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Long-circulating monensin nanoparticles for the potentiation of immunotoxin and anticancer drugs

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Abstract

The carboxylic ionophore monensin was formulated into long-circulating nanoparticles with the help of polyethylene glycol/poly (DL-lactide-co-glycolide) diblock copolymers, in an attempt to enhance the cytotoxicity of a ricin-based immunotoxin, anti-My9, and anticancer drugs like adriamycin and tamoxifen. This study looked into various aspects involving the preparation (using a homogenizer and an EmulsiFlex homogenizer-extrusion device) and lyophilization of long-circulating monensin nanoparticles (LMNP) of particle size < 200 nm in diameter. The particle size of LMNP was reduced from 194 nm to 160 nm by passing the nanoparticles through an EmulsiFlex, before freeze-drying. There was a 4.8–83.7 % increase in the particle size of LMNP after freeze-drying, which was dependent upon the manufacturing conditions such as use of the EmulsiFlex for size reduction before freeze-drying, the freezing method (rapid/slow) and the concentration of lyoprotectant (mannitol or trehalose) employed for freeze-drying. LMNP freeze-dried with 2.4 % of trehalose showed minimal size change (< 9 %) after freeze-drying. Further, the freezing method was found to have negligible effect on the particle size of LMNP freeze-dried with trehalose in comparison with mannitol. The entrapment efficiency of monensin in LMNP was found to be 14.2 ± 0.3 %. The LMNP were found to be spherical in shape and smooth in surface texture as observed by atomic force microscopy. In-vitro release of monensin from LMNP in phosphate buffered saline (PBS) pH 7.4 or PBS supplemented with 10 % human serum indicated that there was an initial rapid release of about 40 % in the first 8 h followed by a fairly slow release (about 20 %) in the next 88 h. In-vivo studies conducted with Sprague-Dawley rats showed that 20 % of monensin remained in circulation 4–8 h after the intravenous administration of LMNP. An in-vitro dye-based cytotoxicity assay (MTS/PMS method) showed that there was 500 times and 5 times potentiation of the cytotoxicity of anti-My9 immunotoxin by LMNP (5×10^{-8} M of monensin) in HL-60 sensitive and resistant human tumour cell lines, respectively. Further, LMNP (5×10^{-8} M of monensin) potentiated the cytotoxicity of adriamycin in MCF 7 and SW 620 cell lines by 100 fold and 10 fold, respectively, and that of tamoxifen by 44 fold in MCF 7 cell line as assessed by crystal violet dye uptake assay. Our results suggest that it is possible to prepare LMNP possessing appropriate particle size (< 200 nm), monensin content and in-vitro and in-vivo release characteristics with the help of a homogenizer and an EmulsiFlex homogenizer-extrusion device. LMNP can be freeze-dried with minimal increase in particle size by using a suitable concentration of a lyoprotectant like trehalose. Furthermore, LMNP could potentiate the cytotoxicity of immunotoxin, adriamycin and tamoxifen by 5–500 fold in-vitro, which will be further investigated in-vivo in a suitable animal model.

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Introduction

An immunotoxin is a chimeric molecule, composed of a toxin and a monoclonal antibody designed to kill target cells in a highly efficient manner (Thrush et al 1996). Ricin is the most commonly used toxin because of its potent cytotoxicity and low immunogenicity in man. Ricin is composed of two chains, A and B, linked by a disulphide bond. The ricin A-chain immunotoxins have been proposed as a new class of anticancer agents (Vitetta et al 1987) and the emerging role of ricin A-chain immunotoxins for the treatment of leukaemia has been reviewed (Engert et al 1998). The anti-My9 immunotoxin is ricin based and designed to selectively destroy acute myeloid leukaemia cells in-vitro and in-vivo. The anti-My9 monoclonal antibody reacts with the CD 33 antigen found on clonogenic acute myeloid leukaemia cells from greater than 80 % of cases and does not react with normal pluripotent stem cells (Roy et al 1991). The anti-My9 immunotoxin has shown a very high potency against HL-60 human tumour cells at a concentration of 10^{-8} mol L^{-1} (more than 4 logs of cell kill). However, it has shown limited activity in clinical trials, which could be attributed to the suboptimal delivery of immunotoxin to the site of action.

A major factor limiting the efficacy of immunotoxins in general is the lack of ricin A-chain potency in-vivo. There is a specific need for nontoxic potentiating agents, which can increase the cytotoxicity of an immunotoxin without increasing its toxicity. Various chemical and biological agents which have been studied for enhancing the cytotoxicity of immunotoxins include ammonium chloride, carboxylic ionophores, calcium-channel antagonists, viruses and cytokines (Wu 1997). The carboxylic ionophore monensin has been studied extensively for its potentiation of immunotoxins because it holds several advantages over other agents (Colombatti et al 1990). Monensin's activity is not limited by intracellular or extracellular pH and it may also exert its function in low pH areas of growing tumour mass. Further, monensin functions at very low concentration to produce a significant potentiation in the activity of immunotoxins (Colombatti et al 1990).

Monensin has been shown to potentiate the effects of immunotoxins against leukaemia (Raso & Lawrence 1984), breast carcinoma (Griffin et al 1987), colorectal carcinoma (Griffin et al 1988) and small cell lung carcinoma (Derbyshire et al 1992) cell lines in-vitro. However, monensin, being lipophilic in nature, has a short half-life in-vivo and therefore needs to be formulated in a suitable drug delivery system. Previous studies on monensin delivery, as a conjugate of human serum albumin (Colombatti et al 1990) or as a lipid–water emulsion (Griffin & Raso 1991), have shown limited success in-vivo. Liposomal delivery of monensin showed considerable potentiation of the antitransferrin receptor immunotoxin (Griffin et al 1993) and monoclonal antibody targeted monensin liposomes have been found to potentiate the 454-A immunotoxin, increasing its activity by a factor of 100 compared with liposomal monensin against various tumour cell lines in-vitro (Singh et al 1994). Subsequent studies carried out in our laboratory on the delivery of monensin in poly (DL-lactide-co-glycolide) (PLGA) nanoparticles (conventional) demonstrated potentiation of the activity of a ricin-based immunotoxin against HL-60 and HT-29 cell lines by a factor of 40–50 (Ferdous et al 1998). Further, monensin is reported to modulate adriamycin resistance in Ehrlich ascites tumour cells (Sehested et al 1988) and to re-sensitize resistant human breast tumour cell lines (MCF 7R) to chemotherapy (Schindler et al 1996). In our previous study (Singh et al 1999), we reported that it was possible to potentiate the cytotoxicity of adriamycin against the MCF 7R cell line in-vitro by using stealth monensin liposomes. However, liposomal formulations are limited by aggregation, poor entrapment and leakage of monensin, oxidation and hydrolysis of phospholipids on long-term storage.

The anthracycline anticancer agent adriamycin is currently being used for a variety of cancers (breast, ovarian, leukaemia and lymphomas). Tamoxifen is an effective agent in the treatment of patients with breast cancer whose tumour is estrogen-receptor positive. The ability of monensin to potentiate the cytotoxicity of immunotoxins may be extended to the currently used anticancer drugs like adriamycin and tamoxifen for a better therapeutic response.

In this communication, long-circulating monensin nanoparticles (LMNP) prepared from polyethylene glycol/poly(DL-lactide-co-glycolide) (PEG/PLGA) diblock copolymers were considered as an alternative drug-delivery system for monensin, in an attempt to eliminate the shortcomings observed with stealth monensin liposomes (Singh et al 1999) and also to improve the half-life of conventional monensin nanoparticles (Ferdous et al 1998). Therefore, we have studied the effects of certain manufacturing steps such as the utility of employing a high-pressure homogenizer equipped with an extrusion unit, EmulsiFlex (C5) (Avestin Inc. Ottawa, ON, Canada), which will be subsequently referred to as EmulsiFlex, the role of different freezing methods (rapid/slow), lyoprotectants and the concentration of lyoprotectant on the particle size of LMNP before and after freeze-drying. We have also studied the

concentration of monensin in serum following the administration of LMNP to rats and the potentiating effect of LMNP on the cytotoxicity of anti-My9 immunotoxin against HL-60 sensitive and resistant human tumour cell lines. In addition, we also report on the cytotoxic potentiation (by LMNP) of adriamycin against MCF 7 and SW 620 human tumour cell lines and tamoxifen against MCF 7 cell line.

Materials and Methods

Materials

Polyethylene glycol 3000-poly(DL-lactide-co-glycolide), 80:20 (inherent viscosity 0.7 dL g^{-1}), polyethylene glycol 5000-poly(DL-lactide-co-glycolide) 50:50 (inherent viscosity 1.28 dL g^{-1}) were obtained from Birmingham Polymers, Inc. (Birmingham, AL). Poly-(vinyl alcohol), 98% hydrolyzed, was obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI). ^{3}H-monensin with a specific activity of 5 Ci $mmol^{-1}$ was obtained from American Radiolabeled Chemicals Inc. (St Louis, MO). Monensin, D-Mannitol, D-(+)-trehalose, sodium carboxymethylcellulose (50–250 centipoise), human serum (Type AB from Male AB plasma) and all tissue-culture media were obtained from Sigma Chemical Company (St Louis, MO). All cancer cell lines were obtained from American Type Culture Collection (Rockville, MD). Anti-My9 immunotoxin was a generous gift from ImmunoGen Inc. (Norwood, MO) and is directed against the CD33 antigen found on the clonogenic acute myeloid leukaemia cells. Anti-My9 immunotoxin is composed of an altered ricin derivative that has its non-specific binding site eliminated by chemically blocking the galactose binding domains of the B chain. Tamoxifen base was obtained from Sifa Ltd Fine Chemical Manufacturers (Ireland). De-ionized distilled water was used throughout the study. Buffers and other chemicals were of reagent grade.

Preparation of LMNP

The emulsification–solvent evaporation method reported previously (Ferdous et al 1998) was used with some modifications. Briefly, 60 mg of PEG 3000-PLGA 80:20, 15 mg of PEG 5000-PLGA 50:50 and 15 mg of monensin were dissolved in 20 mL of ethyl acetate by sonication at low temperature (4°C). ^{3}H-monensin (20 μCi) was added to the formulation for the purpose of quantification. The polymer solution containing monensin was added to the aqueous phase (0.8% w/v poly(vinyl alcohol) in 50 mL water) drop-wise and the mixture was homogenized with a cyclone IQ^{2} homogenizer (The Virtis Company, Gardiner, NY). The organic solvent was evaporated by gentle magnetic stirring overnight. The suspension of nanoparticles was then passed through the EmulsiFlex, which was fitted with various polycarbonate membranes (0.6 μm, two cycles; 0.4 μm, two cycles; and 0.2 μm, four cycles). The sample was then mixed with water, purified and concentrated by cross-flow filtration using Sartocon-Micro ultrafiltration unit (Sartorius Corporation, Edgewood, NY) fitted with a 0.2-μm membrane.

Freeze-drying of LMNP

The samples of LMNP (after cross-flow filtration) were freeze-dried using various concentrations of mannitol or trehalose and 0.1% of sodium carboxymethylcellulose. The samples were either initially frozen in liquid nitrogen and subsequently kept at −80°C overnight (rapid freezing method), or were frozen at −80°C as for the rapid freezing method but omitting the initial freezing step in liquid nitrogen (slow freezing method). Subsequently, LMNP were freeze-dried (FD 3.0, Appropriate Technical Resources, Inc., Laurel, MD). The system comprised of a 12-port floor model, which was connected to a secondary vacuum trap. A Fisher Maxima pump (Model D8C) was used for vacuum. The samples were freeze-dried for 48 h.

Particle size analysis

The particle size and size distribution of LMNP dispersions in water which had been passed through 0.2-μm membranes was determined at room temperature by photon correlation spectroscopy with a BI 90 particle sizer (Brookhaven Instruments Corp., Holtsville, NY). The particle size of LMNP was determined at various stages during their preparation (i.e., after homogenization, extrusion through the EmulsiFlex, and pre and post freeze-drying procedure.

Zeta-potential analysis

The zeta potential of each formulation of freeze-dried LMNP was determined by Zeta Plus, zeta potential analyser (Brookhaven Instruments Corp., Holtsville, NY). The zeta potential of nanoparticles was measured in water passed through 0.2-μm membranes.

Atomic force microscopy (AFM)

The samples for AFM were prepared by placing a drop of a diluted suspension of nanoparticles in water over a glass slide and subsequent drying of the samples in a vacuum desiccator overnight. The images of nanoparticles were taken with AFM (Dimension 3000, Digital Instruments, Santa Barbara, CA). The images were scanned by tapping mode using a cantilever, which oscillated vertically with a piezoelectric driver at a frequency of 350 kHz.

Determination of monensin in LMNP

The monensin content in LMNP was determined by the method reported by Singh et al (1999). The method involves mixing 25 μL of sample with 50 μL of polysorbate 20, followed by bath sonication for a few minutes. Scintillation fluid (3 mL; ScintiVerse II, Fisher, Fair Lawn, NJ) was added to the mixture, vortexed for 2 min and the radioactivity was determined using a LKB Wallac 1219 Rackbeta Liquid Scintillation Counter (Wallac, MD). The encapsulation efficiency was calculated based on the amount of monensin entrapped in each batch of lyophilized nanoparticles compared with the initial amount employed in the formulation. Monensin content was determined in triplicate for each batch of freeze-dried LMNP.

In-vitro release study

The release of monensin from the LMNP was studied by suspending the nanoparticles containing about 60 μg of monensin in 5 mL of phosphate-buffered saline (PBS) pH 7.4 and the suspension was filled in a dialysis bag (Spectra/Por, MWCO 12–14000; Spectrum Laboratories Inc., Los Angeles, CA). The dialysis bag was placed into 500 mL of PBS or PBS containing 10% human serum taken in a dissolution vessel (USP 1000 mL). The dissolution medium was maintained at 37°C and stirred by paddle at 50 rev min^{-1} (USP apparatus II, Vankel VK7000, Cary, NC). At periodic intervals, samples (5 mL) were taken from the outer solution and then replaced with the same volume of the dissolution medium. The concentration of monensin in the dissolution samples was determined by scintillation counting as described previously.

In-vitro degradation of nanoparticles

Fifty milligrams of empty nanoparticles (without monensin) were suspended in 5 mL of PBS pH 7.4 and kept at 37°C for a period of 6 weeks. The hydrolytic degradation of polymer was assessed by measuring the amount of lactic acid released using the enzymatic/colorimetric method for lactate (Lactate Reagent, Cat No. 735–10, Sigma Diagnostics, St Louis, MO) at weekly intervals (Peracchia et al 1997).

Concentration of monensin in serum after administration of LMNP or monensin solution to rats

Male Sprague-Dawley rats (285 g) were injected in the tail vein with a suspension of freeze-dried LMNP in water. At periodic intervals, blood was collected by heart puncture after anaesthesia with halothane and the rats were sacrificed by overdose of halothane. The blood was centrifuged to separate the serum. One hundred microlitres of Solvable (Packard Instruments, Meriden, CT) was added to 100 μL of serum, followed by the addition of 3 mL of scintillation fluid (ScintiVerse II), and the concentration of monensin in serum samples was determined by scintillation counting.

In-vitro potentiation of anti-My9 immunotoxin (by LMNP) against HL-60 sensitive and resistant cell lines

Ten thousand cells per well (100 μL of cell suspension in RPMI medium) were plated in a 96-well plate and incubated in a CO_2-jacketed incubator at 37 ± 0.2°C overnight. Various dilutions of anti-My9 immunotoxin, alone and in combination with a fixed amount of LMNP producing a final concentration of 5.0×10^{-8} M of monensin, were added to the cells, which were then incubated for three days. The cytotoxicity assay was performed with the MTS/PMS method, using an MTS/PMS kit from Promega (Madison, WI).

In-vitro potentiation of adriamycin against MCF 7 and SW 620 cell lines and of tamoxifen against MCF 7 cell line (in combination with LMNP)

Ten thousand cells per well (in 100 μL of medium) were plated in a 96-well plate and incubated overnight in a CO_2-jacketed chamber at 37 ± 0.2°C. Subsequently, various dilutions of adriamycin or tamoxifen, alone and in combination with a fixed amount of LMNP (5.0×10^{-8} M monensin), were added to the cells and the cells were incubated for three days, after which the cells were fixed by 0.25% aqueous glutaraldehyde (100 μL per well) and incubated at room temperature for 30 min,

washed with water and dried. The cells were stained with 0.1 % crystal violet (100 μL per well) and the excess stain removed by washing with water. After drying at room temperature, 100 μL of a 0.05 M sodium phosphate–ethanol (1 : 1 v/v) solution was added to each well and the plates were read using a KC3 microplate reader (Bio-Tek Instruments, Winooski, VT) at a wavelength of 540 nm (Ferdous et al 1998; Singh et al 1999).

Data analysis

The results are expressed as mean $\pm$ s.d. Where appropriate, the data were subjected to Student's t-test. The data were considered to be significant if $P < 0.05$.

Results and Discussion

Preparation of LMNP and the effect of extrusion of LMNP through the EmulsiFlex on their particle size

Several experiments were initially performed to determine the appropriate homogenization conditions (homogenization speed and time and the concentration of polyvinyl alcohol) employed for the emulsification of polymer-monensin solution in ethyl acetate with the aqueous phase so as to obtain an initial particle size of < 200 nm. In addition, the nanoparticle suspension obtained after evaporation of the organic solvent was passed through the EmulsiFlex fitted with various polycarbonate membranes (0.6 μm, 0.4 μm and 0.2 μm) to reduce the particle size. After passing through the EmulsiFlex, the LMNP were subjected to cross-flow filtration, which has been found to be suitable for the removal of polyvinyl alcohol from the raw nanoparticle suspensions (Allemann et al 1993; De Jaeghere et al 1999). The particle size of LMNP was not altered by cross-flow filtration. The extrusion process and the cross-flow filtration steps were the modifications made in this study leading from a previous study dealing with the preparation of monensin-loaded PLGA nanoparticles (Ferdous et al 1998).

We have demonstrated that it is possible to further reduce the size and size distribution of LMNP after the initial homogenization step by passing through the EmulsiFlex. The particle size of LMNP (Batch 1), which was not passed through the EmulsiFlex, had an average size of 194 nm (Table 1) and a polydispersity of 0.4 before freeze-drying. Extrusion of LMNP through 0.6-μm and 0.4-μm membranes reduced the mean particle size to 190 nm (with a polydispersity of 0.2) and 185 nm (with a polydispersity of 0.1), respectively. The mean particle size did not change between the extrusion cycles for either the 0.6-μm or the 0.4-μm membranes ($P > 0.05$). For each extrusion cycle with 0.6-μm and 0.4-μm membranes, the polydispersity of LMNP decreased by 0.1 and 0.05, respectively. Further extrusion of LMNP through the 0.2-μm membrane reduced the mean particle size to about 160 nm (157–165 nm) in Batches 2–12

Table 1 Effect of formulation variables on the particle size and zeta potential of LMNP.

Batch no.	EmulsiFlex	Freezing method	Lyoprotectant	Concn of lyoprotectant (% w/v)	Particle size (nm)		% Size change[a]	Zeta potential (mV)
					BFD	AFD		
1	(−)	Rapid	Mannitol	2.4	194 ± 3.8	340 ± 9.9	75.2	−36.7 ± 4.8
2	(+)	Rapid	Mannitol	2.4	161 ± 1.7	203 ± 3.4	26.1	−45.8 ± 7.8
3	(+)	Rapid	Mannitol	1.2	160 ± 2.9	294 ± 7.0	83.7	−47.1 ± 2.3
4	(+)	Rapid	Trehalose	2.4	160 ± 2.5	174 ± 2.6	8.7	−33.1 ± 7.0
5	(+)	Rapid	Trehalose	1.2	159 ± 2.1	203 ± 3.5	27.7	−37.1 ± 5.6
6	(+)	Rapid	Trehalose	0.6	161 ± 2.6	219 ± 3.8	36.0	−34.3 ± 5.5
7	(+)	Rapid	Trehalose	0.3	160 ± 3.2	255 ± 7.6	59.4	−41.9 ± 3.1
8	(+)	Slow	Trehalose	2.4	165 ± 2.1	173 ± 5.2	4.8	−27.7 ± 3.2
9	(+)	Slow	Trehalose	1.2	160 ± 2.6	207 ± 2.6	29.4	−36.9 ± 5.5
10	(+)	Slow	Trehalose	0.6	161 ± 2.9	222 ± 7.9	37.9	−26.9 ± 4.8
11	(+)	Slow	Trehalose	0.3	159 ± 2.3	256 ± 3.5	61.0	−31.9 ± 3.3
12	(+)	Slow	Mannitol	2.4	157 ± 2.1	247 ± 5.6	57.3	−33.5 ± 2.1

(−), Nanoparticles not passed through EmulsiFlex; (+), nanoparticles passed through EmulsiFlex. [a]With respect to initial size before freeze-drying. BFD, before freeze-drying; AFD, after freeze-drying. Particle size and mean zeta potential are expressed as means ± s.d. ($n = 4$ and $n \leqslant 4$, respectively).

(Table 1) and the polydispersity to 0.07 (0.06–0.1), before freeze-drying. This reduction in the particle size may be attributed to the high-pressure homogenization coupled with the extrusion process. The particle size of LMNP (before freeze-drying) obtained by extrusion of nanoparticles through the EmulsiFlex was found to be comparable with a previous study involving the preparation of monensin-loaded PLGA nanoparticles, prepared by homogenization followed by simultaneous stirring and sonication (Ferdous et al 1998). Furthermore, extrusion of nanoparticles through the EmulsiFlex was found to be beneficial in minimizing the particle size change due to freeze-drying. After freeze-drying, the particle size of Batch 1 (which was not passed through the EmulsiFlex) increased by 75.2% in comparison with a 26.1% increase in Batch 2 (passed through the EmulsiFlex), freeze-dried with 2.4% mannitol (Table 1). These results indicate the utility of extrusion of LMNP through the EmulsiFlex in reducing the particle size before freeze-drying and minimizing the particle size change after freeze-drying. The EmulsiFlex homogenizer has been used in the formulation of solid lipid nanoparticles of azidothymidine palmitate (Heiati et al 1997) and paclitaxel emulsion (Constantinides et al 2000). Recently, it has also been used to prepare PulmoSpheres for the aerosol delivery of anti-asthmatic drugs (Dellamary et al 2000) and immunoglobulin (IgG) (Bot et al 2000). However, this is the first study utilizing the EmulsiFlex homogenizer-extrusion device for the preparation of polymer-based nanoparticles.

Effect of lyoprotectant (mannitol or trehalose) concentration and the freezing method on the particle size of LMNP

It is evident from Table 1 that there was an increase in particle size after freeze-drying of LMNP of all batches, with the size change ranging between 4.8 and 83.7% depending on the manufacturing conditions. Apart from extrusion of nanoparticles through the EmulsiFlex, the lyoprotectant (mannitol or trehalose) played an important role in minimizing the size change in freeze-drying of LMNP. The particle size of LMNP freeze-dried with 2.4% and 1.2% mannitol (Batches 2 and 3, rapid freezing method) was found to be 203 nm and 294 nm, respectively (Table 1). Under similar manufacturing conditions, the particle size of LMNP freeze-dried with 2.4% and 1.2% trehalose (Batches 4 and 5) was found to be 174 nm and 203 nm, respectively. As the trehalose concentration was decreased from 2.4% to 0.3%, the particle size change increased from 8.7% to 59.4% and from 4.8% to 61% in LMNP freeze-dried using rapid freezing (Batches 4–7) and slow freezing method (Batches 8–11), respectively (Table 1). It is evident from this data that the freezing method had a negligible effect on the particle size of LMNP freeze-dried with trehalose. In contrast to this, the freezing method had an effect on the particle size of LMNP freeze-dried with mannitol where rapid freezing was found to have smaller size change in Batch 2 (a 26.1% increase in size after freeze-drying), in comparison with Batch 12 (slow freezing), freeze-dried with 2.4% mannitol (a 57.3% increase in size after freeze-drying). Further, the particle size of LMNP was found to be lower in batches freeze-dried with trehalose than in those free-dried with mannitol prepared under similar manufacturing conditions. These results indicate that trehalose is a better lyoprotectant than mannitol for freeze-drying of LMNP.

The lyoprotective effect of mannitol or trehalose may be explained by extending the concepts that have been proposed for the lyophilization of liposomes and proteins (Crowe et al 1996, 1997). It can be postulated that the lyoprotectant may form an amorphous matrix with water around the particles during freezing, thereby preventing the crystallization of polyethylene glycol chains. There is also a possibility of direct interaction of lyoprotectant with the polar groups (i.e., polyethylene glycol chains) on the surface of PEG-PLGA nanoparticles. A similar kind of interaction has been reported for the stabilization of liposomes (Crowe et al 1996; Tsvetkova et al 1998). Therefore, the difference in the extent of matrix formation and the interaction between lyoprotectant and PEG chains would have contributed to the difference in the extent of lyoprotection of LMNP with mannitol or trehalose. The effect of the concentration of the lyoprotectant on the particle size of LMNP may also be explained by the occurrence of the optimal interaction, when carbohydrate/polyethyleneglycol ratio is optimum (De Jaeghere et al 1999). It was observed that the redispersibility of LMNP in water was greatly enhanced by employing 0.1% sodium carboxymethylcellulose along with the lyoprotectant during freeze-drying.

Morphological characteristics, zeta potential, monensin entrapment and in-vitro monensin release of LMNP

The morphological characteristics of LMNP were studied by AFM and it was observed that LMNP were spherical in shape and smooth in surface texture (Figure

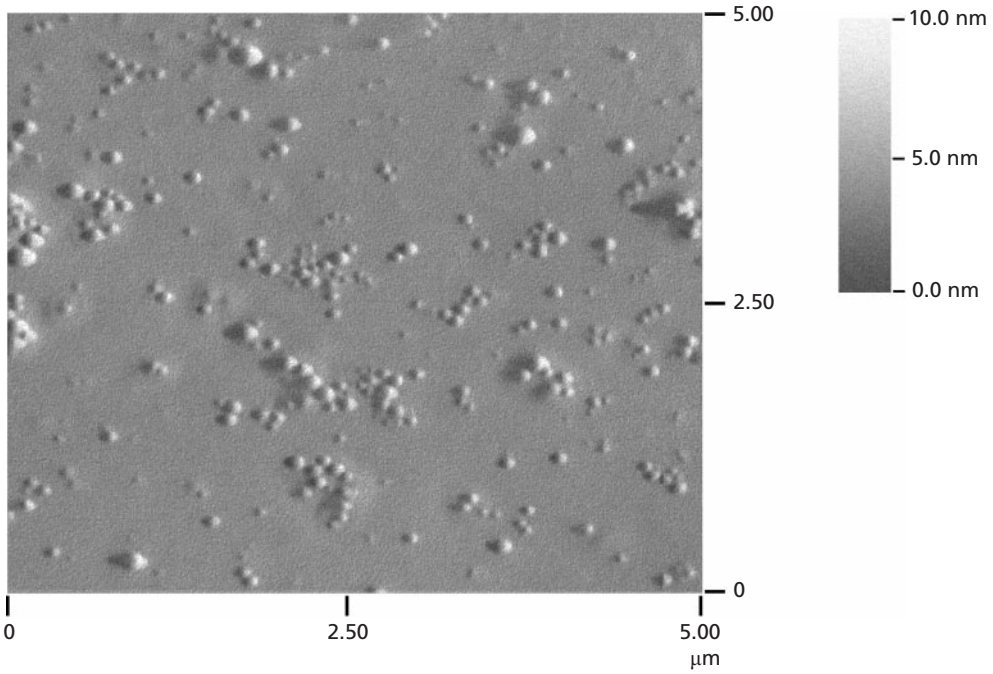

Figure 1 Atomic force microscope (AFM) photograph of LMNP. Scan size, 5 μm; set point, 1.283 V; scan rate, 0.5 Hz; number of samples, 512.

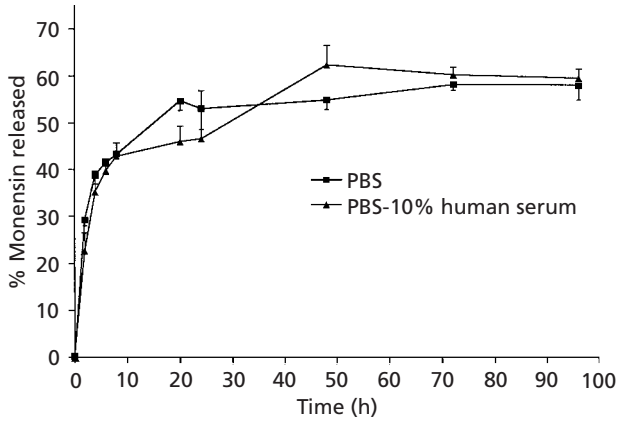

Figure 2 In-vitro release of monensin from LMNP. Data are mean $\pm$ s.d., n = 3.

1). AFM studies also indicated that LMNP were fairly monodisperse and uniform in shape. The zeta potential of LMNP freeze-dried with sodium carboxymethylcellulose and mannitol or trehalose was -40.8 mV (varied between -33.5 and -47.1 mV) and -33.7 mV (varied between -26.9 and -41.9 mV), respectively (Table 1). The high zeta potential of LMNP freeze-dried with lyoprotectant (mannitol or trehalose) and sodium carboxymethylcellulose may be attributed to the presence of carboxyl groups of sodium carboxymethylcellulose on the surface of nanoparticles, as LMNP lyophilized with trehalose (0.3–2.4%) only had a zeta potential of -17 mV (data not shown). It was observed that 14.2 ± 0.3% of monensin could be entrapped in LMNP, under the conditions employed in the study. In-vitro release of monensin from LMNP in PBS or PBS supplemented with 10% human serum showed a rapid release of about 40% in the first 8 h. Subsequently, the release of monensin was found to be slower, with about another 20% releasing in the next 88 h (Figure 2). Human serum was incorporated into dissolution medium to simulate the likely effect of in-vivo conditions on the release of entrapped drug from nanoparticles. From Figure 2, it is evident that there is no significant effect of human serum on the release of monensin from LMNP. Therefore, it may be possible to obtain controlled release of monensin from LMNP for an extended period.

In-vitro degradation of nanoparticles

It is evident from Figure 3 that there was a slow release of lactic acid from nanoparticles in PBS with an average release rate of 14 μg of lactic acid per week in the period between weeks 1 and 6. The percentage of lactic acid released from nanoparticles at the end of 6 weeks was

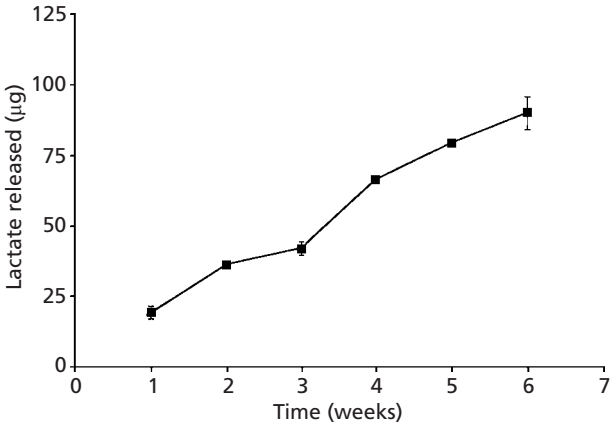

Figure 3 Time course of lactic acid released from empty nanoparticles (without monensin). Data are mean ± s.d., n = 3.

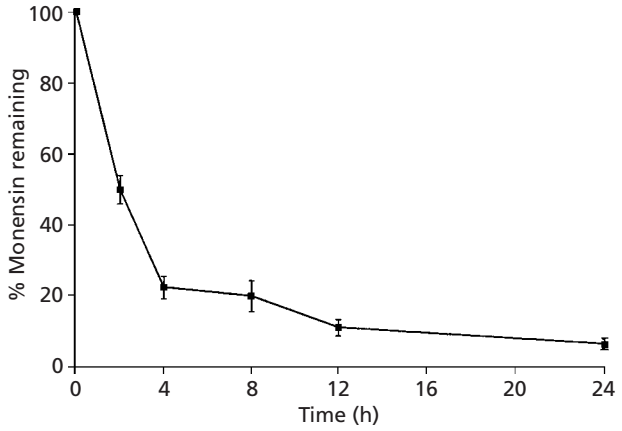

Figure 4 The concentration of monensin in serum (% of injected dose)–time profile, following the intravenous administration of LMNP (monensin 1 mg kg^{-1}) to rats. Data are mean ± s.d., n = 3.

found to be 8.6% when expressed with respect to the total lactic acid obtained after acid hydrolysis of the same amount of nanoparticles as employed for the in-vitro degradation studies. It appears from this study that the rate of degradation of the polymer and the release of monensin from nanoparticles differ from each other and that drug release can take place by diffusion through the partially hydrolyzed matrix. Similar results were reported for the release of lidocaine from PEG-PLGA microspheres (Peracchia et al 1997).

Concentration of monensin in serum following the administration of LMNP or monensin solution to rats

The administration of LMNP to rats resulted in 50% of monensin remaining in circulation after 2 h. Subsequently, monensin levels in serum were maintained at 20% between 4 and 8 h (Figure 4). Further, there was more than 10% of monensin remaining in circulation at both 12 and 24 h after the administration of LMNP to rats (Figure 4). In contrast, the concentration of monensin in serum declined rapidly following the administration of monensin solution or conventional monensin nanoparticles (Ferdous et al 1998), with no monensin remaining in circulation after 4 h. These results indicate that LMNP altered the pharmacokinetics of monensin, resulting in prolonged blood levels. Previous studies conducted on stealth monensin liposomes in mice have shown sustained levels of monensin in serum for up to 24 h (Singh et al 1999). The PEG coating on the surface of LMNP provides highly hydrated and flexible chains of PEG, thereby impairing interaction with circulating opsonins and phagocytic cells, resulting in a longer circulation time (Bazile et al 1995; Vittaz et al 1996). This longer circulation time of LMNP and the controlled release of monensin from LMNP would have contributed to the decreased clearance of monensin from blood. It has been reported that the plasma half-life of PLA-PEG nanoparticles was about 6 h instead of a few minutes for PLA-albumin or PLA-poloxamer-188-coated nanoparticles (Verrecchia et al 1995). Similarly, the circulation time of nanoparticles prepared from PEG-PLGA was significantly increased in comparison with PLGA nanoparticles (Gref et al 1994). The increased blood circulation of stealth nanoparticles from PEG-PLA altered the pharmacokinetics of the encapsulated drug (Verrecchia et al 1995).

In-vitro potentiation of anti-My9 immunotoxin, adriamycin and tamoxifen by LMNP

The cytotoxicity profiles of anticancer agents alone (immunotoxin, adriamycin, tamoxifen) or in combination with LMNP are shown in Figures 5 and 6. The potentiation of the cytotoxicity was calculated from the ratio of the IC50 (the concentration to produce 50% cell kill) value of the anticancer agent alone to that of the combination of anticancer agent and LMNP. The IC50 for immunotoxin alone against HL-60 tumour cells (sensitive) was found to be 3 ± 0.1 ng mL^{-1} and the combination of immunotoxin with LMNP had an IC50 of 0.006 ± 0.0006 ng mL^{-1}, demonstrating a 500-fold potentiation of the activity of immunotoxin by LMNP (Figure 5A). With the resistant HL-60 cell line, the IC50 values for immunotoxin alone and the combination

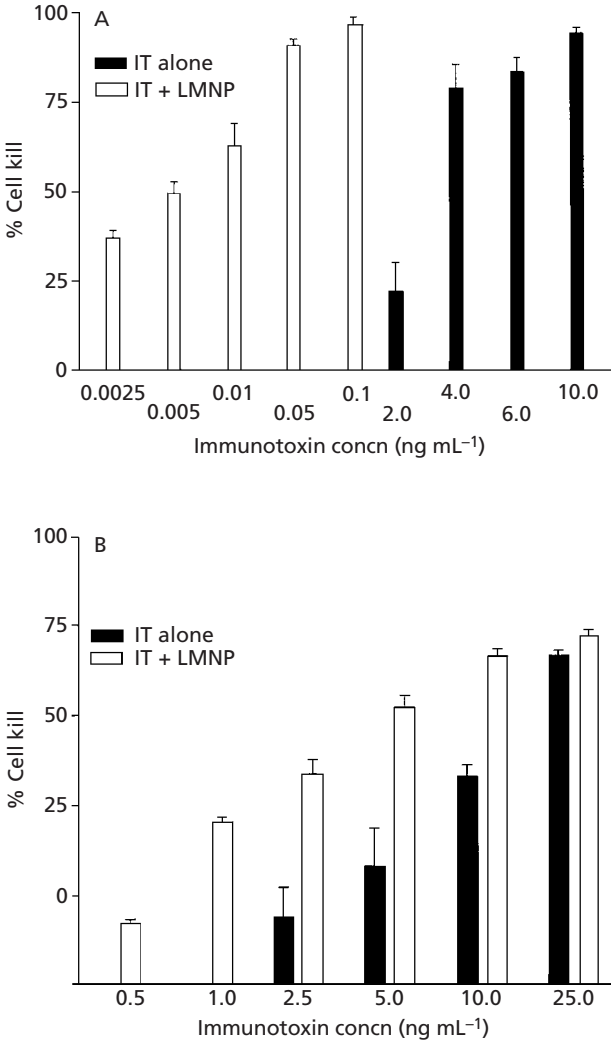

Figure 5 Enhancement of cytotoxicity of anti-My9 immunotoxin by LMNP against HL-60 sensitive cell line (A) and HL-60 resistant cell line (B). Monensin (LMNP) concentration used was 5.0×10^{-8} M. Data are mean $\pm$ s.d., n = 3.

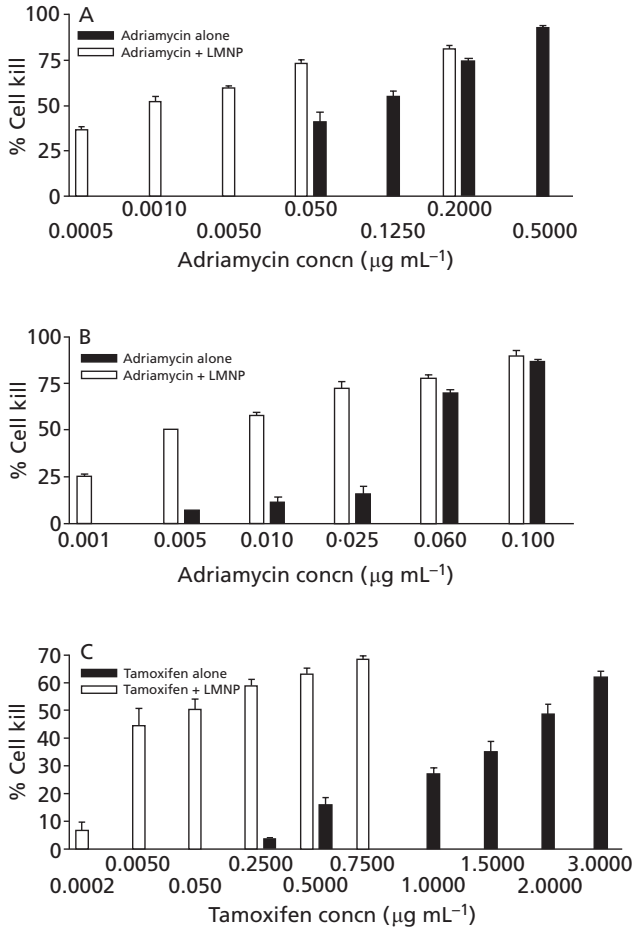

Figure 6 Enhancement of cytotoxicity of adriamycin by LMNP against MCF 7 cell line (A), SW 620 cell line (B) and that of tamoxifen against MCF 7 cell line (C). Monensin (LMNP) concentration used was 5.0×10^{-8} M in all the experiments. Data are mean $\pm$ s.d., n = 3.

of immunotoxin with LMNP were found to be 8 ± 1.4 ng mL^{-1} and 1.6 ± 0.2 ng mL^{-1}, respectively, and thus LMNP could only enhance the activity of the immunotoxin by a factor of 5 (Figure 5B). The cytotoxicity of adriamycin was enhanced 100 fold (IC50 for adriamycin alone, 100 ± 20 ng mL^{-1}; IC50 for adriamycin + LMNP, 1 ± 0.3 ng mL^{-1}) and 10 fold (IC50 for adriamycin alone, 50 ± 2 ng mL^{-1}; IC50 for adriamycin + LMNP, 5 ± 0.1 ng mL^{-1}) against MCF 7 and SW 620 cell lines, respectively (Figures 6A and 6B). The cytotoxicity of tamoxifen was potentiated 44 fold against MCF 7 cell line (Figure 6C; IC50 for tamoxifen alone, 2.2 ± 0.2 μg mL^{-1}; IC50 for tamoxifen + LMNP, 0.05 ± 0.02 μg mL^{-1}). Free monensin (monensin solution) was found to be non-toxic to the cells and showed no potentiation of the cytotoxicity of immunotoxin/adriamycin/tamoxifen at the concentration studied for LMNP (5.0×10^{-8} M).

The ricin A-chain subunits of the immunotoxin kill target cells by inhibiting cellular protein synthesis by the enzymatic removal of adenine from the 28s ribosomal RNA (Endo & Tsurugi 1987). The mechanism by which monensin potentiates the effects of the immunotoxin has not been completely elucidated. However, it has been demonstrated that intracellular trafficking is the rate-limiting step in the tumoricidal effect of immunotoxin (Raso et al 1987). Therefore, it may be possible that monensin affects or retards the intracellular pathways of immunotoxin (Raso et al 1987, Manske et al

1989). It has been reported from our laboratory that incubation of HL-60 cells with monensin liposomes or conventional nanoparticles (with immunotoxin) results in dilation of Golgi, which may allow the transport of toxin molecules to ribosomal RNA (Ferdous et al 1998). Recently, Singh et al (1999) have demonstrated dilation of Golgi apparatus by stealth monensin liposomes in combination with adriamycin in HL-60 resistant cell line. It has been postulated that the dilation of Golgi apparatus has been responsible for altering the intracellular distribution of adriamycin and immunotoxin. Therefore, the enhancement of the cytotoxicity of immunotoxin, adriamycin and tamoxifen by LMNP observed in this study may be presumed to be due to alteration of the intracellular distribution of these anticancer agents. However, further studies are warranted to elucidate the mechanism by which monensin in novel drug delivery systems like long-circulating nanoparticles or liposomes potentiates the cytotoxicity of anticancer agents in sensitive and resistant cell lines.

This is the first study on the preparation, characterization and evaluation of long-circulating nanoparticles of monensin. Based upon our study, it can be concluded that it is possible to prepare LMNP with a particle size of < 200 nm (using a homogenizer and an EmulsiFlex homogenizer-extrusion device) and a monensin content of $> 10\%$. Further, these nanoparticles can be successfully freeze-dried by using a suitable lyoprotectant. It is also possible to provide controlled release of monensin in-vitro and sustained serum concentration of monensin in-vivo from LMNP. The significant potentiation, by a factor of 5 (confidence Interval, 1.6–8.4) 500 (confidence Interval 385.1–614.9), of the cytotoxicity of various anticancer agents like immunotoxin, adriamycin and tamoxifen in-vitro by the administration of monensin through long-circulating nanoparticles will be further evaluated in-vivo in a suitable animal model.

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