applied generally is currently being investigated in other tumour model systems. The Clinical Trials Committee of the Cancer Research Campaign has approved ADR–HPMA copolymer conjugates for human investigation, and phase I/II trials are planned for the near future.

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