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Delivery of an anticancer drug and a chemosensitizer to murine breast sarcoma by intratumoral injection of sulfopropyl dextran microspheres

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Abstract

Intratumoral injection of controlled-release microsphere formulations of anticancer compounds has the potential to selectively increase tumour exposure to drugs. This work aimed to evaluate the therapeutic effect and toxicity of microsphere formulations containing the anticancer drug, doxorubicin, in a murine tumour model. The effect of co-administration of verapamil, a P-glycoprotein modulator or chemosensitizer, was investigated. Initial in-vitro studies confirmed the ability of verapamil to enhance the accumulation of both doxorubicin and [^{99m}Tc]sestamibi, also a P-glycoprotein substrate, in EMT6 murine breast sarcoma cells and a doxorubicin-selected multidrug-resistant variant, EMT6/AR1.0. Ex-vivo studies using confocal microscopy demonstrated release of doxorubicin from microspheres and diffusion of the drug through tissue. For in-vivo studies, EMT6 and EMT6/AR1.0 cells were grown in BALB/c mice. Following intratumoral injection of doxorubicin-loaded microspheres, alone or in combination with verapamil-loaded microspheres, the tumour diameter was measured serially as an indication of therapeutic effect, while the weight, appearance, and behaviour of the mice were monitored as an indication of general toxicity. Intratumoral injections of doxorubicin-loaded microspheres were tolerated much better than systemic administration of equivalent drug concentrations. There was a modest (up to 34%) delay of tumour growth compared with groups receiving no treatment or blank microspheres. Co-injection of verapamil microspheres with doxorubicin microspheres produced a moderate increase in toxicity but no further delay in tumour growth. Controlled-release microsphere formulations of anticancer agents administered intratumorally were an efficient way to deliver high drug doses to the tumour with little systemic toxicity.

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Introduction

Locoregional administration of currently available anticancer agents is a promising way to enhance their therapeutic efficacy (Ensminger & Gyves 1984; Deurloo et al 1991; Anderson et al 1994). This can be achieved by intra-arterial infusion of chemotherapeutic agents upstream of the tumour or by direct injection into the tumour (intratumoral injection), and has been evaluated using a number of anticancer drugs with varying degrees of success (Ensminger & Gyves 1984; Deurloo et al 1991; Anderson et al 1994; Aigner 1998; Lee et al 2002). High intratumoral drug concentrations can be achieved while exposure of normal tissues is reduced (Kato et al 1996; Sugiyama et al 1998; Hagiwara et al 1998).

Chronic exposure of tumour cells to certain anticancer drugs, including doxorubicin, vinblastine, and paclitaxel, in-vitro or in-vivo can lead to the development of multidrug resistance (MDR) (Bradley et al 1988; Cole et al 1992). MDR is characterized by diminished cellular drug accumulation that usually results from an increased rate of drug efflux by specific membrane transporters, such as P-glycoprotein (Pgp) and multidrug resistance-related protein (MRP). Addition of MDR-reversing agents or chemosensitizers, such as verapamil, ciclosporin, and more potent and selective second- and third-generation agents, to conventional chemotherapy has been used for treatment of MDR tumours (Raderer & Scheithauer 1993; Bartlett et al 1994;

Germann et al 1997). However, there has been limited success in clinical trials thus far, especially in the treatment of solid tumours. This is partially a consequence of the inability to achieve effective drug concentrations in the tumours without excessive systemic side effects (Raderer & Scheithauer 1993). Since the locoregional delivery of microsphere (MS) formulations of anticancer drugs has been successful in increasing the intratumoral drug concentration (Codde et al 1990; Chen et al 1992), simultaneous delivery of chemosensitizing and antineoplastic agents could, in theory, increase tumour cell killing for MDR tumours.

Previous work in this laboratory has shown sulfopropyl dextran microspheres to be suitable drug carriers due to their biocompatibility as well as high drug loading capacity for both anticancer drugs (e.g. doxorubicin and vinblastine) and chemosensitizers (e.g. verapamil and quinidine) (Liu et al 1997, 1999, 2001b). The microspheres alone are not cytotoxic in-vitro and drugs released from the microspheres are not altered in chemical structure or potency (Liu et al 1997, 1999, 2001b). Above all, in an MDR cell culture, it has been demonstrated that chemosensitization can be obtained by simultaneous delivery of vinblastine-loaded microspheres and verapamil microspheres (Liu et al 1999) or Triton-X-100 modified polymers and vinblastine (Liu et al 2001a). The focus of this work was in-vitro and in-vivo evaluation of microsphere-loaded doxorubicin (Dox-MS) and verapamil (Ver-MS) in a murine tumour model using the EMT6 mouse mammary sarcoma cell line and its doxorubicin-selected MDR derivative EMT6/AR1.0 (Twentyman et al 1990).

Materials and Methods

Materials

Cross-linked dextran microspheres containing sulfonic groups (Sephadex SP C-25, Pharmacia & Upjohn, Kalamazoo, MI) were washed several times with deionized water before use. Doxorubicin HCl and verapamil HCl were purchased from Sigma Chemical Company (St Louis, MO). Pluronic F-127 was a gift from BASF Corporation (Mount Olive, NJ). [^{14}C]Doxorubicin HCl (sp. act. 1.85 GBq mmol^{-1}) was purchased from Amersham Life Sciences (Amersham, Bucks, UK). [^{99m}Tc]Sestamibi was prepared from kits (Cardiolite, DuPont Pharma, Billerica, MA).

Characterization of particle size and distribution of microspheres

To evaluate whether the microspheres had suitable sizes for injection, their particle size and size distribution were determined with a particle sizer. The microspheres were dispersed in an isotonic salt solution with addition of a small amount of a nonionic dispersant (Coulter type IA), and the particle size and size distribution were measured by a Coulter Counter (R) Multisizer (Coulter Electronics Inc., Hialeah, FL) with aperture size of 560 nm and aperture current of 3200 μA.

Drug loading into the microspheres and in-vitro drug release

In a typical loading process, dry ionic microspheres (30 mg) were added to 10 mL deionized water containing 1 mg mL^{-1} doxorubicin HCl. After incubation at room temperature for predetermined time intervals, samples of microsphere suspension were centrifuged and the residual drug concentration in the supernatant was analysed by absorbance at 540 nm. When drug incorporation reached equilibrium, as indicated by no further reduction in doxorubicin concentration in the supernatant, the microspheres were harvested by centrifugation, washed extensively with deionized water and lyophilized. The amount of drug loaded was calculated from the difference between the initial and final drug concentration in the supernatant after incubation with the microspheres. The parameters of the standard curve were: slope, 30.77; intercept, 0.0268; $r^2 = 0.9985$. Verapamil loading was carried out using the same method with its concentration in solution being monitored by UV absorbance at 278 nm (Liu et al 1999). The parameters of the standard curve were: slope, 4.7503; intercept, 0.0513; $r^2 = 0.9831$. Drug loading was expressed as weight of drug per unit weight of microspheres $\times$ 100% (% w/w). Release studies of doxorubicin or verapamil from loaded microspheres were carried out under sink conditions at 37 °C with a UV cuvette containing 0.15 mg dry microspheres in 3 mL pH 7.4 phosphate buffer (0.05 M). The mixtures were gently stirred magnetically while the absorbance of the sample was measured as a function of time spectrophotometrically as above (Liu et al 1999).

To develop an injectable microsphere formulation, Pluronic F-127 was used. This non-ionic surfactant can form a viscous gel at body temperature thus providing an additional release barrier. An aqueous solution ($\sim$20%) of Pluronic F-127 was prepared at 4 °C. This solution undergoes gelation at temperatures higher than 10 °C. Microspheres (Sephadex SP C-25) loaded with ionic drugs (e.g. verapamil or doxorubicin) were added to the cold solution with stirring. The suspension showed the same reversible thermal gelation as the parent Pluronic F-127 solution, as demonstrated in heating and cooling cycles. It was syringe-injectable at lower temperatures. A 1-g sample of the gel with 5% w/w microspheres loaded with 29% w/w verapamil was transferred into a dialysis tubing (molecular weight cut-off: 6000), which was then clamped and placed in 200 mL phosphate buffer (0.05 M, pH 7.4). The amount of doxorubicin released was monitored as described above.

Tissue cell culture and drug accumulation studies

The parental EMT6 and multidrug-resistant EMT6/AR1.0 (EMT6/AR) murine breast sarcoma cell lines, originally obtained from Dr P. Twentyman, University of Cambridge, UK (Twentyman et al 1990), were gifts from Dr I. F. Tannock, Ontario Cancer Institute, Toronto, Canada. The EMT6/AR resistant variant had been

selected by growth in the presence of 1.0 μg mL^{-1} doxorubicin and originally showed 70-fold resistance to doxorubicin compared with the parental cell line (Twentyman et al 1990). Cells were grown in 25-cm^2 plastic tissue culture flasks in alpha minimum essential medium (α-MEM), containing 10% foetal bovine serum and 0.5% penicillin–streptomycin (growth medium) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Cultures were re-established from frozen stock after approximately three months. To characterize the cell lines, in-vitro drug accumulation studies were performed as described previously (Bendayan et al 1994; Liu et al 1999). Cell suspensions (0.5 mL, 1.2×10^6 cells mL^{-1}) were seeded on 24-well plates and grown to confluence (2–3 days). Drug accumulation was initiated by addition of 0.5 mL Earle's Balanced Salt Solution (EBSS) containing 0.2 kBq (5 μg) [^{14}C]doxorubicin or 100 kBq [^{99m}Tc]sestamibi (Ballinger et al 1995). The cells were then incubated for up to 2 h at 37 °C in an atmosphere of air. Drug accumulation by the cells was rapidly stopped by aspirating the media and by washing the attached cells twice with ice-cold saline. The cells were then lysed with NaOH, neutralized with HCl and the ^{14}C and ^{99m}Tc activities were measured by liquid scintillation and gamma counting, respectively. Protein determination was performed for each plate by a standard colorimetric method (Bradford 1976; Bendayan et al 1994). Correction for non-specific binding to cells, determined by measuring the radioactivity in samples at time zero, accounted for 1 to 2% of the cellular uptake. The accumulation of [^{14}C]doxorubicin or [^{99m}Tc]sestamibi over time by the parental and resistant cells was determined in the presence or absence of 50 μM verapamil to evaluate the chemosensitization effect of verapamil. Results were expressed as radioactivity accumulated normalized to protein concentration.

Selection of doxorubicin and verapamil dosages and formulations for in-vivo studies

Doxorubicin is a toxic substance with an LD50 of 21.1 mg kg^{-1} for intravenous administration to albino mice (Swiss strain) (Bertazzoli et al 1970). The dose of doxorubicin used in chemotherapy is typically 5 mg kg^{-1} for a mouse tumour model, or 100 μg per 20-g mouse (Nahabedian et al 1988). Verapamil at doses of 8, 16, and 32 mg kg^{-1} has been shown to be safe in animals when given subcutaneously together with 5 mg kg^{-1} doxorubicin (Giri & Marafino 1984); therefore, 30 mg kg^{-1} or 600 μg verapamil per 20-g mouse was selected for co-administration with 100 μg doxorubicin. Three microsphere formulations were prepared: Dox-MS-33 containing 33% w/w doxorubicin, Dox-MS-10 containing 10% w/w doxorubicin, and Ver-MS consisting of 30% w/w verapamil.

Ex-vivo studies of doxorubicin release and diffusion in tissue

Samples of 100 μL Dox-MS-33 were injected into the livers of two female BALB/c mice. After 10 or 60 min, the mouse was killed by cervical dislocation, and the liver was excised and then snap frozen. Cryostat sections of 10-μm thickness were prepared and examined under a confocal microscope (MRC 600, Bio-Rad Laboratories, Hercules, CA).

In-vivo treatment of mice bearing EMT6 or EMT6/AR tumours

Ten-week-old female BALB/c mice, two weeks after being received from Charles River Lab (Boston, MA), were inoculated intramuscularly in the hind leg with 2×10^5 EMT6 or EMT6/AR cells in 50 μL of the growth medium (Nahabedian et al 1988). Treatments were begun 5–7 days post-inoculation when the tumour-plus-leg diameters were 9–10 mm. All animal handling was conducted under an approved protocol from the Animal Care Committee at the Ontario Cancer Institute following Canadian Council on Animal Care (CCAC) guidelines. The various treatment regimens are outlined in Table 1. All the groups designated "A" represent controls, either without injection (groups 1A, 5A) or with injection of blank microspheres (groups 2A, 3A, 4A). The blank microspheres or microspheres containing doxorubicin or verapamil were suspended in a solution of 9% Pluronic F-127 in deionized water. A sample of 50–100 μL of this suspension was then injected intratumorally using a syringe with a 20-gauge needle. Starting on the day of treatment, the tumour-plus-leg diameter was measured serially, at least every other day, by passing the tumour-bearing leg through a series of circular holes of decreasing diameter at 0.5-mm intervals in a plastic plate. When the tumour-plus-leg diameter exceeded 15 mm, corresponding to a tumour weight of 1.7 g, the animal was killed (Siemann et al 1977). The general toxicity of each treatment was evaluated in a semi-quantitative manner through five parameters, each assessed on a scale of 0, 1, or 2: weight loss (0: no weight loss; 1: 1–2 g weight loss; 2: >2 g weight loss), observation of fur roughing of the animal, decreased water and food intake, general activity, and tissue damage at the site of injection. Thus, a general toxicity score had a maximum value of 10 and minimum value of 0. In experiments 4 and 5 the method of assessing tumour-plus-leg diameter was changed to measure higher up the leg because of a concern about animal tumour burden. This resulted in a shortening of the time for control tumour-plus-leg diameter to reach 15 mm by approximately two days compared with experiments 1–3. However, because the control group within each experiment was assessed in the same manner as treatment groups within the experiment, comparisons between groups within an experiment are still valid. The tumour-plus-leg diameter measurements were converted to tumour weight using a previously determined calibration curve which was done with the measurement technique used in experiments 1–3, Table 1. Thus, the tumour weights in experiments 4 and 5 were slightly overestimated. From the plots of tumour weight vs time, prolonged survival (P - S) was computed as:

$$\text{P - S} = (T_{treat} - T_{control})/T_{control} \times 100\%$$

Table 1 Summary of treatment protocols, tumour growth times, prolonged survival, and toxicity assessment.

Experiment number	Tumour type	Group	Type of microsphere	Microsphere/ doxorubicin dose (μg)[a] × number of injections	Time (days) to 1.5 g[b] (mean ± s.d.)	P-S[c]	Total toxicity score[d]
1	EMT6	1A	None	—	10.0 ± 0.8		0
		1B	Blank	300	10.4 ± 1.1	+4%	0
2	EMT6	2A	Blank	750	10.6 ± 2.7		0
		2B	Dox-MS-33	750/250	13.1 ± 3.2	+24%	0
3	EMT6/AR	3A	Blank	750	10.6 ± 2.0		0
		3B	Dox-MS-33	750/250	9.6 ± 2.5	−9%	0
4	EMT6	4A	Blank	300	7.0 ± 1.1		0
		4B	Dox-MS-33	300/100 × 5	9.4 ± 1.2	+34%[e]	1
		4C	Dox-MS-33 + Ver-MS	300/100 + 1800/600 × 5	9.8 ± 1.4	+40%[e]	4
5	EMT6	5A	None	—	8.1 ± 0.5		0
		5B	Dox-MS-10	1000/100 × 5	9.1 ± 1.2	+12%	3

[a]The dose of microspheres/dose of doxorubicin in μg per injection of each. Multiple injections given on days 0, 2, 4, 6, and 8 are denoted × 5. [b]Tumour-plus-leg diameters were measured every other day and converted to tumour weight using a calibration curve. From a plot of tumour weight as a function of time, the point at which a size of 1.5 g was reached was interpolated to the nearest 0.5 day. In experiments 4 and 5 a more conservative measurement was taken, resulting in a shortening of time to reach 1.5 g by approximately two days. [c]Prolonged survival $(P\text{-}S) = (T_{treat} - T_{control})/T_{control} \times 100\%$, where T_{treat} and $T_{control}$ denote the mean number of days for the tumour weight to reach 1.5 g for the treatment and control groups, respectively. [d]Toxicity assessments were performed in a semi-quantitative manner on a scale of 0–2 for the parameters: weight loss, fur roughing, decreased water and food intake, reduced general activity, and tissue damage at the site of injection. Total toxicity is the sum of the scores for the five categories. [e]One-way analysis of variance, $P < 0.01$.

where T_{treat} and $T_{control}$ denote the mean number of days for the tumour weight to reach 1.5 g for the treatment and control groups, respectively.

Statistics

Time for the tumour weight to reach 1.5 g was expressed as the mean ± s.d., where the mean values were calculated from all animals (4–5 mice) in each group. Differences between two groups were determined using the Student's *t*-test for unpaired experimental values. Differences among three groups (i.e. experiment 4) were determined using one-way analysis of variance. A value of $P \leq 0.05$ was considered statistically significant.

Results

In-vitro drug accumulation studies

Doxorubicin and [^{99m}Tc]sestamibi were substrates for Pgp (Coley et al 1989b; Piwnica-Worms et al 1993; Ballinger et al 1995) and were used to characterize the present EMT6 and EMT6/AR cell lines with respect to differences in accumulation of Pgp substrates and effect of verapamil on accumulation levels. As shown in Figure 1A, doxorubicin accumulation in MDR cells (EMT6/AR) after 120-min incubation was 35% of that in parental EMT6 cells in control conditions. In the presence of 50 μM verapamil, there was a 40% and 120% increase in the accumulation of doxorubicin in parental and MDR cells, respectively. However, even in the presence of 50 μM verapamil the accumulation of doxorubicin in EMT6/AR cells remained lower compared with EMT6 cells i.e. verapamil at this concentration was unable to completely reverse the MDR-related accumulation deficit. Figure 1B demonstrates that [^{99m}Tc]sestamibi accumulation in MDR cells was 40% of that in the parental cells at 60 min. In the presence of 50 μM verapamil, [^{99m}Tc]sestamibi accumulation in parental cells increased by 300%, while that in MDR cells increased by 550%. Thus, [^{99m}Tc]sestamibi showed a greater percent increase in accumulation than doxorubicin to the addition of verapamil in both cell lines and verapamil was able to restore [^{99m}Tc]sestamibi accumulation in EMT6/AR cells to the same levels seen in EMT6 cells.

Size and size distribution of microspheres

The particle size distribution curve of the blank microspheres in saline solution was bell shaped with a mean diameter of 36.8 μm, standard deviation 4.3 μm (data not shown). Thus, most of the microspheres in the hydrated state were between 19 and 70 μm in diameter, with only a small fraction of larger microspheres. This information suggested that the microspheres in an aqueous medium could go through a 20-gauge needle (i.e. inner diameter = 0.9 mm) without damage, which resulted in the selection of this needle size for in-vivo injection of the microsphere preparations.

In-vitro drug release from microspheres

Figure 2 displays the fractional release of verapamil from the microspheres into pH 7.4 buffer at 37 °C. In the

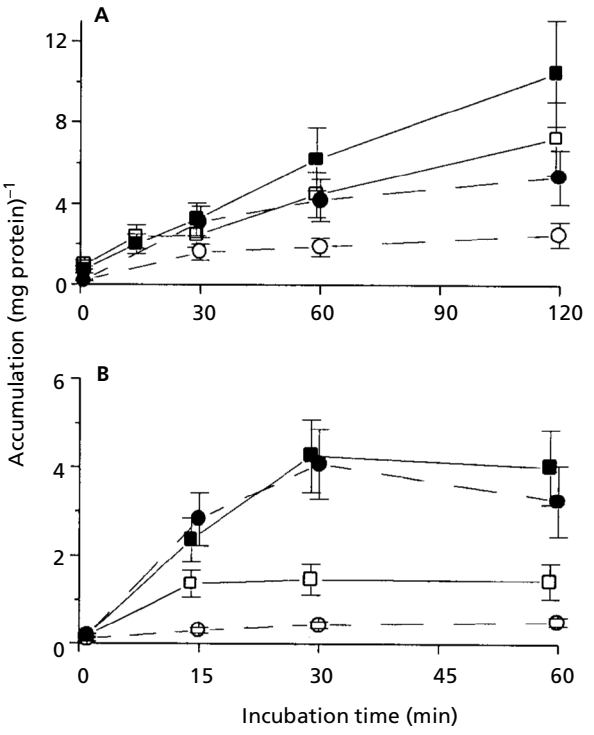

Figure 1 Time course of accumulation of [^{14}C]doxorubicin (A) or [^{99m}Tc]sestamibi (B) by the parental murine breast sarcoma cell line EMT6 (squares) or the MDR derivative cell line EMT6/AR (circles) under control conditions (open symbols) or in the presence of 50 μM verapamil (closed symbols). The drug accumulation value was defined as relative radioactivity in the cells per mg of protein. Data points and bars represent the mean of three experiments ± s.d.

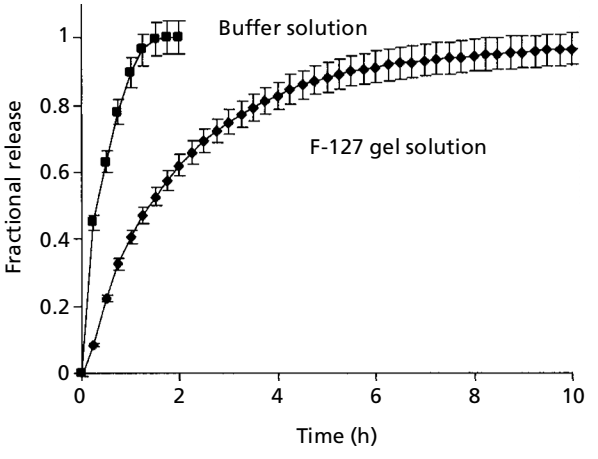

Figure 2 Fractional release as a function of time of verapamil loaded at 30% w/w in a pH 7.4 (0.05 M) phosphate buffer solution at 37 °C from microspheres or from microspheres-20% Pluronic F-127 suspension in a dialysis tubing. Fractional release was calculated as the amount of drug released divided by the initial total drug content of the microspheres. Data points and bars represent the means of three experiments ± s.e.

control situation of buffer alone, verapamil was completely released within 2 h, while in a 20% Pluronic F-127 gel solution the drug release was significantly slower with a completion of release over 10 h. The same reduction effect of the gel solution on doxorubicin release was observed also (data not shown). This result indicated that drug release rate could be tailored by addition of Pluronic F-127 in the formulation. In the later in-vivo study, F-127 was employed at a concentration of only 9% owing to consideration that the osmotic effect of a concentrated F-127 solution would cause animal discomfort. Osmotic pressure is directly proportional to solute concentration for a given solute and temperature.

Previous work showed little influence of the verapamil content in the microspheres (i.e. % w/w) on the drug release profile (Liu et al 1999). Doxorubicin release from microspheres, however, was more complicated. As depicted in Figure 3, at a lower loading of 10% w/w, the release rate was faster and complete release of the drug was achieved within approximately 1 h, with a half-life of release of approximately 20 min. However, at a doxorubicin content of 33% w/w, only ~35% of the microsphere-associated drug was released into the buffer even after 7 h, with a half-life of release of approximately 40 min.

Ex-vivo studies of doxorubicin release and diffusion in tissue

Figure 4A is a confocal microscope image of a section of mouse liver obtained 10 min after interstitial injection of doxorubicin-loaded microspheres. The microspheres were clearly seen against a background of autofluorescence. There was a narrow penumbra of released doxorubicin that had diffused only a short distance. In Figure 4B, where the mouse was killed 60 min after injection of doxorubicin-loaded microspheres, the doxorubicin fluorescence in the microspheres was less intense, indicating release of doxorubicin, and there was evidence of diffusion of doxorubicin into surrounding tissue to a much greater distance.

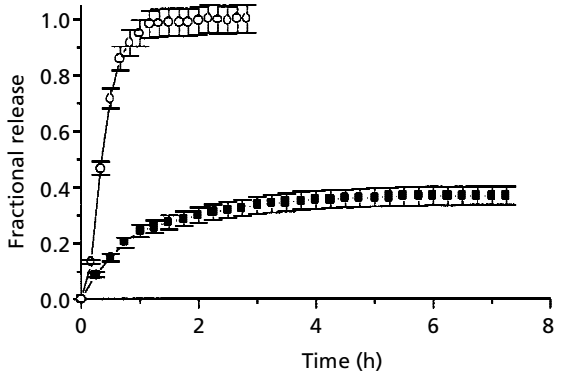

Figure 3 Fractional release as a function of time of doxorubicin in pH 7.4 (0.05 M) phosphate buffer at 37 °C from two microsphere formulations with doxorubicin loadings 33% w/w (Dox-MS-33, closed squares) or 10% w/w (Dox-MS-10, open circles).

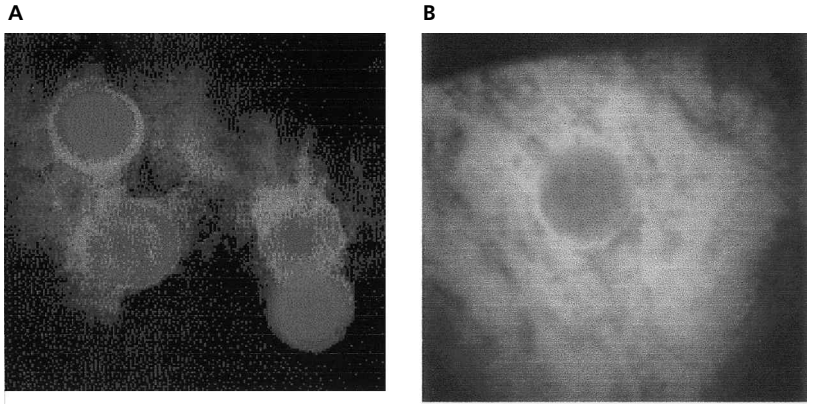

Figure 4 Confocal microscope images of mouse liver obtained 10 min (A) or 60 min (B) after interstitial injection of doxorubicin-loaded microspheres. The mean hydrated particle diameter was 37 μm.

In-vivo treatment of mice bearing EMT6 or EMT6/AR tumours

The effect of blank microspheres, doxorubicin-loaded microspheres alone or with verapamil-loaded microspheres on tumours growing in-vivo was assessed using tumour growth curves and prolonged-survival (P-S; Table 1). General toxicity to the animal was assessed using various parameters for local and systemic toxicities as described in Materials and Methods. A series of five experiments were carried out as described below.

Experiment 1: blank microspheres in EMT6 tumours
Initially, the effect of blank microspheres on tumour growth and animal survival was studied in two groups of four mice bearing EMT6 tumours. Group 1A received no treatment as the control while group 1B received intratumoral injection of 300 μg blank microspheres every other day for a total of five injections (days 0, 2, 4, 6, and 8). As shown in Table 1, there was little difference between the two groups in terms of tumour growth and animal survival. The average time for tumour weight to reach 1.5 g for control and blank microsphere groups were 10.0 ± 0.8 and 10.4 ± 1.1 days (mean $\pm$ s.d., n = 4; P = not significant), respectively, and the toxicity scores showed that the blank microspheres caused no toxicity to the animals (Table 1). These results suggested that the microspheres were an inert, nontoxic carrier.

Experiment 2: doxorubicin single injection in EMT6 tumours
The effect of a single injection of doxorubicin-loaded microspheres was evaluated in two groups of five mice bearing EMT6 tumours. Group 2A received an intratumoral injection of 750 μg blank microspheres while group 2B received 750 μg Dox-MS-33 containing 250 μg doxorubicin. The results in Table 1 and Figure 5 indicated that tumour growth in the Dox-MS-33-treated group appeared to be somewhat slower compared with the blank microsphere group. The average number of days for tumour weight to reach 1.5 g for the blank microsphere and Dox-MS-33 groups were 10.6 ± 2.7 and 13.1 ± 3.2, respectively. Thus, the average survival time of the treatment group was 24% longer than the control group, though the difference was not statistically significant. Toxicity assessment indicated little, if any, adverse effect caused by the Dox-MS-33 treatment (Table 1).

Experiment 3: doxorubicin single injection in EMT6/AR tumours
Two groups of animals bearing multidrug-resistant EMT6/AR tumours were treated with microspheres in the same manner as above. Compared with the blank microsphere group, tumour growth of the Dox-MS-33 group was not delayed at all (groups 3A and 3B of Table 1),

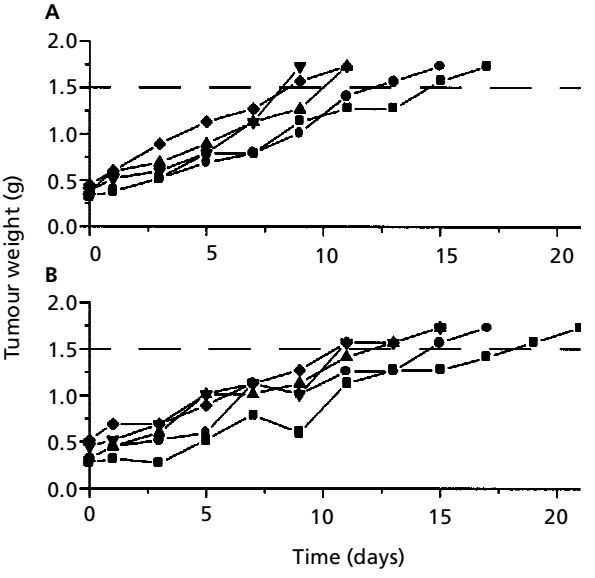

Figure 5 Tumour weight as a function of time for individual mice after a single intratumoral dose on day 0 of 750 μg blank microspheres (A) or 750 μg Dox-MS-33 containing 250 μg doxorubicin (B) in experiment 2. Horizontal line indicates weight of 1.5-g tumour.

with average time to reach 1.5 g for the blank microsphere and Dox-MS-33 groups of 10.6 ± 2.0 and 9.6 ± 2.5 days, respectively (P = not significant). Thus, the EMT6/AR tumour was refractory to the Dox-MS-33 treatment. There was no sign of toxicity shown in either group (Table 1).

Experiment 4: five doses of doxorubicin ± verapamil in EMT6 tumours

A single intratumoral dose of 750 μg Dox-MS-33 containing 250 μg doxorubicin had a small effect on the EMT6 tumour (experiment 2, Figure 5 and Table 1), while no effect was observed in the EMT6/AR tumour (experiment 3, Table 1). In both cases, no side effects were seen. Based on these results, it was postulated that a larger therapeutic effect might be achieved by using a smaller dose of doxorubicin (in Dox-MS-33) given multiple times, resulting in administration of a greater total dose of drug. In addition, instead of treating both types of tumours, we focused on EMT6, which showed some response to the above treatment. The modest degree of response may be partially attributable to the presence of small but significant levels of Pgp activity in EMT6 tumours evident in the in-vitro experiments (Figure 1). Thus, addition of microsphere-encapsulated verapamil to the Dox-MS-33 regimen could potentially improve the therapeutic effect.

Accordingly, in experiment 4, five injections of a small dose of Dox-MS-33 (100 μg doxorubicin in 300 μg of microspheres) plus Ver-MS (600 μg verapamil in 1800 μg of microspheres) given on days 0, 2, 4, 6, and 8 were used in the treatment of EMT6 tumours in-vivo (group 4C). As a comparison group, an equal dose of Dox-MS-33 alone was administered on the same schedule (group 4B), while the control group received only 300 μg blank microspheres every other day (group 4A). As shown in Figure 6 and Table 1, there was a statistically significant delay in tumour growth in the two treatment groups compared with the control group. The average survival days for blank microspheres, Dox-MS-33, and Dox-MS-33 plus Ver-MS groups were 7.0 ± 1.1, 9.4 ± 1.2 and 9.8 ± 1.4, respectively. There was a 34% and 40% increase in the average days to reach 1.5 g tumour for the group receiving either Dox-MS-33 or Dox-MS-33 plus Ver-MS, respectively, compared with the control group (one-way analysis of variance, $P < 0.01$). Toxicity assessment indicated that there were few side effects for the Dox-MS-33 group, while the Dox-MS-33 plus Ver-MS group showed moderate toxicity (Table 1).

Experiment 5: fast-release doxorubicin microspheres in EMT6 tumours

In-vitro experiments showed that Dox-MS-33 released the drug at a lower rate and to a lesser extent than Dox-MS-10. In all of the above experiments, the slower-release microsphere formulation was used. Thus it was of interest to evaluate the therapeutic effect of Dox-MS-10. Tumour growth curves for animals treated with five injections of Dox-MS-10 (100 μg doxorubicin in 1 mg microspheres) given on days 0, 2, 4, 6, and 8 are shown as group 5B in Table 1, compared with the group without treatment (group 5A). The average time to reach 1.5 g tumour for the control and Dox-MS-10 groups was 8.1 ± 0.5 and 9.1 ± 1.5 days, respectively. There was a 12% increase in the surviving time of the group receiving faster-releasing Dox-MS-10 compared with the group receiving no treatment (P = not significant). Toxicity assessment (Table 1) indicated that there were some side effects apparent for the Dox-MS-10 group, which suggested that the faster-release Dox-MS-10 could cause higher systemic exposure to the animal compared with the slower-release Dox-MS-33, both of which were administered as five intratumoral injections (once every other day) containing 100 μg doxorubicin.

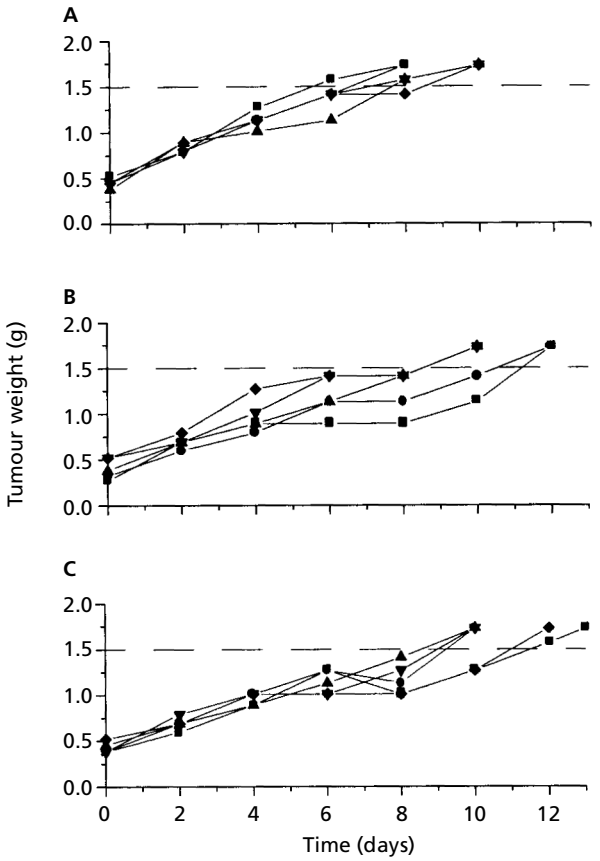

Figure 6 Tumour weight as a function of time for individual mice after multiple doses on days 0, 2, 4, 6, and 8 of 300 μg blank microspheres (A), 300 μg Dox-MS-33 containing 100 μg doxorubicin (B), or 300 μg Dox-MS-33 containing 100 μg doxorubicin plus 1800 μg Ver-MS containing 600 μg verapamil (C) in experiment 4. Horizontal line indicates weight of 1.5-g tumour.

Discussion

Doxorubicin and [^{99m}Tc]sestamibi are substrates for Pgp and MRP (Coley et al 1989b; Piwnica-Worms et al 1993; Ballinger et al 1995), but in this study they exhibited different kinetics of accumulation in EMT6 and EMT6/AR

cells, with the more diffusible [^{99m}Tc]sestamibi accumulating rapidly and reaching a plateau within 30 min while doxorubicin accumulation continued over 120 min (Figure 1). Though both showed accumulation levels in MDR cells, which were reduced to 35–50% of those seen in the parental cell line, there was a striking difference between doxorubicin and [^{99m}Tc]sestamibi in MDR modulation by verapamil, which has been shown to partially reverse MDR caused by either Pgp or MRP in-vitro (Bradley et al 1988; Vergote et al 1998). Doxorubicin accumulation by EMT6/AR cells could be restored by 50 μM verapamil to 80% of the level of EMT6 cells under control conditions and 50% of the level in EMT6 cells treated with doxorubicin (Figure 1A). In contrast, verapamil abolished the accumulation deficit for [^{99m}Tc]sestamibi and levels in EMT6/AR cells in the presence of 50 μM verapamil were similar to those in EMT6 cells (Figure 1B). Coley et al (1989a, b) observed that there were additional resistance mechanisms other than Pgp in EMT6/AR cells. The discrepancy between doxorubicin and [^{99m}Tc]sestamibi accumulation by EMT6/AR cells in the presence of verapamil might be explained by differing sensitivities to the multiple resistance transporters operating in EMT6/AR cells. A similar disparity between doxorubicin and [^{99m}Tc]sestamibi was reported in a different cell system, where doxorubicin was measured by flow cytometry rather than ^{14}C counting (Muzzammil et al 2001). The in-vitro experiments highlight the significant level of transporter function in the parental cell line and validate the use of these cell lines in the proposed work.

Previous work showed that doxorubicin could be loaded readily onto ion exchange microspheres (Codde et al 1990; Chen et al 1992; Leo et al 1999; Liu et al 2001b). However, incomplete release of the loaded content in various buffers was generally observed. Only with concentrated electrolyte solutions, additional divalent ions such as Ca^{++}, or refreshing the electrolyte solutions could complete drug release from microspheres be achieved (Sawaya et al 1988; Liu et al 2001b). Also, we observed that the doxorubicin release rate depended on the drug content within the microspheres. It has been reported that doxorubicin, once trapped in liposomes, can form molecular aggregates in the form of linear, curved and circular bundles of fibres and the aggregation is concentration-dependent (Li et al 1998). It appears that a similar phenomenon occurs with the drug bound ionically within the microspheres. At higher drug content within the microspheres, the drug appears to form stronger interactions with itself and stick firmly to the microspheres. Therefore, as shown in Figure 3, only a partial release in phosphate buffer was observed for microspheres of high doxorubicin content (Dox-MS-33). Nevertheless, complete release of the loaded drug in-vivo was expected even for the microspheres of high doxorubicin content upon the removal of released doxorubicin by the circulation and the eventual biodegradation of the dextran matrix (Artursson et al 1987; Chakravarthy et al 1994).

The microspheres were formulated in Pluronic F-127 to modulate the rate of drug release by increasing viscosity. It has been reported that gelation of F-127 was dependent on concentration and temperature, with gelation temperature increasing as concentration decreased. For example, a solution of 20% Pluronic F-127 formed a gel at $> 10\,^{\circ}$C, while with 9% F-127 gelation occurred at $> 18\,^{\circ}$C (Cabana et al 1997; Bohorquez et al 1999). The lack of effect of the microspheres on gelation was observed visually by comparing the flow behaviour of the preparation with a Pluronic F-127 solution without microspheres. When kept refrigerated at approximately 4 °C each solution flowed as a liquid, whereas when warmed to 20 °C each turned to a jelly-like solid.

Doxorubicin is very toxic to animals, with an acute intravenous LD50 of 21.1 mg kg^{-1} in mice, or 422 μg per 20-g mouse (Bertazzoli et al 1970). However, animals receiving a total of 500 μg doxorubicin in Dox-MS-33 given in five doses on alternate days showed few signs of toxicity (experiment 4B). Moderate toxic signs were observed in animals receiving Ver-MS in addition to Dox-MS-33 (experiment 4C) or the faster release Dox-MS-10 (experiment 5B). Even in these cases, however, no tissue damage was seen at the site of injection. Idani et al (2000) observed tissue necrosis following subcutaneous injection of as little as 12.5 μg free doxorubicin. Only some marginal fur roughing, weight loss and slightly subdued activity were found in a minority of the animals. The obvious advantage of using Dox-MS over the drug solution is the reduction of side effects. Since drug loaded in microspheres is released gradually, the acute exposure to normal organs should be lower in intratumoral Dox-MS as compared with intratumoral drug solutions. Therefore, animals receiving intratumoral controlled-release drug formulations can tolerate much higher drug doses (up to eight times higher), as observed by Deurloo et al (1991).

Treatment of tumours grown from the EMT6 cell line by intravenous injections of doxorubicin solution was shown to cause little delay in tumour growth, although no observation on animal toxicity was mentioned (Nahabedian et al 1988). Serious problems of extravasation with injected solutions of doxorubicin have been reported (Bertazzoli et al 1970). To the best of our knowledge, there have been no previous reports on intratumoral injections of either drug solutions or controlled-release formulations in this animal tumour model.

Concerning the therapeutic effects, the EMT6 tumour growth was delayed by the intratumoral injection of a single dose of 250 μg doxorubicin in microspheres (750 μg Dox-MS-33), with a 24% increase in the average survival time (experiment 2B). Growth of the MDR tumour EMT6/AR was not delayed at all (experiment 3B), as would be predicted for an MDR tumour greater than 100-times more resistant to doxorubicin than its wild-type parent (R. Cheung, unpublished colony assay results in this laboratory).

Multiple injections of smaller doses of Dox-MS-33 (300 μg microspheres containing 100 μg doxorubicin every other day) were used also (experiment 4B), based on the observation of a higher growth rate two days after a single injection. The 34% increase in survival times for the treated animals over the corresponding control group ($P < 0.02$) was slightly greater than that observed in the

single-injection experiments (24% increase, $P = 0.07$, experiment 2B). Furthermore, using the faster-release Dox-MS-10 (1000 μg microspheres containing 100 μg doxorubicin every other day for five doses) instead of the slower one resulted in a smaller percentage increase (12%) in the average animal survival times compared with the control group (experiment 5). Moderately increased toxicities were also observed in the latter case. The residence times of drug inside the tumour after intratumoral injection were probably in the following order: slower-release Dox-MS-33 (longest), faster-release Dox-MS-10 (middle), doxorubicin in solution (shortest), though pharmacokinetics were not measured in this study. Idani et al (2000) demonstrated such an effect in comparing liposomal doxorubicin with free doxorubicin. Therefore, the prolongation of survival decreased and toxicity increased in the above order.

The co-administration of Ver-MS with Dox-MS-33 (experiment 4C) seemed to have no additional therapeutic benefit on inhibition of tumour growth compared with Dox-MS-33 alone, despite the fact that a modest in-vitro chemosensitization of EMT6 tumour cells was observed in the presence of verapamil (Figure 1). These results are in agreement with previous reports that verapamil was ineffective in potentiating doxorubicin activity in tumours that did not respond to intravenous treatment with doxorubicin (Formelli et al 1988; Nahabedian et al 1988). Even for drug-sensitive tumours, responses were heterogeneous in their susceptibility to modulation by verapamil.

Co-administration of 100 μg doxorubicin in Dox-MS-33 every other day with Ver-MS delivering a verapamil dose of 200 or 400 μg seemed safe in mice (data not shown). However, at a higher verapamil dose of 600 μg toxicity was apparent (experiment 4C, Table 1). Monti et al (1988) reported that myocardial cells in the Langendorff isolated rat heart model showed significantly enhanced doxorubicin uptake when verapamil was co-administered. They suggested that combined therapy with doxorubicin and calcium-modulating agents might enhance the drug-induced cardiotoxicity. Additionally, Arvelo et al (1995) reported that verapamil modestly delayed the plasma clearance of doxorubicin. Therefore, enhanced doxorubicin toxicity to critical organs and elevated plasma doxorubicin levels were probably the cause for the side effects associated with co-administration of verapamil. This problem may be overcome by using more potent, less toxic chemosensitizers, especially those which would not alter the pharmacokinetics of doxorubicin.

Some recent in-vitro findings may explain the limited success of chemosensitization in-vivo. Tunggal et al (1999a, b, 2000) found that the efficacy of Pgp reversal agents decreased with increased cell density, presumably due to higher concentration of Pgp molecules. Solid tumours contain approximately 10^9 cells mL^{-1} whereas in tissue culture the concentration is often 10^4–10^7 cells mL^{-1}. The same authors reported that the presence of a chemosensitizer resulted in decreased penetration of anticancer drugs through multicellular layers formed from Pgp-expressing cells. The implications for the present study on intratumoral drug delivery are profound since in-vitro work showed that even parental EMT6 cells have significant Pgp expression. Based on these results, two factors may have contributed to the failure of in-vivo chemosensitization after co-administration of Dox-MS and Ver-MS. Firstly, verapamil may be less effective in the solid tumour compared with cell culture due to the cell density effect and its rapid removal from the tumour site by the circulation. The latter was evidenced by higher systemic toxicity with the use of verapamil. Secondly, since effective treatment required that the localized doxorubicin was released from microspheres and diffused out to reach tumour cells distal from the site of injection, impediment of drug penetration by verapamil due to higher proximal cellular drug uptake may have presented a serious obstacle. In addition, tissue penetration of doxorubicin itself is known to be poor (Kerr & Kaye 1987). Future remedies for the above problems may involve using more efficient chemosensitizers (e.g. valspodar, biricodar, elacridar, and LY335979) (Germann et al 1997) to offset the problems arising from high tumour cell density (Tunggal et al 1999a) and a penetration enhancer to help the drug distribute evenly throughout the tumour (Kerr & Kaye 1987; Tannock 1998; Tunggal et al 2000). In hindsight, the selection of a tumour more sensitive to doxorubicin may have yielded more convincing evidence of therapeutic benefit. Nevertheless, this work demonstrated the potential of the present approach.

Conclusions

In-vitro and in-vivo sustained release of doxorubicin and verapamil was achieved by using sulfopropyl dextran microspheres. The use of Pluronic F-127 solution in the formulation further prolonged the drug release. The blank microspheres and the excipient did not cause adverse effects on the animals. Treatment of EMT6 tumours in-vivo by intratumoral injection of the slow-release Dox-MS-33 formulation increased the animal survival by up to 34% with minimal toxicity. Qualitatively similar results were obtained with Ver-MS co-administered with Dox-MS, although moderately increased toxicity was observed. It was evident that the use of controlled-release microsphere formulations of doxorubicin were able to increase modestly the therapeutic effect while decreasing toxicity, even in the EMT6 tumour model that showed little response to treatment by intravenous injection of doxorubicin (Nahabedian et al 1988).

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