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# Reduced cardiotoxicity of doxorubicin given in the form of *N*-(2-hydroxypropyl)methacrylamide conjugates: an experimental study in the rat

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**Summary.** A rat model was used to evaluate the general acute toxicity and the late cardiotoxicity of 4 mg/kg doxorubicin (DOX) given either as free drug or in the form of three *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer conjugates. In these HPMA copolymers, DOX was covalently bound via peptide linkages that were either non-biodegradable (Gly-Gly) or degradable by lysosomal proteinases (Gly-Phe-Leu-Gly). In addition, one biodegradable conjugate containing galactosamine was used; this residue was targeted to the liver. Over the first 3 weeks after the i.v. administration of free and polymer-bound DOX, all animals showed a transient reduction in body weight. However, the maximal reduction in body weight seen in animals that received polymer-bound DOX (4 mg/kg) was significantly lower than that observed in those that received free DOX (4 mg/kg) or a mixture of the unmodified parent HPMA copolymer and free DOX (4 mg/kg; $P < 0.01$). Throughout the study (20 weeks), deaths related to cardiotoxicity were observed only in animals that received either free DOX or the mixture of HPMA copolymer and free DOX; in these cases, histological investigations revealed marked changes in the heart that were consistent with DOX-induced cardiotoxicity. Sequential measurements of cardiac output in surviving animals that received either free DOX or the mixture of HPMA copolymer and free DOX showed a reduction of ~30% in function beginning at the 4th week after drug administration. The heart rate in these animals was ~12% lower than that measured in age-matched control rats ($P < 0.05$). Animals that were given the HPMA copolymer conjugates containing DOX exhibited no significant change in cardiac output throughout the study ($P < 0.05$). In addition, no significant histological change was observed in the hearts of animals that received DOX in the form of HPMA copolymer conjugates and were killed at the end of the study. However, these animals had shown a significant increase in heart rate beginning at 8 weeks after drug administration ($P < 0.01$). This study demonstrates that covalent binding of DOX to HPMA copolymer conjugates via both stable and biodegradable peptidyl linkages considerably reduces both the general acute toxicity and the late cardiotoxicity of DOX in the rat and could offer the potential for improving the therapeutic index in the clinical application of DOX.

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## Introduction

The anthracycline antibiotic doxorubicin (DOX) shows a valuable broad spectrum of antitumour activity in man [2]. However, its clinical use is often limited by peripheral toxicity, particularly cardiotoxicity, the onset of which is delayed and can lead to congestive heart failure [22]. In view of this limiting toxicity, it is recommended that the cumulative dose given to patients be restricted to 550 mg/m$^2$ [19], although a recent study has demonstrated a significant fall in the left ventricular ejection fraction even after a lower cumulative dose of 300 mg/m$^2$ [31].

Several approaches have been proposed to increase the therapeutic index of anthracyclines, some of which were specifically designed to abrogate the associated cardiotoxicity. These include the synthesis of novel analogues that produce reduced cardiotoxicity, such as epirubicin (EpiDox) [36], the use of cardioprotective agents [14] and the manipulation of the schedule or route of drug administration [16, 29]. An alternative approach involves the modification of pharmacokinetics using drug-delivery systems such as immunoconjugates, liposomes, nanoparticles, erythrocyte ghosts and macromolecular conjugates [24, 40]. Such delivery systems can reduce DOX deposition in the heart and also, in certain cases, increase the tumour-specific accumulation of the drug.

In this respect, *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer conjugates containing anthracyclines represent a particularly promising approach. The drug is

**Table 1.** Characteristics of polymers used

| Polymer code[a] | Side-chain structure | Side-chain characteristics |
|---|---|---|
| 1 | P-Gly-Gly-DOX | Non-biodegradable |
| 2 | P-Gly-Phe-Leu-Gly-DOX | Biodegradable |
| | Gly-Phe-Leu-Gly-DOX | Biodegradable; |
| | / | |
| 3 | P | targeting to |
| | \ | |
| | Gly-Phe-Leu-Gly-galactosamine | hepatocytes |
| | Gly-Gly-aminopropanol | |
| | / | |
| 4 | P | Non-biodegradable |
| | \ | |
| | $TyrNH_2$ | |

[a] Only copolymers 1–3 contain doxorubicin
DOX, Doxorubicin; P, polymer backbone; Gly, glycine; Phe, phenylalanine; Leu, leucine

covalently bound to the polymer via oligopeptide spacers designed specifically for stability in the circulation [26] and for terminal cleavage by lysosomal, thiol-dependent enzymes following pinocytic internalisation of the conjugates [7]. Such conjugates exhibit antitumour activity in vitro [8] and in vivo [9–11], and there is evidence that administration of DOX in the conjugate form reduces the drug's general toxicity; the $LD_{50}$ value (lethal dose for 50% of the group) for conjugates is 5- to 10-fold that obtained for free DOX [10]. In addition, drug conjugation has resulted in a 100-fold decrease in the DOX levels measured in heart tissue [3, 28], suggesting that conjugates may also have considerable potential for abrogating the late onset of DOX-associated cardiotoxicity.

In the present study, an established, clinically relevant model system in the rat was used to examine the cardiotoxicity of HPMA copolymer conjugates containing DOX. This method has successfully been used to compare the relative cardiotoxicity of DOX and EpiDOX [38] and involves non-invasive techniques to assess sequential changes in cardiac output and heart rate after drug administration. Histological examinations were also carried out using the method of Bertazzoli et al. [1] in animals that died and in those that were killed after 20 weeks at the end of the study. The HPMA copolymers containing DOX were synthesised to contain oligopeptide spacers that were either non-degradable (Gly-Gly) or degradable by lysosomal enzymes (Gly-Phe-Leu-Gly). In addition, galactosamine, a terminal moiety that can be used to target polymer conjugates to liver hepatocytes [23; (Seymour et al., in press)], was incorporated into one copolymer conjugate. The effects of HPMA copolymer conjugates containing 4 mg/kg DOX were compared with those of free DOX and those of a mixture of an HPMA copolymer and free DOX.

## Materials and methods

A total of 50 male Sprague-Dawley rats aged 14 weeks and weighing 400–450 g were used in this study. The animals were caged in groups of three and were fed 41-B cubed diet and water ad libitum. These animals were sub-divided into seven groups comprising a minimum of seven animals each. Three groups of animals received single i.v. doses of one of the three copolymers (Table 1) containing 4 mg/kg DOX (Farmitalia, Milan, Italy). The other groups received either saline (control group), 4 mg/kg free DOX, a mixture of HPMA copolymer 4 (54.8 mg/kg) and free DOX (4 mg/kg) or 54.8 mg/kg HPMA copolymer 4 alone (Table 1). Free DOX and HPMA conjugates were dissolved in sterile water to give a DOX concentration of 2 mg/ml. Injections were given via the femoral vein at a rate of 2 ml/min. For drug administration and for subsequent measurements of cardiac output and heart rate, the animals were anaesthetised with chloral hydrate (300 mg/kg i.p.). DOX and HPMA conjugates were always given between 1330 and 1530 hours so as to minimise any effects of circadian timing [39].

The methods used to prepare the monomers, HPMA, methacryloyltyrosinamide and methacryloylated peptidyl *p*-nitrophenyl esters have been described elsewhere [6, 27, 32]. Polymer precursors were then synthesised by copolymerisation of HPMA with the appropriate methacryloylated monomers [27]. DOX and (in the case of copolymer 3) galactosamine were bound by consecutive aminolysis [25].

The methods used to assess general acute and late cardiotoxicity in the rat after drug administration have been described elsewhere [38]. Briefly, the animals were examined and weighed daily for up to 3 weeks after drug administration. The mean maximal reduction in body weight observed over this period was used to assess general acute toxicity. Relative changes in cardiac function were assessed at 4-week intervals for up to 20 weeks by the measurement of cardiac output and heart rate in drug-treated animals as compared with rats that received saline.

Cardiac output was determined by an external counting technique: a 0.2 ml bolus of the radioactive tracer sodium pertechnetate Tc 99m (sp. act., 2 mCi/ml) was injected into the femoral vein and the activity-time curve over the heart was monitored for 40 s using an NaI detector. The heart rate was determined concomitantly using a modified human ECG monitor (Hewlett Packard 7830 A) coupled to a scope memory and a chart recorder.

Postmortem examinations were carried out in animals that died during the course of the study and in those that were killed at the end of the experiment at 20 weeks. The hearts were fixed in 10% formal saline, dehydrated, and embedded in hydroxyethylmethacrylate. Sections measuring 1 μm in thickness were cut and stained with toluidine blue (0.1%) in 1% Borax buffer prior to examination by light microscopy. The atrial and ventricular lesions observed were separately scored using the method of Bertazolli et al. [1]. Animals that died without fulfilling the criteria for heart failure were excluded from the final analysis [38, 39]. Animals were considered to have died of heart failure when they showed signs of congestive heart failure at death (e.g. pleural effusion, ascites and/or general oedema) or had shown a reduction of >40% in cardiac output prior to death and/or histological evidence of anthracycline-associated myocardial lesions together with signs of pulmonary congestion. In the present study, statistical differences between group mean values were analysed using Student's *t*-test.

## Results

In the first 2–3 weeks after drug administration, the majority of animals showed no overt sign of toxicity. Only one animal died during this period; it had received free DOX (4 mg/kg) and showed clinical signs of congestive heart failure, with haemorrhagic pleural effusion (10 ml), ascites and general oedema on its death. Both treated and control animals showed a transient reduction in body weight. The maximal reduction in body weight was recorded after approximately 6 days for animals that received free DOX or the mixture of copolymer 4 and free DOX. The body weight of these animals returned to pre-treatment values between days 14 and 21. In animals that received either saline, conjugates containing DOX (copolymers 1–3) or

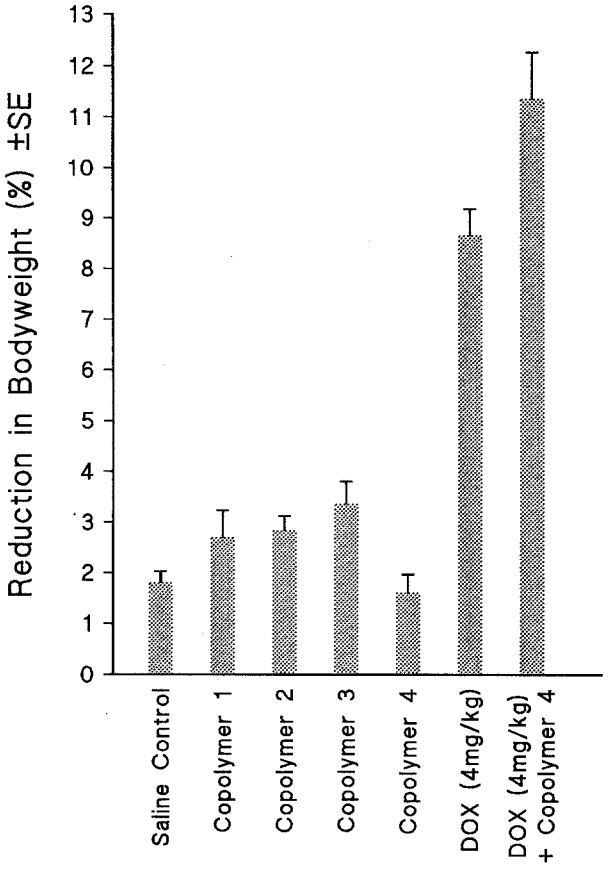


**Fig. 1.** Histogram showing the maximal reduction in body weight (%± SE) in the first 2–3 weeks after drug administration in the different groups of animals. Copolymers 1–3 were DOX-bound conjugates and were given at a dose of 4 mg/kg DOX

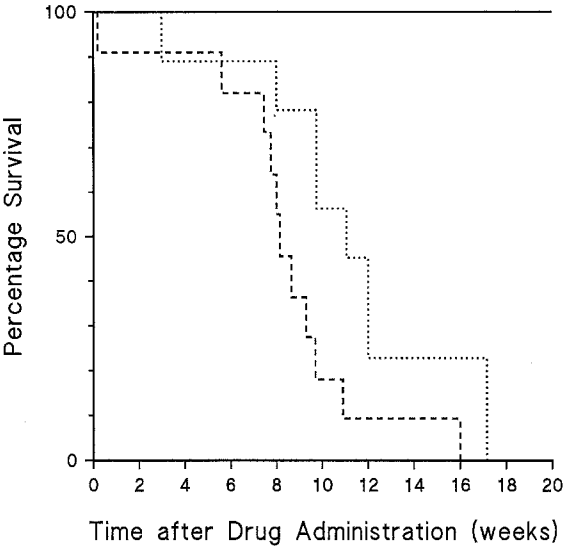


**Fig. 2.** Time-related changes in animal survival for up to 20 weeks after the administration of free DOX (– – – –), copolymer 4+ free DOX (. . .), copolymers 1–4 or saline (———)

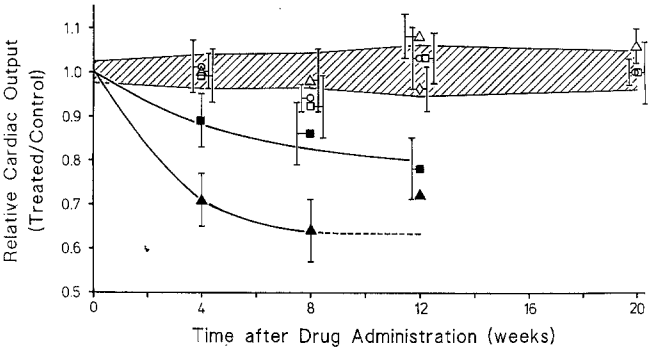


**Fig. 3.** Time-related changes in the relative cardiac output of rats (± SE) after single intravenous doses of drug. □, Copolymer 1; ○, copolymer 2; △, copolymer 3; ◇, copolymer 4; ■, mixture of copolymer 4+ free DOX; ▲, free DOX. The *hatched area* represents the SE for age-matched control animals

HPMA copolymer 4 alone, the maximal reduction in body weight was seen on day 2 or day 3. In all of these groups, the body weight had returned to pre-treatment values by day 4 or 5.

For comparative purposes, the maximal reduction in body weight in each group was expressed as a percentage of their respective pre-treatment values (Fig. 1). The greatest reduction in body weight was seen in animals that were given either free DOX or the mixture of HPMA copolymer and free DOX, the values obtained being 8.64%±0.52% and 11.32%±0.92%, respectively. This decrease was significantly greater than that observed in rats that received the HPMA copolymer-DOX conjugates, saline, or copolymer 4 alone ($P$ <0.001). The reduction in body weight in animals that were given the biodegradable copolymers 2 and 3 was slightly, albeit significantly, greater than that in animals that received saline ($P$ <0.01). The reduction in body weight did not differ significantly among animals that received saline, copolymer 4 alone or the non-degradable copolymer 1 ($P$ >0.1).

Almost all animals survived without exhibiting apparent adverse symptoms for the first 6 weeks following drug administration. However, the condition of rats that received free DOX or the mixture of HPMA copolymer and free DOX declined rapidly thereafter. Most of these animals died of heart failure at between 6 and 12 weeks after drug administration (Fig. 2). At death, their body weight was reduced by up to ~40% of the pre-treatment value, and on postmortem examination, ~50% of them showed signs of congestive heart failure with haemorrhagic pleural effusion, ascites and/or general oedema. In marked contrast, the animals that received the HPMA copolymers conjugated with DOX, copolymer 4 alone or saline survived for 20 weeks. All of these animals appeared healthy and continued to gain weight throughout the study, although rats that received the three conjugates containing DOX displayed a lower body weight (approximately 10%) than did the age-matched controls. However, this difference was not significant ($P$ >0.05), and no gross abnormality was observed on postmortem examination at 20 weeks after drug administration.

Sequential measurements of cardiac output showed a progressive decline in function in animals that received free DOX or the mixture of HPMA copolymer and free DOX (Fig. 3). The decline was significantly greater in animals that were given free DOX ($P$ <0.05). Cardiac out-

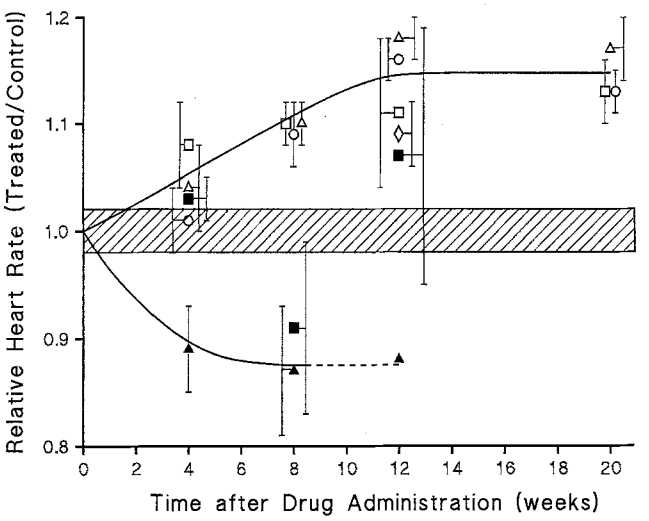


**Fig. 4.** Time-related changes in the relative heart rate of rats (± SE) after single intravenous doses of drug. For key to symbols, see Fig. 3

put measured in survivors from these two groups of animals at 12 weeks was ~75% of that determined in age-matched control rats. However, the loss of animals due to heart failure during the study indicates that these data underestimate the true effect of the drug [33]. Animals that received HPMA copolymer-conjugated DOX, (copolymers 1–3) or copolymer 4 alone showed no change in cardiac output throughout the study.

Rats that received free DOX or the mixture of HPMA copolymer and free DOX also showed a progressive reduction in heart rate; at 8 weeks, it was reduced to ~90% of the values measured in control animals that received saline (Fig. 4). However, the reduction was significant only in animals that received free DOX ($P < 0.05$). At 12 weeks, this heart rate of the sole survivor in this group was maintained at this reduced level. However, in the two survivors that received the mixture of HPMA copolymer and free DOX, we found an increase in heart rate in one animal and a normal value in the other. All groups that received HPMA copolymer-conjugated DOX exhibited a progressive increase in heart rate that was ~15% higher than age-matched control values at 12 weeks, and the mean heart rate remained elevated at this level until the end of the study at 20 weeks after drug administration. Measurement of the heart rate in animals that received copolymer 4 alone revealed an increase of ~9% at 12 weeks ($P < 0.01$).

Histological examination of the heart specimen was carried out in animals that died during the course of the experiment after receiving free DOX or the mixture of HPMA copolymer and free DOX. However, a few of these animals exhibited such severe postmortem changes that histological examination was not possible. The rest of the animals examined exhibited lesions in the myocardium that were typical of those induced by DOX in the rat. Their features included vacuolisation of myocytes, loss of myofilaments, interstitial oedema, fibrosis and necrosis. The extent of damage involved ranged from the presence of single, scattered myocytes to the occurrence of widely spaced foci of damaged myocytes. Based on an arbitrary

**Table 2.** Histological grading of cardiac lesions after single doses of either HPMA copolymers 1–3 conjugated to DOX, free DOX or of copolymer 4 in the rat

| Compound | Histological score[a] | | Hearts examined |
|---|---|---|---|
| | Atrial lesions | Ventricular lesions | |
| Free DOX | 3.1 (2–6) | 2.6 (1–4) | 8 |
| Copolymer 4+ free DOX | 4.8 (4–6) | 4.8 (4–6) | 5 |
| Copolymer 1 | 0.1 (0–0.5) | 0.3 (0–0.5) | 6 |
| Copolymer 2 | 0.3 (0–0.5) | 0.1 (0–1.5) | 7 |
| Copolymer 3 | 0.3 (0–0.5) | 0.5 (0–2) | 7 |
| Copolymer 4 | 0.1 (0–0.5) | 0.8[b] (0–1) | 5 |

[a] Average grade of lesions scored according to Bertazzoli et al. [1]; associated ranges of values are shown in parentheses

[b] Non-specific interstitial myocarditis

histological scoring system, the mean score for the atria and ventricles of animals that received free DOX was 3.1 (range, 2–6) and 2.6 (range, 1–4), respectively. For animals that were given the mixture of HPMA copolymer and free DOX, both the atria and the ventricular scores were 4.8 (range, 4–6; Table 2). Histological examination of hearts from animals that were killed at 20 weeks after they received the DOX conjugates revealed a very low score for cardiac lesions in both the atria and the ventricles (<0.5). In all groups the number of damaged myocytes was low and the severity of myocyte vacuolisation was moderate. The hearts of four of the five animals that were given copolymer 4 alone showed focal areas of non-specific interstitial myocarditis.

## Discussion

Although the mechanisms responsible for DOX cardiotoxicity are not completely understood, recent studies suggest that damage may result from free radical generation and/or from the formation of doxorubicin-iron complexes [5, 20]. Changes noted in the heart after DOX administration include lipid peroxidation [21], a modification in enzyme activity [4, 13] and changes in the calcium balance [18]. It is likely that a combination of these effects plays a role in the induction of cardiotoxicity. However, there is a general consensus that anthracycline-induced cardiotoxicity is related both to the peak concentration of the drug in the heart [31] and to the rate of drug elimination [33]. Free DOX enters cells by diffusion, and the heart concentration would thus be expected to be related to the plasma concentration. However, this relationship is highly complex.

The potential for accomplishing a reduction in anthracycline-related cardiotoxicity by the use of drug-delivery systems has been an attractive prospect for some time. The entrapment of drug within particulate carriers such as liposomes or the synthesis of macromolecular conjugates should in theory reduce drug access to tissues by abolishing membrane diffusion; such vehicles can enter cells only by the endocytic route. In previous studies, a 3- to 6-fold decrease in DOX levels in the hearts of mice has been

achieved by the binding of anthracycline to liposomes via a stable formulation [12]. Niosomal [17] and cyanoacrylate nanoparticulate [35] formulations of DOX have achieved a similar effect. However, the simple physical entrapment of DOX has a disadvantage: there is always a potential for the drug to escape from the carrier by diffusion and be released in a random and uncontrollable fashion [34].

The covalent binding of DOX to HPMA copolymers produces a formulation that is stable in circulation, and when a desired peptide linkage is implemented, DOX can be released in a more controllable manner [7, 8, 28]. It has recently been shown [28] that copolymer binding of DOX essentially abolishes the appearance of free drug in the heart. In view of the fact that two such polymer conjugates will soon be subjected to clinical evaluation, it was considered important that the degree of cardiotoxicity resulting from their use be investigated using a clinically relevant assay in the rat [37–39]. The present study demonstrated that DOX that has been covalently bound to HPMA copolymer and given i.v. is much less cardiotoxic than free DOX. A dose of 4 mg/kg free DOX significantly reduced the cardiac output of rats, with ~90% of the animals dying of heart failure within 12 weeks of drug administration. At death, all of these animals exhibited histological evidence of severe DOX-induced cardiotoxicity. However, when the same dose of DOX was given in the HPMA copolymer form, cardiac output was not significantly modified in the first 20 weeks after drug administration. All animals thus treated survived the 20-week observation period, and at the end of the experiment, their heart tissue showed no significant histological change.

Administration of DOX as a simple mixture with an HPMA copolymer indicated that a small degree of cardioprotection was provided by the polymer. Animals that received the mixture showed a slight but insignificant increase in life span after drug administration as compared with those that received free DOX alone ($P > 0.05$); moreover, the decrease in cardiac output measured in the former animals was significantly lower than that observed in the latter group ($P < 0.05$). The mechanisms leading to this slight cardioprotective effect of the HPMA copolymers are unknown, but when DOX was given with an HPMA copolymer, the solution injected was more viscous than that given as free DOX in sterile water. This could have slightly modified the initial biodistribution of DOX, resulting in a subsequent change in cardiotoxicity.

As indicated by cardiac output measurements and confirmed by histological observations, the marked reduction in cardiotoxicity produced by HPMA copolymer-conjugated DOX is consistent with the pharmacokinetic measurements reported elsewhere [28]. Lysosomal proteinase-mediated drug release from conjugates containing the spacer Gly-Phe-Leu-Gly has been followed in the liver after the i.v. injection of the galactosamine-targeted formulation (copolymer 3), and it is known that drug is released slowly within the hepatocyte over approximately 24 h (Seymour et al., in press). Copolymer 2 is likely to release anthracycline even more slowly following non-selective fluid-phase pinocytic capture by both tumour [3, 28] and normal cells. However, in theory, extracellular release could also occur if the appropriate proteolytic enzymes were present. It is clear from the results of the present study that such a slow release of DOX does not compromise cardiac function at a dose of 4 mg/kg. However, it is necessary that the dose dependency of conjugate toxicity be carefully investigated, and these experiments are currently under way. A reduction in the cardiotoxicity of free DOX due to a decrease in the peak blood concentration has been reported following the continuous infusion of the drug [16].

Although no significant modifications in cardiac output were observed in animals that received HPMA copolymer-conjugated DOX, a significant increase of up to ~15% in the heart rate occurred during the course of the study. The pattern of change equally applied to conjugates containing the biodegradable spacers (copolymers 2 and 3) and to those containing the non-biodegradable spacers (copolymer 1). This was somewhat unexpected, particularly in the case of copolymer 1, which released DOX at a rate of <1% over 24 h on incubation in vitro with isolated lysosomal enzymes (Subr et al., in press) and does not show antitumour activity in vivo [10]. HPMA copolymer alone produced an increase of ~9% in the heart rate after 12 weeks ($P < 0.01$).

A pattern of change in the heart rate similar to that produced by HPMA copolymer-conjugated DOX was found in animals receiving a lower dose of free DOX (1 mg/kg) (Yeung, unpublished data). This contrasts with the present findings in animals that received 4 mg/kg free DOX or the mixture of HPMA copolymer and free DOX, which revealed a substantial fall in heart rate, probably reflecting extensive and terminal cardiac failure. The increase in heart rate measured in animals that were given the conjugates is not fully understood, although it might reflect the cardiac reserve's compensating for a subtle level of heart damage that is undetectable by the measurement of cardiac output alone. Further studies are required to elucidate this phenomenon.

The present study also confirmed previous reports [10] concerning the protective effects of the binding of DOX to HPMA copolymers on other toxicities of the drug. The regular monitoring of body weight during the first 3 weeks after the administration of HPMA copolymer-conjugated DOX showed a significant reduction in general toxicity. Moreover, monitoring of body weight in these animals at 4 week intervals for 20 weeks also demonstrated an improvement in their general condition; their growth was comparable with that observed in age-matched control rats. This indicates another potential advantage of the clinical use of these HPMA copolymers conjugated to DOX. In the treatment of malignant disease, the initial toxic effects of DOX on the bone marrow and epithelial tissues has limited the use of the drug to a 3- to 4-week cycle.

Thus, in summary, the cardiotoxicity of DOX can be significantly reduced when the drug is conjugated to HPMA copolymers. Although the magnitude of this effect is unknown, it would appear to be greater than that achieved by the administration of EpiDOX as an analogue of DOX [38]. The reduced general acute toxicity of DOX conjugated to HPMA copolymers may represent another clinical advantage of the use of these compounds, which seems unlikely to be the case for other approaches design-

ed to reduce cardiotoxicity alone [15]. This factor and the potential for tumour-specific targeting of DOX may offer the possibility of achieving a substantial increase in the therapeutic index of DOX in cancer therapy.

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