JPP
Journal of
Pharmacy and Pharmacology

JPP 2009, 61: 303–310

Received May 22, 2008
Accepted November 17, 2008
DOI 10.1211/jpp/61.03.0004
ISSN 0022-3573

Research Paper

Galactose-grafted chylomicron-mimicking emulsion: evaluation of specificity against HepG-2 and MCF-7 cell lines

Vikas Jain[a], Banashree Nath[a], Girish K. Gupta[a], Parag P. Shah[b], Maqsood A. Siddiqui[b], Aditya. B. Pant[b] and Prabhat R. Mishra[a]

[a]Department of Pharmaceutics, Central Drug Research Institute, Lucknow, India and
[b]*In Vitro* Toxicology Unit, Indian Institute of Toxicological Research, Lucknow, India

Abstract

Objectives A chylomicron-mimicking lipid emulsion was prepared and loaded with paclitaxel (paclitaxel-CM) and was further grafted with galactose (paclitaxel-GCM) using palmitoyl-galactosamine, which was synthesized by reacting galactosamine hydrochloride with *N*-hydroxy succinimide ester of palmitic acid. Palmitoyl-galactosamine was used as a ligand for asialoglycoprotein receptors.

Methods The uptake characteristics of the emulsions were evaluated in HepG-2 cells (human hepatocarcinaoma), which express asialoglycoprotein receptors, and MCF-7 (breast cancer) cells, which are devoid of these receptors.

Key findings The incorporation efficiency of paclitaxel-CM was 68.05 ± 4.80% and that of paclitaxel-GCM was 72.10 ± 3.93% when the emulsion was prepared with 7.5% (w/w) paclitaxel/lipid phase. The globule size of paclitaxel-GCM and paclitaxel-CM was 124 ± 8.67 and 96.45 ± 5.78 nm, respectively. The release of paclitaxel from both of the formulations was fairly sustained: 50 ± 3.2% of paclitaxel in 24 h. The cytotoxicity and uptake of paclitaxel-GCM were significantly higher ($P < 0.05$) in HepG-2 cells than MCF-7 cells, while for paclitaxel-CM cytotoxicity and uptake were similar in the two cell lines. This study clearly demonstrates that upon surface modification palmitoyl-galactosamine remains an integral part of the formulation. Paclitaxel solubility can be improved using optimum paclitaxel/lipid phase ratios. The paclitaxel-GCM formulation recognizes asialoglycoprotein receptors over-expressed on HepG-2 cells.

Conclusions Under our experimental conditions, the proposed paclitaxel-GCM formulation is an ideal delivery vehicle for specific targeting to liver cancer cells, which is anticipated to result in improved efficacy and reduced toxicity to normal cells.

Keywords chylomicron; cytotoxicity; paclitaxel; surface modification; tumour targeting

Introduction

The detrimental effects of chemotherapeutic drugs on normal tissue in cancer patients is a key issue being addressed worldwide. Paclitaxel is most commonly used to treat breast and ovarian cancers.[1] Recently, paclitaxel was shown to significantly inhibit the growth of human hepatoma tumours in nude mice.[2] However, paclitaxel has disadvantages of low aqueous solubility and non-specificity towards liver cancer cells, which are main constraints to the development of effective formulations. Furthermore, marketed formulations based on a cremophor EL/ethanol mixture have been associated with serious adverse effects, and the solution tends to precipitate on storage, so in-line filtering is required during administration to patients.[3,4] Targeted delivery of paclitaxel to tumour cells would enable reduction of the dose and associated toxic side-effects. Attempts have been made to prepare safe formulations of paclitaxel by incorporating into lipid emulsions,[5] mixed micelles,[6,7] liposomes,[8] taxol–albumin conjugates[9] and poly (γ-glutamic acid)-poly (lactide) nanoparticles.[10] All of these formulations have disadvantages, however, and there is plenty of scope to develop safer and more selective formulations.

Recent results suggest that chylomicron-mimicking lipid emulsions can provide an effective delivery vehicle for poorly water-soluble drugs, particularly to the liver. When injected intravenously, the reconstituted chylomicrons were preferentially taken up by liver hepatocytes.[11] Cell-specific drug targeting to liver parenchymal cells has also been achieved using apolipoprotein (apo)-E associated emulsions.[12] The surface of the lipid emulsion was

Correspondence: Dr P. R. Mishra, Department of Pharmaceutics, Central Drug Research Institute, Chattar Manzil Palace, UP 226001, Lucknow, India.
E-mail: mishrapr@hotmail.com
CDRI communication number 7495

modified with galactose using a synthetic galactosylated cholesterol derivative [cholesten-5-yloxy-*N*-(4-((1-imino-2-D-thiogalactosylethyl) amino) butyl) formamide (Gal-C4-Chol)], which resulted in improved uptake of lipid emulsion in HepG-2 cells via asialoglycoprotein-receptor-mediated endocytosis.[13] Thus, the use of ligand-directed chylomicrons for specific delivery of paclitaxel to the liver parenchyma could be a big boon for the treatment of liver cancer.

In this study we have developed a chylomicron-mimicking lipid emulsion bearing paclitaxel (paclitaxel-CM), and further modified the surface with a galactose moiety using synthetic palmitoyl-galactosamine (paclitaxel-GCM). The main aim of our study was to determine whether paclitaxel-GCM could be actively targeted to HepG-2 cells via asialoglycoprotein receptors. These formulations were characterized in terms of paclitaxel incorporation efficiency, globule size, zeta potential, viscosity and their in-vitro release profiles. The cytotoxicity of the prepared paclitaxel-CM and paclitaxel-GCM formulations was evaluated in HepG-2 (human hepatocarcinoma) and MCF-7 (human breast cancer) cell lines and compared with that of a paclitaxel formulation similar in composition to the commercially available formulation (paclitaxel-M, containing 6 mg paclitaxel, 527 mg cremophor EL and 49% (v/v) ethyl alcohol per ml). Uptake of paclitaxel-CM and paclitaxel-GCM was also determined in HepG-2 and MCF-7 cell lines after labelling with rhodamine and quantified using flow cytometry.

Materials and Methods

Materials

Paclitaxel (purity > 99%) was a gift from Dabur Research Foundation (Ghaziabad, India). Castor oil (refined), cholesterol, cholesteryl palmitate, galactosamine, palmitic acid *N*-hydroxysuccinimide ester, triethylamine, DMSO and lecithin were all obtained from Sigma-Aldrich (St Louis, MO, USA). l-α-phosphatidylcholine and lyso-phosphatidyl choline were gifts from the Lipoid AG (Ludwigshafen, Germany). All other reagents and chemicals were of analytical grade.

For cell culture, Dulbecco's modified Eagle medium (DMEM) with glutamate, fetal bovine serum (FBS) and antibiotic solution (penicillin/streptomycin, 0.1% v/v) were purchased from Sigma. Trypan blue solution, Giemsa stain and MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) were also from Sigma. Well plates for cytotoxicity and uptake studies were from Greiner Bio One (Frickenhausen, Germany). All materials were used without further purification. The water used in all experiments was prepared in a three-stage Millipore Milli-Q plus 185 purification system (Bedford, MA, US) and had a resistivity greater than 18.2 mΩ/cm.

Synthesis of N-palmitoyl galactosamine

Galactosamine HCl (100 mg) was dissolved in an appropriate quantity of DMSO and triethylamine (108 μl). Palmitic acid *N*-hydroxy succinimide (142 mg) dissolved in 2 ml chloroform was added to the mixture. The mixture was stirred at room temperature for 72 h and protected from light. Finally, the chloroform was evaporated off at room temperature and the remaining liquid freeze dried. The dried powder was washed with water and again freeze dried. The palmitoyl-galactosamine conjugate formed was analysed by ^{1}H NMR (Avance DRX-300, Brucker, Switzerland) and fast-atom bombardment (FAB) mass spectrometry on a ZAB-SE mass spectrometer (MSroute JMS-600H, Jeol, Japan).

Preparation of chylomicron-mimicking emulsion

The emulsion was prepared by mixing castor oil, lecithin, lysophosphatidylcholine, cholesteryl palmitate and cholesterol in a glass vial in the weight ratio of 70 : 22.7 : 2.3 : 3 : 2. Chloroform was added to dissolve the contents. The chloroform was removed by blowing nitrogen gas into the vial, followed by hydration with Tris-saline buffer over 24 h. The emulsion formed was vortex mixed for 60 s, followed by sonication for 10 min. This emulsion was then passed through liposoFast (Avestin Inc., Ottawa, Canada) 50 times to get a fine emulsion of the desired size.[14] In order to incorporate paclitaxel into the formulation, different concentrations of paclitaxel were dissolved in the lipid mixture. To incorporate the conjugate (palmitoyl-galactosamine), it was dispersed in Tris-buffer by alternate cycling of warming and sonication until completely dispersed. To perform uptake studies, the emulsion was labelled with the fluorescent marker rhodamine 123 (Sigma-Aldrich) (0.01 mM) calculated on the basis of oil volume.

Measurement of paclitaxel

Paclitaxel concentrations in the samples were measured by reverse-phase HPLC. The HPLC system was equipped with 10 ATVP binary gradient pumps (Shimadzu, Japan), a rheodyne model 7125 injector (Cotati, CA, US) with a 20 μl loop and SPD-M10 AVP UV detector (Shimadzu). Separation was achieved on a Lichrosphere Lichrocart C_{18} column (250 mm, 4 mm, 5 μm) (Merck, Darmstadt, Germany). The mobile phase was a mixture of acetonitrile and triple-distilled water (55: 45 v/v) delivered at a flow rate of 1 ml/min. The injection volume was 20 μl and detection was at 227 nm. Data were acquired and processed using class-VP (Shimadzu) software.

Measurement of incorporation efficiency

Paclitaxel incorporation efficiency and drug loading was estimated by separating unincorporated paclitaxel by ultracentrifugation at 48 000*g* at 4°C for 30 min in a Beckman Optima MAX ultracentrifuge (Beckman Coulter, Fullerton, CA, US).[15] The concentration of free paclitaxel in the supernatant was then estimated. Incorporation efficiency (% IE) was calculated using the equation IE (%) = [(A2 − A1) / A2] × 100, where A1 is the amount of free paclitaxel and A2 is the total paclitaxel taken up during formulation. Drug loading (DL) was calculated using the equation DL = (total paclitaxel taken − free paclitaxel) / total amount of lipid.

Formulation characterization

The zeta potential and mean globule size of the emulsion were measured using the Malvern Zetasizer (Malvern, UK). Each sample of emulsion was diluted in water to an appropriate concentration before measurement at room temperature.

The viscosity of the formulation was measured using a rotational viscometer parallel plate and cone (20 mm diameter, 4° angle) at a constant shear rate at room temperature (25 ± 1°C).

In-vitro drug release (dialysis method)

The in-vitro release was determined using dialysis tubing (Sigma). Free drug was removed from the formulation by exhaustive dialysis against phosphate-buffered saline (PBS) for 24 h at 4°C. For the release experiment, 1 ml emulsion was pipetted into a dialysis bag (molecular weight cut off 12 000 Da; Sigma) at room temperature. The dialysis bags were kept in 500 ml PBS at pH 7.4. The temperature was maintained at 37°C and the shaking frequency at 150 rpm in dissolution apparatus.[16] Samples (2 ml) were taken at 0, 15 and 30 min and 1, 2, 4, 6, 12 and 24 h and replaced with an equivalent volume of fresh PBS. The concentrations of released paclitaxel were determined by HPLC.

Cytotoxicty studies

The in-vitro cytotoxicity of formulations was measured by the MTT proliferation assay.[17] The experiments were carried out on cells in the exponential growth phase. HepG-2 cells and MCF-7 cells were seeded into 24-well plates at 5×10^4 cells/well and were allowed to adhere overnight. The growth medium was replaced with a fresh medium and then cells were incubated for 24 h with different formulations (paclitaxel-M, paclitaxel-CM, paclitaxel-GCM) containing equivalent amounts of paclitaxel in the concentration range (0.25–8 mg/ml). Cells were then washed twice with 1 ml PBS. Cells were then incubated in a growth medium containing 1 mg/ml MTT for 4 h at 37°C, and 500 μl DMSO was added to each well to ensure solubilization of the formazan crystals. The optical density was measured using a multiwell scanning spectrophotometer (MRX Microplate Reader, Dynatech Laboratories Inc., Chantilly, VA, US) at a wavelength of 570 nm.

In-vitro uptake studies

The HepG-2 cells were plated onto a 12-well cluster dish at a density of 2×10^5 cells/well and cultivated in 800 μl DMEM supplemented with 10% FBS (Sigma, US). After 24 h the culture medium was replaced with fresh culture medium containing CM and GCM emulsions incorporating rhodamine 123 (0.2, 0.5 and 1% w/v). After incubation for 1 h at 37°C, the solution was removed by aspiration, and the cells were washed five times with ice-cold Hank's balanced salt solution. For separation of the internalized and surface-bound emulsions, the cells were washed three times with acetate buffer (pH 4.0). After detachment from the cell surface in phenol-red-free DMEM, and relative fluorescence was measured by flow cytometry (Becton Dickinson, Oxford, UK) at an excitation wavelength of 511 nm and an emission wavelength of 534 nm.

Measurement of haemolysis

The degree of haemolysis induced by the emulsions and the other emulsion components on fresh citrated human blood was determined.[18] Lipid emulsion (0.2 ml) containing equivalent amounts of paclitaxel (50 μg/ml) were incubated with 1 ml blood from male Wistar rats at 37°C for 45 min, then centrifuged at 10 000 rpm for 20 min. The haemoglobin released in the supernatant was measured spectrophotometrically at 550 nm. The absorbance at this wavelength is directly proportional to the haemoglobin concentration, which is in turn proportional to the number of intact red blood cells that were not destroyed by the added sample. For the control, blood was similarly incubated with 0.9% (w/v) saline. Blood was also incubated with the same volume of distilled water (100% haemolysis). The percentage haemolysis was determined from the haemoglobin released into the supernatant.

Statistical analysis

All results are given as means ± SD ($n = 3$). Differences between formulations were compared using one-way analysis of variance (ANOVA) followed by the Tukey–Kramer multiple comparison test, using Graph pad Instat software (Graph Pad Software Inc. CA, US). $P < 0.05$ denotes significance in all cases. The effect of formulation type on paclitaxel release at each time point, and the effect of % paclitaxel/lipidic phase on incorporation efficiency and paclitaxel loading were analysed using the Kruskal–Wallis test followed by Fisher's post-hoc test.

Results

Synthesis of the palmitoyl-galactosamine anchor

The anchor palmitoyl-galactosamine was synthesized by reacting activated esters of *N*-hydroxy succinimide palmitate specifically with the amino group of galactosamine, as shown in Figure 1. The formation of conjugate was confirmed by FAB mass spectrometry and ^{1}H NMR. The FAB analysis showed the presence of mass ion peaks (M^{+1}) at 418 nm, other mass fragments were at 400 nm (%, M^+ -OH), 238 nm (100, M^+ -galactosamine), 176 nm (%, M^+ -palmitoyl-CH_3-CH-COOH). An NMR spectrum confirmed the formation of complexes, as it exhibited peaks corresponding to the ^{1}H NMR of palmitoyl-galactosamine, showing peaks at 0.92 (t, 3H, J = 7.5 Hz, $CH_{3\text{-}a}$), 1.28–1.56 (m, 24 H (CH_2) X12), 1.76 (q, 2H, J = 7.5 Hz, $CH_{2\text{-}b}$), 2.61 (t, 2H, J = 7.5 Hz, $CH_{2\text{-}d}$), 3.40 (ddd, 1H, J = 9.0 Hz, 12.6 Hz, J = 3.06, H-5), 3.70 (d, 2H, J = 12.6 Hz, H-6), 3.82 (t, 2H, J = 11.1 Hz, H-4), 4.06 (t, 1H, J = 11.1 Hz, H-3), 4.83 (t, 1H, J = 8.4 Hz, H-2), 5.42 (d,1H, J = 3.0 Hz, H-1).

Development of the formulation

Attempts were first made to solubilize paclitaxel in different oils such as soybean oil, castor oil and olive oil. The solubility

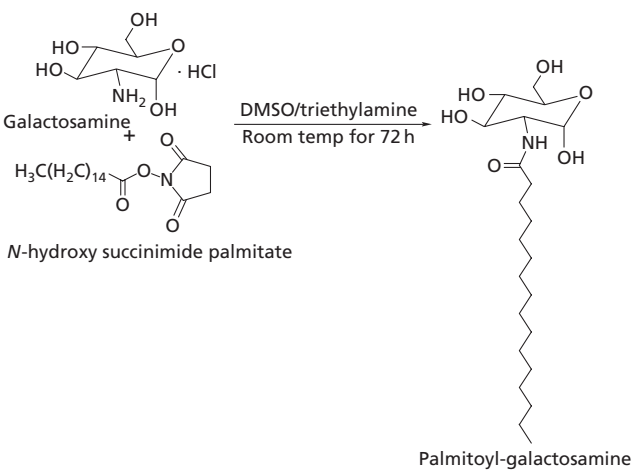

Figure 1 The synthesis of palmitoyl-galactosamine.

of paclitaxel was highest in castor oil: 1.32 ± 0.4 mg/g, compared with 0.15 mg/g in soyabean oil and 0.35 mg/g in olive oil. Castor oil was therefore selected as the oil phase for developing the formulation. The chylomicron-mimicking emulsion was prepared by mixing castor oil, lecithin, lysophosphatidylcholine, cholesterol oleate and cholesterol at a fixed weight ratio of 70: 22.7: 2.3: 3 : 2. The surface of the chylomicrons was further modified with galactose using palmitoyl-galactosamine (5%) to enable active targeting to hepatoma cells via the asialoglycoprotein receptors. An emulsion of required globule size of about 100 nm was obtained by extruding it through filters.

Paclitaxel incorporation efficiency

In order to determine the incorporation efficiency of paclitaxel, the amount of paclitaxel in the lipid phase of the emulsion was gradually increased (i.e. 2.5, 5, 7.5, 10 and >10% w/w). Drug loading and entrapment improved up to 7.5% paclitaxel, beyond which there was decrease in drug loading. The amount of paclitaxel loaded was 0.47, 0.82, 1.02 and 0.84% with the 2.5, 5, 7.5 and 10% paclitaxel/lipid phases, respectively (differences not significant). Thus it can be concluded that solubility reaches saturation when the paclitaxel/lipid phase ratio reaches 7.5%, above which precipitation occurs. The incorporation efficiency with 7.5% (w/w) paclitaxel/lipid phase was 68.05 ± 4.08% in paclitaxel-CM and 72.10 ± 3.93% in paclitaxel-GCM. Thus, the CM prepared with 7.5% (w/w) paclitaxel (vs total lipids 100 mg/ml) had the highest amount of paclitaxel incorporated, which was estimated to be 1.02–1.09 mg paclitaxel per ml aqueous solution. This is approximately 35-fold higher than the original paclitaxel solubility of 0.03 mg/ml.[19]

Formulation characterization

The developed formulation was characterized in terms of globule size, zeta potential and viscosity (Table 1). Paclitaxel-CM had a globule size of 96 nm and paclitaxel-GCM had a globule size of 124 nm. When stored at 4°C, the size of the emulsion particles did not change significantly in a 2-week short-term stability study (data not shown). The zeta potentials of the paclitaxel-CM and paclitaxel-GCM were −22 mV and −14 mV, respectively. The viscosity of paclitaxel-CM and paclitaxel-GCM systems were less than 3.9 cps at 25 ± 1°C. Therefore, these formulations appeared to be safe and convenient for intravenous administration.

In-vitro release

The release of paclitaxel *in vitro* was determined using the dialysis bag method. As shown in Figure 2, paclitaxel is released slowly from both formulations (with and without conjugate) compared with paclitaxel suspended in 0.5% Tween 80, which was used as the control. The cumulative drug release after 24 h was 55.4 ± 3.0% from paclitaxel-CM and 47.8 ± 3.5% from paclitaxel-GCM.

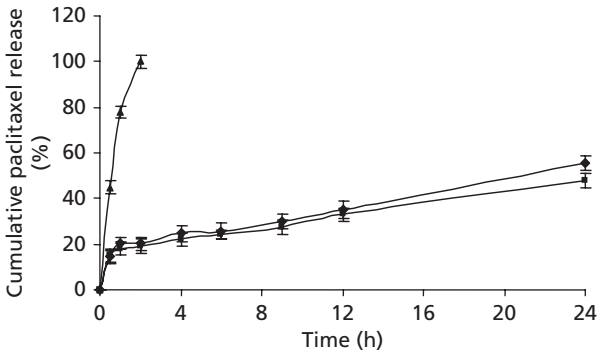

Figure 2 In-vitro release of paclitaxel from different formulations. PTX-CM (-◆-), paclitaxel-loaded chylomicron emulsion; PTX-GCM (-■-), paclitaxel-loaded galactose-grafted chylomicron emulsion; control (-▲-), paclitaxel suspended in phosphate-buffered saline containing 0.5% Tween 80. Data are means ± SD (n = 3 experiments).

Cell viability studies

Cytotoxicity of the paclitaxel-CM and paclitaxel-GCM was evaluated *in vitro* using the MTT assay and compared with paclitaxel-M. The study was carried out on HepG-2 cells, which express asialoglycoprotein receptors, and MCF-7 breast cancer cells, which do not express asialoglycoprotein receptors. When exposed to paclitaxel-loaded emulsions at different concentrations, dose-dependent increases in cytotoxicity were seen in both cell lines. Inhibition of cell growth was greater with paclitaxel-CM and paclitaxel-GCM than paclitaxel-M ($P < 0.05$). Paclitaxel-GCM exhibited significantly higher inhibitory activity than paclitaxel-CM in HepG-2 cells ($P < 0.05$), as shown in Figure 3a. The two emulsions had similar effects in MCF-7 cells and there were no significant difference in inhibitory activity between paclitaxel-GCM and paclitaxel-CM (Figure 3b). This indicates that hepatoma cells (HepG-2) tend to recognize palmitoyl-galactosamine-terminated CM via the asialoglycoprotein receptors located on their surfaces. At a paclitaxel concentration of 8 μg/ml, paclitaxel-GCM killed more than 50% of HepG-2 cells, whereas paclitaxel-M and paclitaxel-CM killed 20% and 35% of cells, respectively. The activity of paclitaxel-M and paclitaxel-CM at 10 μg/ml was slightly higher ($P < 0.05$) against MCF-7 than HepG-2 cells. In the case of paclitaxel-M, cell killing was 45% against MCF-7 and only 37% against HepG-2 cells. This agrees with data reported by Gao *et al.*[20] that paclitaxel is more effective against breast cancer. However, its efficacy against

Table 1 Characteristics of the paclitaxel-loaded chylomicron formulations

Formulations	Average globule size (nm)	Zeta potential (mV)	Viscosity (cps)	Incorporation efficiency (%)
PTX-CM	96.45 ± 5.78	−22.2 ± 2.78	2.4 ± 0.42	68.05 ± 4.08
PTX-GCM	124.35 ± 8.67	−14.1 ± 1.50	2.7 ± 0.37	72.10 ± 3.93

PTX-CM, paclitaxel-loaded chylomicron-mimicking emulsion; PTX-GCM, paclitaxel-loaded galactose-grafted chylomicron-mimicking emulsion. Values are means ± SD of three experiments.

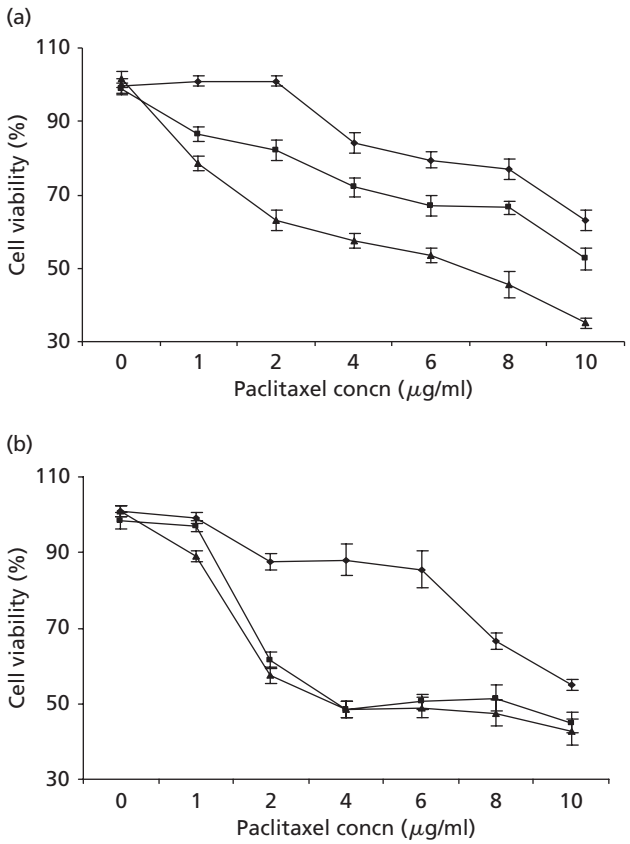

Figure 3 Viability of (a) HepG-2 cells and (b) MCF-7 cells (determined by MTT assay) following exposure to paclitaxel-loaded formulations. PTX-CM (–■–), paclitaxel-loaded chylomicron emulsion; PTX-GCM (–▲–), paclitaxel-loaded galactose-grafted chylomicron emulsion; PTX-M (–◆–), paclitaxel formulation similar to marketed preparation containing cremophor and ethanol. Data are means ± SD (n = 3 experiments).

hepatic cell lines can be improved tremendously by active targeting using paclitaxel-GCM. As demonstrated in Figure 3a and Figure 3b, the cell killing of paclitaxel-GCM against MCF-7 was nearly 50%, while it was more than 65% against HepG-2 cell lines. There were no significant differences between paclitaxel-CM and paclitaxel-GCM against MCF-7 cell lines. These data clearly indicate that paclitaxel-GCM is preferentially targeted to asialoglycoprotein receptors in HepG-2 cells.

In-vitro uptake studies

As observed in the cell viability studies, paclitaxel-GCM has a more pronounced cytotoxic effect in HepG-2 cells than MCF-7 cells, whereas paclitaxel-CM had comparable cytotoxicity in both cell lines, which indicates active uptake of paclitaxel-GCM in HepG-2 cells. In order to confirm specificity of paclitaxel-GCM towards HepG-2 cells, an in-vitro uptake study was carried out using rhodamine 123-labelled CM and GCM formulations. For the uptake study, emulsions of both the formulations containing oil in the concentration range 0.01–0.05% were incubated with HepG-2 and MCF-7 cells for 2 h. As shown in Figure 4, cellular uptake increased proportionately with increasing lipid concentration in both MCF-7 and HepG-2 cell

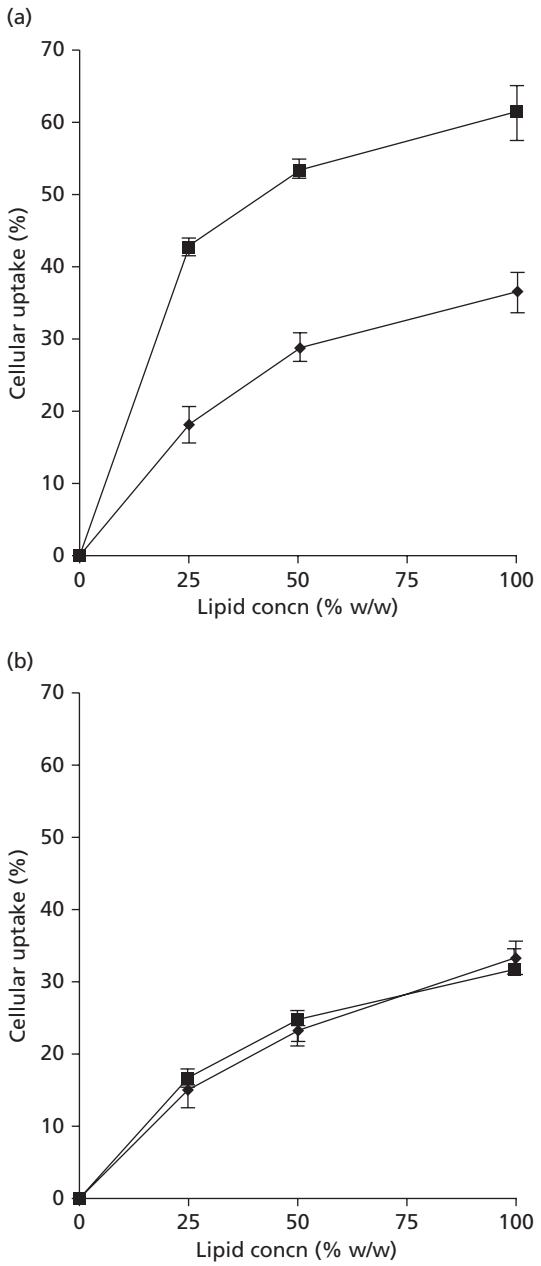

Figure 4 Cellular uptake of chylomicron emulsions labelled with rhodamine in (a) HepG-2 cells and (b) MCF-7 cells at different lipid concentrations. CM (–◆–), chylomicron-mimicking emulsion; GCM (–■–), galactose-grafted chylomicron-mimicking emulsion. Data are means ± SD (n = 3 experiments).

lines. From these data it seems that the cellular uptake depends on the lipid concentration – as lipid concentration increases, the concentration of fluorescent marker increased correspondingly, owing to its hydrophobicity. Uptake of the rhodamine-labelled GCM formulation was more than double that of the CM formulation in HepG-2 cells ($P < 0.05$) (Figure 4a). In contrast, cellular uptake was nearly 30% with both formulations in MCF-7 cells (Figure 4b). These observations agree with the cytotoxicity data, providing evidence of active receptor-mediated cellular uptake of the GCM formulation.

Figure 5 shows representative flow cytometry images for the uptake of rhodamine-loaded CM and GCM in HepG-2 cells after 24 h. Each shows two scattered zones M2 and M1, which correspond to the number of cells with and without uptake of the formulation, respectively. The broad M2 scattered zone indicates the heterogenous population of HepG-2 cells with different numbers of internalized formulations. The ratio M2/(M1 + M2) represents the percentage of cells with internalized formulations. The cellular uptake for GCM was 65%, which is almost double that with the CM formulation (35% cellular uptake after 24 h).Unlike with HepG-2 cells, the active uptake of formulations was not observed in MCF-7 cells, since there was no significant difference in fluorescence intensity between CM and GCM, as shown in Figure 4b.

To evaluate the safety profile of the developed formulation, the haemolytic activity was measured with and without paclitaxel incorporated into the formulations. The plain lipid CM emulsion without drug showed haemolytic activity of 5.6 ± 2.5%, suggesting that phosphatidylcholine was moderately toxic to erythrocytes. Surface modification of CM with palmitoyl-galactosamine had no notable effect on haemolytic activity, which was 7.1 ± 2.9% for GCM. The impact of paclitaxel loading on haemolytic behaviour was also evaluated. Paclitaxel alone caused 4.5 ± 1.6% haemolysis, while paclitaxel-CM and paclitaxel-GCM formulations had little effect on erythrocytes.

Discussion

Paclitaxel has shown therapeutic potential against breast and ovarian cancers, and it is well known that various cancer cells, including hepatoma cells, can also be killed effectively using paclitaxel. Scientists throughout the world have endeavored to develop better and improved formulations that are suitable for different applications, including targeted delivery systems to increase the therapeutic potential of paclitaxel.[21] A variety of paclitaxel preparations have been developed as possible alternatives to cremophor EL-based formulations. However, the utility of most of these preparations has been limited by the poor solubility of paclitaxel. We have therefore attempted to develop a paclitaxel-loaded chylomicron-mimicking emulsion, and we also grafted this with a galactose-based anchor and evaluated its receptor-mediated specificity using HepG-2 and MCF-7 cell lines. We hypothesized that a galactose-grafted chylomicron-mimicking emulsion with a high payload of paclitaxel would not only improve its solubility but also exhibit receptor-mediated specificity towards hepatoma cells. The asialoglycoprotein receptors for sugar such as galactose are usually over expressed on hepatocytes.[22]

Many authors have reported use of emulsions as drug delivery vehicles for paclitaxel, as the drug can be completely carried in the oil phase and, because it does not possess a strong amphiphilic character, it is mainly retained in the oil phase and does not localize in the oil–water interface.[23] The CM emulsion was developed using commercially available lipids and oil that are, or can be readily metabolized into, naturally occurring and biocompatible molecules. We kept the ratio of the lipid component almost constant, and, most importantly, used the most clinically acceptable oil that could dissolve the paclitaxel completely.

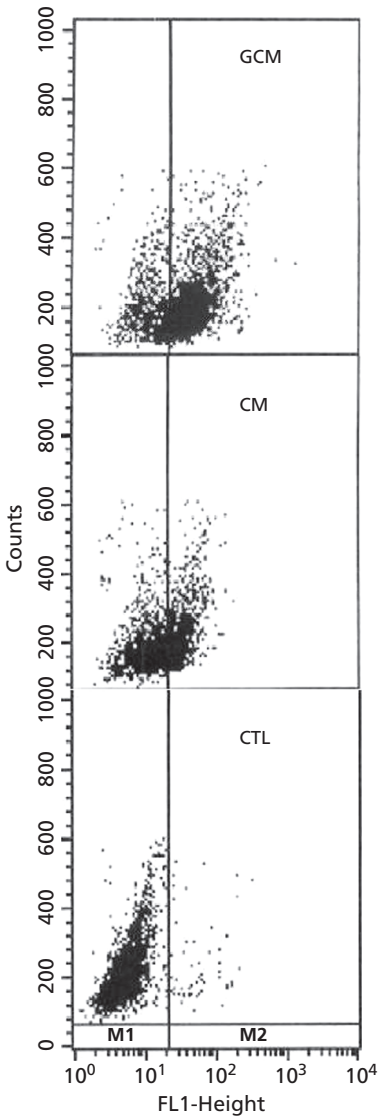

Figure 5 Flow cytometry diagram of HepG-2 cells exposed to rhodamine-labelled chylomicron-mimicking emulsions. M2 and M1 scattered zones represent cells that have and have not taken up the formulation, respectively. CTL, control cells; CM, chylomicron-mimicking emulsion; GCM, galactose-grafted chylomicron-mimicking emulsion.

Among different oils used, castor oil showed maximum solubility, which can be attributed to the intrinsic polarity of the poorly water-soluble drugs favoring solubilization in small/medium molecular volume oils such as medium-chain triglycerides or mono- or diglycerides.[24] Thus, castor oil was selected as the oil phase for the development of formulation. The US Food and Drug Administration has categorized castor oil as 'generally recognized as safe and effective' (GRASE). In order to prepare the galactose-grafted chylomicron-mimicking emulsion formulation, the anchor palmitoyl-galactosamine was successfully synthesized using a reported method with slight modification.[25]

The low aqueous solubility of paclitaxel (about 0.03 mg/ml) often results in low encapsulation in structure-based vehicles.

However, encapsulation of paclitaxel in this prototype formulation was significantly increased, which may be attributed to improved solubility in the oil phase. The physicochemical parameters like globule size, zeta potential and viscosity which usually determine suitability for parenteral administration and fate after administration[26] were evaluated. The particle size was within acceptable range (< 100 nm). However, the globule size of the paclitaxel-GCM emulsion was relatively larger than paclitaxel-CM, which is likely to be due to the galactosamine moiety, which may project out on the surface of the emulsion particles, whereas palmitoyl and oil-soluble components are likely to be entrapped inside the lipid phase of the chylomicrons.

The relatively smaller globule-size formulation can be beneficial for efficient targeting to liver parenchymal cells. It is widely reported in the literature that the diameter of the fenestra of the liver sinusoids is usually less than 200 nm. Thus, globules larger than this do not reach liver parenchymal cells and are readily scavenged non-specifically by monocytes and the reticuloendothelial system. It has been reported that smaller particles tend to accumulate at the tumour sites because of the enhanced permeability and retention effect.[27]

The surface of the chylomicron emulsion was negatively charged, which may further improve its tendency to accumulate in liver hepatocytes, as it has been reported that chylomicrons accept apolipoproteins (i.e. apo-E1) in the blood, and adsorption of apo-E depends on surface charge of the system. The negatively charged globule adsorbs maximum apo-E, followed by neutral and positive globules, and it is reported that chylomicrons are taken up by liver hepatocytes via apoprotein receptors.[28] There was a minor reduction in the negative zeta potential in the case of paclitaxel-GCM, which may be due to a camouflage effect of the palmitoyl-galactosamine incorporated in the system.

The in-vitro release study suggests that paclitaxel is successfully retained in the oil matrix and subsequent in-vitro release was fairly sustained. Thus, with only small amounts of paclitaxel being released *in vitro* compared with the control, it can be concluded that after intravenous injection the majority of paclitaxel incorporated in the emulsion will remain inside the emulsion until it is taken up by the liver. It has been reported that when administered intravenously, 70% of chylomicron coated with apo E was taken up by mouse liver within 20 min.[29] Even without apo E protein coating, about 30% of the reconstituted chylomicron accumulated in mouse liver 30 min after injection.

The in-vitro cell toxicity and uptake studies provides further evidence that GCM interacted with HepG-2 cells via ligand (asialoglycoprotein) receptor recognition. It can therefore be implied that the GCM emulsion would have first been internalized into HepG-2 cells via the asialoglycoprotein receptors, followed by paclitaxel release inside the cytoplasm, inhibiting the growth of the cells. Thus, the active-targeting nature of the GCM may lead to high selectivity to hepatic tumours and enhance their cellular uptake, with a consequent decrease in systemic toxicity.

The formulations developed seem to be safe for systemic administration, causing minimum haemolysis, and the castor oil used to solubilize the paciltaxel is safer than cremophor EL, a polyethoxylated derivative of castor oil, which is used in the marketed formulation. Castor oil is categorized by the US FDA as GRASE, whereas cremophor is reported to be more toxic because of its surfactant properties; it enters biological membranes and/or induces peroxidation of polyunsaturated fatty acids and inflicts direct damage to vital cell constituents such as lipids, proteins and DNA.[30] Further, concentration of castor oil used in our formulations is unlikely to cause toxic reactions.

Conclusions

In the present investigation paclitaxel-loaded chylomicron-mimicking emulsions were successfully developed. By exploiting the presence of specific receptors for sugar recognition on liver parenchymal cells, this surface-modified chylomicron-mimicking system can target anticancer drugs to hepatocytes, in localized as well as metastasized cancer. The in-vitro uptake study suggests that this galactose-grafted prototype formulation holds promise for preferential paclitaxel delivery to liver parenchymal cells.

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

Dr P. R. Mishra is grateful to Department of Science and Technology, New-Delhi, India, for providing financial support under the Fast Track Scheme. Girish K Gupta and V. Jain are thankful to the Council of Scientific and Industrial Research, New-Delhi, India, for providing a Research Associateship and Senior Research Fellowship, respectively.

References

1. Fuchs J *et al.* Paclitaxel: an effective antineoplastic agent in the treatment of xenotransplanted hepatoblastoma. *Med Pediat Oncol* 1999; 32: 209–215.
2. Yuan JH *et al.* Growth-inhibiting effects of Taxol on human liver cancer *in vitro* and in nude mice. *World J Gastroenterol* 2000; 6: 210–215.
3. Rowinsky EK *et al.* Clinical toxicities encountered with paclitaxel (Taxol). *Semin Oncol* 1993; 20: 1–15.
4. Sarosy G, Reed E. Taxol dose intensification and its clinical implications. *J Natl Med Assoc* 1993; 85; 427–431.
5. Lundberg BB. A submicron lipid emulsion coated with amphipathic polyethylene glycol for parenteral administration of paclitaxel (Taxol). *J Pharm Pharmacol* 1997; 49: 16–21.
6. Onyuksel A *et al.* A mixed micellar formulation suitable for the parenteral administration of taxol. *Pharm Res* 1994; 11: 206–212.
7. Ramaswamy M *et al.* Human plasma distribution of free paclitaxel and paclitaxel associated with diblock copolymers. *J Pharm Sci* 1997; 86: 460–464.
8. Sharma A, Straubinger RM. Novel taxol formulations: preparation and characterization of taxol-containing liposomes. *Pharm Res* 1994; 11: 889–896.
9. Dosio F *et al.* Poly (ethylene glycol)-human serum albumin–paclitaxel conjugates: preparation, characterization and pharmacokinetics. *J Control Release* 2001; 76: 107–117.
10. Liang HF *et al.* Paclitaxel loaded poly (γ -glutamic acid)-poly (lactide) nanoparticles as a targeted drug delivery system

against cultured HepG-2 cells. *Bioconj Chem* 2006; 17: 291–299.
11. Rensen PC *et al*. Selective liver targeting of antivirals by recombinant chylomicrons – a new therapeutic approach to hepatitis B. *Nature Med* 1995; 1: 221–225.
12. Rensen PC *et al*. Particle size determines the emulsion for the LDL receptor versus hepatic remnant receptor *in vivo*. *J Lipid Res* 1997; 38:1070–1084.
13. Managit C *et al*. Uptake characteristics of galactosylated emulsion by HepG2 hepatoma cells. *Int J Pharm* 2005; 301: 255–261.
14. Dierling AM, Zhengrong C. Targeting primaquine into liver using chylomicron emulsions for potential vivax malaria therapy. *Int J Pharm* 2005; 303: 143–152.
15. Song C *et al.* Arterial uptake of biodegradable nanoparticles for intravascular local drug delivery: results with an acute dog model. *J Control Release* 1998; 54: 201–211.
16. D'souza SS, DeLuca PP. Development of a dialysis *in vitro* release method for biodegradable microspheres. *AAPS Pharmaceutical Science Technol* 2005; 6: E323–E328.
17. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity. *J Immunol Methods* 1983; 65: 55–63.
18. Wang JJ *et al*. Submicron lipid emulsion as a drug delivery system for nalbuphine and its prodrugs. *J Control Release* 2006; 115: 140–149.
19. Nicolaou KG *et al*. Chemistry and biology of taxol. *Angew Chem Int Ed Engl* 1994; 33: 12–44.
20. Gao Z *et al*. PEG-E/Phosphatidylcholine mixed immunomicelles specifically deliver encapsulated taxol to tumor cells of different origin and promote their efficient killing. *J Drug Target* 2003; 11: 87–92.
21. Lee IH *et al*. Stable paclitaxel formulations in oily contrast medium. *J Control Release* 2005; 102: 415–425.
22. Hashida M *et al*. Cell-specific delivery of genes with glycosylated carriers. *Adv Drug Deliv Rev* 2001; 52: 187–196.
23. Kan P *et al*. Development of nonionic surfactant / phospholipid o/w emulsion as a paclitaxel delivery system. *J Control Release* 1999; 58: 271–278.
24. Tamilvanan S. Oil-in-water lipid emulsions: implications for parenteral and ocular delivering systems. *Prog Lipid Res* 2004; 43: 489–533.
25. Lapidot Y *et al*. Use of esters of *N*-hydroxysuccinimide in the synthesis of *N*-acylamino acids. *J Lipid Res* 1967; 8: 142–145.
26. Chansri N *et al*. Inhibition of liver metastasis by all-*trans* retinoic acid incorporated into O/W emulsions in mice. *Int J Pharm* 2006; 321: 42–49.
27. Maeda H. The enhanced permeability and retention (EPR) effect in tumor vasculature: the key role of tumor-selective macromolecular drug targeting. *Adv Enzyme Regul* 2001; 41:189–207.
28. Wall DA *et al*. The galactose-specific recognition system of mammalian liver: the route of ligand internalization in rat hepatocytes. *Cell* 1980; 21: 79–93.
29. Rensen PC *et al*. Design and synthesis of novel N-acetylgalactosamine-terminated glycolipids for targeting of lipoproteins to the hepatic asialoglycoprotein receptor. *J Med Chem* 2004; 47: 5798–5808.
30. Gelderblon H *et al*. Cremophor EL: the drawbacks and advantages of vehicle selection for drug formulation. *Eur J Cancer* 2001; 37: 1590–1598.