International Journal of Pharmaceutics 367 (2009) 187–194

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Formulation and pharmacokinetics of lipid nanoparticles of a chemically sensitive nitrogen mustard derivative: Chlorambucil

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ARTICLE INFO

Article history:
Received 25 March 2008
Received in revised form
13 September 2008
Accepted 16 September 2008
Available online 26 September 2008

Keywords:
Chlorambucil
Solid lipid nanoparticles
Lipid nanoparticles
Dimethyl dioctadecyl ammonium bromide
Pharmacokinetics
Stability

ABSTRACT

Lipid nanoparticles of the cancer drug Chlorambucil (CLB) were prepared by ultrasonication, using stearic acid as the core lipid. Four types of lipid nanoparticle formulations were studied: (i) stearic acid solid lipid nanoparticles (SLN); (ii) sterically stabilized SLN with pegylated phospholipids as stabilizer; (iii) nanostructured lipid complexes with oleic acid as adjunct lipid; (iv) lipid nanocomplexes with dimethyl dioctadecyl ammonium bromide (DDAB) as surface modifier (LN). Lipid nanoparticles were characterized for particle size, assay and encapsulation efficiency, particle morphology and physico-chemical stability over 90 days. All of the formulations were physically stable, with an average particle size of 147 (±10) nm. The drug encapsulation efficiency (DEE) of all the formulations except LN decreased significantly over time ($p < 0.05$), probably due to the expulsion of CLB upon crystallization. This indicated that the presence of DDAB in stearic acid nanoparticles increases DEE, preventing CLB degradation in the aqueous disperse phase. Pharmacokinetic studies of the intravenous LN formulation revealed plasma clearance kinetics were comparable to that of CLB solution ($p > 0.01$), indicating electrostatic charge mediated clearance, as reported earlier. In tissue and tumor distribution studies, lower AUC values of CLB were observed for LN compared to CLB solution in liver, kidneys, heart and lungs. However, higher AUC values of LN formulation as compared to CLB solution ($p < 0.01$) in tumors suggested that the presence of DDAB on the lipid nanoparticles resulted in greater accumulation of the drug in tumors.

1. Introduction

Nanotechnology-based drug delivery systems (NBDDS) for anticancer therapy are designed to alter the unfavorable properties of 'free' drug molecules (anticancer agents), such as poor specificity and high toxicity. Over the past decade solid lipid nanoparticles (SLN) have emerged as potential NBDDS for delivery of anticancer drugs. The advantages offered by SLN include ease of scale up, production without organic solvents, use of GRAS listed excipients and a wide spectrum of application (dermal, oral, intravenous) (Mehnert and Mader, 2001; Muller and Keck, 2004; Muller et al., 2000, 2002b). Moreover, most of the anticancer drugs are lipophilic and SLN can be used to deliver such drugs more effectively. To enhance the selectivity of anticancer agents, newer generation SLN such as polymer–lipid hybrid nanoparticles, nanolipid complexes and long circulating SLN have been developed (Wong et al., 2007). There are many published reports where SLN have been utilized for encapsulation of anticancer drugs such as camptothecin (Yang et al., 1999), doxorubicin (Fundaro et al., 2000), etoposide (Harivardhan Reddy et al., 2005) and paclitaxel (Lee et al., 2007; Yuan et al., 2008).

SLN made from solid lipids show drug expulsion after long term storage, resulting in low drug encapsulation efficiency (Jores et al., 2004). Drug expulsion occurs due to the tendency of the lipid to form perfect crystals on re-crystallization (Muller et al., 2002a). Use of either complex lipids (with less ordered structure such as compritol 888 ATO) or a mixture of solid lipid and oil (nanolipid complexes) has been suggested to overcome the drug expulsion and drug loading problems of SLN. The idea behind these approaches is that complex lipids have inbuilt imperfections (being a triglyceride with different fatty acids) in their lattice structure, and inclusion of oil with a solid lipid can create separation in the fatty acid chain packing, making more room for the drug (Saupe et al., 2005).

The aim of the present study was to determine the efficiency of solid lipid nanoparticles to encapsulate a poorly water soluble drug, Chlorambucil (CLB) (4-[bis(2-chlorethyl)amino]benzenebutanoic acid), which is a nitrogen mustard derivative and a quite widely used antineoplastic drug (Fig. 1). CLB is a DNA alkylating agent, with a low melting point (64–66 °C) and high solubility in lipids (The Merck Index, 2006). Despite its low solubility in water, it undergoes hydrolysis in aqueous media (Bosanquet and Clarke, 1986; Owen and Stewart, 1979). This hydrolysis is affected by several factors including pH and the ionic species present in the vicinity of the CLB molecule (Chatterji et al., 1982; Ehrsson et al., 1980).

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doi:10.1016/j.ijpharm.2008.09.032

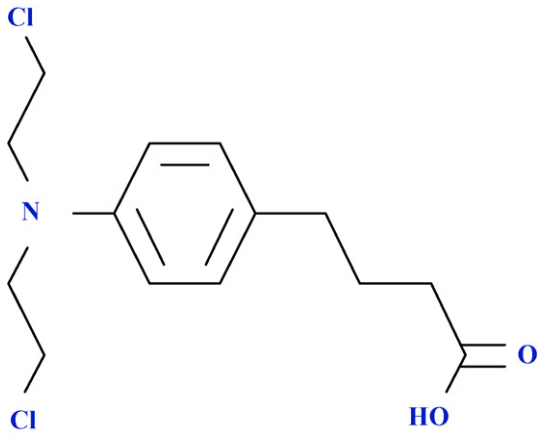

Fig. 1. Structure of Chlorambucil (4-[bis(2chlorethyl)amino]benzenebutanoic acid).

The chemical instability of CLB is a major limitation in achieving the optimum therapeutic performance (Salmaso et al., 2007). Due to these reasons, encapsulating CLB in a solid lipid based formulation has the potential to enhance both its stability and tumor specificity. In the current study, selection of the core lipid for preparation of solid lipid nanoparticles was based on the solubility of CLB in the different lipids available (discussed later). Developed lipid nanoparticles were evaluated for physico-chemical properties, physical and chemical stability and their pharmacokinetics and biodistribution after intravenous administration in mice. The pharmacokinetics and biodistribution of the stable lipid nanoparticle formulation were compared to that of CLB solution (CLB-Sol).

2. Materials

Chlorambucil (CLB), praziquantel (internal standard, I.S.), stearic acid, Tween 60, tyloxapol and oleic acid were purchased from Sigma Chemicals (USA). Distearoyl phosphatidyl ethanolamine polyethylene glycol 2000, poloxamer F 68, sodium glycocholate, precirol ATO 5, compritol ATO 888, dynasan 114, glyceryl monostearate were received as gift samples from Lipoid GmbH (Germany), BASF (USA), New Zealand Pharmaceuticals Limited (New Zealand), Gattefossé (France) and Sasol (Germany), respectively. All the reagents used in mobile phase preparation for HPLC were of AR grade and solvents were of HPLC grade. Water used in the preparation of formulation and buffers was obtained by reverse osmosis (MilliQ unit) of demineralized water.

3. Methods

3.1. Selection of lipid

Lipid selection was based on the solubility of CLB to give a visually clear solution in lipid melt under normal light when seen with the naked eye Shah et al., 2007. The lipids used for this study were compritol 888 ATO, precirol ATO 5, dynasan 114, glyceryl monostearate and stearic acid. CLB (10 mg) and varying quantities of selected lipids in 10 ml glass vials were heated 5 °C above the melting point of the lipid in a controlled temperature water bath (Water Circulator, Julabo, GmbH, Germany). After melting, the solubility of the CLB was observed visually in the melt.

3.2. Preparative technique

Lipid nanoparticles were prepared by the controlled temperature probe sonication technique. An accurately weighed amount of lipid (5.0 g)/CLB (0.010 g) mixture was placed in a standard 20 ml glass vial, in a stainless steel cylindrical jacketed (in house) assembly whose temperature was controlled by a circulating water bath (Water Circulator, Julabo, GmbH, Germany) at 75 °C. When the lipid/CLB mixture was melted to give a clear solution, a hot (75 °C) surfactant solution (5 ml) as the aqueous phase was added under ultrahigh sonication, using a probe sonicator (Ultrasonic Processor, Hielscher, Germany). The resulting hot emulsion was always filtered through 0.2 μm filter and then cooled much below the crystallization temperature of the lipid (up to 20 °C) to give lipid nanoparticles. Immediately after preparation there was always an increase in the volume (5.2 ml) of the formulation (due to the presence of melted oil droplets and high temperature). However, after filtration, cooling and keeping the formulation for 1 h at 25 °C, the volume of the formulation was always 5 (±0.1) ml. The amplitude and time of ultrasonication was optimized based on the particle size required and the gel formation propensity of the final formulation.

Four types of nanoparticles were prepared; solid lipid nanoparticles or SLN, pegylated solid lipid nanoparticles or PEG-SLN [using Distearoylphosphatidylethanolamine Polyethylene Glycol 2000 (PEG_{2000}DSPE with a stearic acid domain)], nanolipid complexes or NLC (using oleic acid as adjunct lipid) and lipid nanoplexes or LN [using dimethyl distearyl ammonium bromide (DDAB)]. The lipid concentration (stearic acid) was kept constant at 5% (w/v) in a 5 ml batch size. In LN formulations, 1% composition of the lipid was also tested. In all formulations, CLB was mixed with the lipid phase. In case of PEG-SLN, PEG_{2000}DSPE was added to the lipid phase. Compositions of SLN, PEG-SLN, NLC and LN are shown in Table 1. Different concentrations and mix of lipids, surfactant/cosurfactant and CLB were studied for their effect on particle size and the physical nature of the final formulation. Only final composition of individual formulation is mentioned in Table 1.

3.3. Analytical techniques

3.3.1. Evaluation of particle size and zeta potential

The particle size analysis of lipid nanoparticle formulations was performed by dynamic light scattering using a Malvern Hydro 2000SM particle size analyzer (Malvern Instruments, UK). The formulations were added dropwise to the sample dispersion unit. The laser obscuration range was always maintained between 10% and 20%. As it is difficult to obtain the absolute refractive index of indi-

Table 1
Composition of different lipid formulations.

Type	SLN	NLC[a]	PEG-SLN	LN
Lipid/adjunct lipid	SA (5%)	SA/OA (5%)	SA (5%)	SA (1 and 5%)
Surfactant	Poloxamer (2%)	Tyloxapol (2%)	Poloxamer (2%)	Poloxamer (2%)
Cosurfactant	SGC (1%)	SGC (1%)	SGC (1%)	Tween 60 (1%)
Functional excipient	–	–	PEG_{2000}DSPE (0.2%)	DDAB (0.0006%)

SA: stearic acid; OA: oleic acid; SGC: sodium glycocholate; PEG_{2000}DSPE: Distearoylphosphatidylethanolamine Polyethylene Glycol 2000; DDAB: dimethyl distearyl ammonium bromide.

[a] 20% (w/w) (with respect to lipid amount) of OA was used in SA + OA mix.

vidual formulations, a refractive index value of 1.5 was used for particle size analysis. The analysis was performed in triplicate and the average values of volume distribution were used for analysis. The average particle size is expressed as *d* (0.5) (50% of the particle volume below this value). A *d* (0.9) values indicate the 90% of particle volume below a particular size. For the zeta potential, samples were diluted with double-distilled water and placed in the electrophoretic cell of the Zetasizer (Malvern Instruments, Malvern, UK).

3.3.2. Evaluation of drug entrapment efficiency and high pressure liquid chromatography (HPLC) method

Two methods were used for estimation of drug entrapment efficiency (DEE) *viz.* ultrafiltration and direct solubilization.

3.3.2.1. Ultrafiltration method. In ultrafiltration method, a formulation is membrane filtered by applying hydrostatic pressure (against the membrane) resulting in the separation of particles (depending upon the size of the filter) and clear fluid. In the present study, ultrafiltration was performed using Centrisart tubes (Sartorius, AG, Germany), which consists of a filter membrane (molecular weight cut-off 20,000 Da) at the base of the sample recovery chamber. About 1 ml of undiluted sample of CLB loaded formulation was placed in the outer chamber and the sample recovery chamber placed on top of the sample. The unit was centrifuged at 3500 rpm for 15 min. The lipid nanoparticles along with encapsulated drug remained in the outer chamber while the aqueous phase moved into the sample recovery chamber through the filter membrane. The concentration of the CLB in the aqueous phase was estimated using HPLC. The DEE (%) was estimated using the formula

$$\mathrm{DEE}(\%) = \left(\frac{W_{initial} - W_{obtained}}{W_{initial}}\right) \times 100$$

where "$W_{obtained}$" is the amount of drug in the aqueous phase calculated from HPLC and "$W_{initial}$" is the amount of drug present initially in the formulation.

3.3.2.2. Direct solubilization. The DEE of the drug was determined by measuring the concentration of CLB remaining (undegraded) in the formulation. To determine (DEE), a definite volume of formulation was taken (in triplicate) and diluted with methanol to give a final concentration of 10 μg/ml (theoretically) and analyzed by HPLC. DEE was calculated as follows:

$$\mathrm{DEE}(\%) = \left(\frac{W_{obtained}}{W_{initial}}\right) \times 100$$

where "$W_{obtained}$" is the amount of drug calculated from HPLC and "$W_{initial}$" is the amount of drug present initially in the formulation. CLB and the excipients used in the study were soluble in methanol. Because CLB is unstable in aqueous surfactant solution, any decrease in the DEE is indicative of loss of drug from the lipid nanoparticles.

A validated reverse phase HPLC method was used for the analysis of CLB in sample. An Agilent® series 1100 LC comprising of a quaternary pump, a thermostatted column compartment, online degasser, an autosampler, and an electrochemical PDA detector were used for the analysis of the drug. For instrument control, data acquisition and processing, the chromatographic system was interfaced to Agilent Chemstation® LC/MS software (Agilent Technologies, Germany). Chromatographic separation was performed using a Gemini analytical column (250 mm × 4.6 mm, particle size 5 μm) from Phenomenox, USA and a C18 precolumn of the same packing (12.5 mm × 4.6 mm). Mobile phase comprising of acetonitrile and 0.2% dilute acetic acid solution was used in a composition of 65:35 (v/v) at an isocratic flow rate of 1 ml/min. Before use, the mobile phase was always filtered through 0.45 μm nylon filters (Millipore, USA). The volume of samples injected was 50 μl and analysis was carried out at a wavelength of 258 nm. Auto sampler temperature was maintained at 10 °C. The HPLC method was validated for linearity (from 0.1 to 30 μg/ml), specificity (by peak purity and resolution), accuracy (at 0.1, 15 and 30 μg/ml) and precision. The HPLC method was found to be linear in the range of 0.1–30 μg/ml ($R^2 > 0.999$). Intraday variability of quality control standard at 0.1, 15 and 30 μg/ml was 2.2%, 0.7% and 1.3% RSD, respectively. The interday variability at the above concentration was 4.5%, 1.9% and 1.3% RSD, respectively. The accuracy values at 0.1, 15 and 30 μg/ml were 5%, 1.3% and 1.0% (for intraday) and 10%, 2.6% and 1.7% (for interday) of the true values.

HPLC method used for CLB determination was stability indicating with respect to CLB peak as determined by Agilent Chemstation® software.

3.4. Physical and chemical stability

Stability studies of lipid formulations (SLN, PEG-SLN, NLC and LN) were performed for 90 days. All the stability samples were prepared in triplicate and were kept at 4–8 °C (Stability Chamber, WTC, Binder, Germany). Evaluation parameters for the stability study were selected depending upon the study objective. Particle sizing was performed for the evaluation of the physical stability of the formulation. For establishing the stability of the drug in the lipid nanoparticle formulation, DEE was evaluated using HPLC analysis. At each stability time point, samples were evaluated for particle size and DEE.

3.5. Osmolality and pH measurement

Measurement of osmolality was based on the freezing-point method as described in the user's manual (Advanced Instruments). Briefly, after calibration of the osmometer (Model 3D3, Advanced Instruments, Inc., USA) with reference standards (100 and 290 mOsm/kg, Advanced Instruments), the osmolality was recorded with 0.20 ml of sample. The osmolality of the formulation was adjusted to 300.6 ± 2.3 mOsm/kg with mannitol before intravenous administration. The pH was measured using a calibrated Mettler Toledo pH meter (Mettler-Toledo GmbH, Switzerland).

3.6. Pharmacokinetics and tissue distribution

Male mice (C57 BL/6) weighing 25–30 g were obtained from Vernon Jansen Unit, The University of Auckland, New Zealand. The animals were acclimatized for at least 1–2 weeks before experimentation, fed with standard diet and allowed for water *ad libitum*. All animal experiments were evaluated and approved by the Animal Ethics Committee, The University of Auckland, New Zealand.

A dose of 10 mg/kg of each formulation was administered via tail vein (5 ml/kg) with a 1 cm^3 Tuberculin syringe (Terumo Syringe, Laguna, Philippines) fitted with a 26 gauge needle. Control groups received the appropriate vehicles. At predetermined time points (5, 15, 30, 60 and 90 min); three mice were anaesthetized with isoflurane. Blood was collected from retro-orbital sinus into eppendorf tubes containing 7.5% sodium ethylenediamine tetraacetate solution, and centrifuged at 4500 rpm for 15 min for the isolation of the plasma.

The mice were then euthanized by cervical dislocation, and the liver, kidney, heart, and lungs were collected, washed, weighed and homogenized (Ultra-Turrax Homogenizer (IKS T10), IKA Werke GmbH & Co., Germany) in 1 ml of PBS (pH 7.4). After collection, both plasma and tissue samples were stored at −20 °C until analysis. Tumors were also collected when carrying out the pharmacoki-

netic study in tumor bearing mice and processed as mentioned above.

3.6.1. Preparation of CLB solution

CLB solution (CLB-Sol) was prepared according to the method reported by (Lee et al., 1986). CLB (20 mg) was dissolved in 1 ml of acidified ethanol (4.8 ml of concentrated hydrochloric acid added to 95% (v/v) ethyl alcohol in a volume of 100 ml) and diluted to 10 ml with propylene glycol/dipotassium hydrogen phosphate buffer (20 g of dipotassium hydrogen phosphate plus 450 ml propylene glycol diluting to 1 l with water for injection), final pH 7.4. Because of the poor stability of CLB solution, it was injected immediately by the i.v. route in a volume of 5 ml/kg body weight.

3.6.2. Plasma and tissue sample analysis

To determine CLB content, 5 μl of I.S. (5 μg/ml) was added to 0.1 ml of plasma or tissue homogenate. Protein precipitation was carried out by addition of 1 ml chilled acetonitrile. After vortexing for 1 min with the VX100 Labnet vortex mixer (Labnet Int., NJ, US), the samples were kept on ice for 30 min and then centrifuged at 4500 rpm for 15 min to precipitate the proteins. The supernatant was then removed to a clean test tube and vacuum dried (Labconco Corporation, Kansas, US). Residues were reconstituted with mobile phase, and 50 μl injected into the HPLC for analysis. The HPLC method previously reported (Ganta et al., 2008) was used and validated for the estimation of CLB in plasma and tissue matrix. The lowest standard (i.e., 0.1 μg/ml) on the calibration curve was identified as the lower limit of quantification as the analyte peak was identifiable and reproducible with a precision of less than 20%. A calibration curve was prepared using six calibration standards (0.1–20 μg/ml, 5 μg/ml I.S.). Intraday and interday accuracy and precision were determined by analysis of the 0.1, 10 and 20 μg/ml concentrations. The precision and accuracy of the method at each concentration was calculated as the percent relative standard deviation (% RSD). CLB extraction efficiency from plasma and tissues was determined by comparing the concentration of extracted samples (0.1, 10 and 20 μg/ml) with the unextracted standards containing the same amount of the analyte. In all the cases five replicate samples were analyzed.

3.6.3. Pharmacokinetics and tissue distribution in tumor bearing mice

Pharmacokinetics and tissue distribution of CLB were investigated in mice (C57 BL/6, male) subcutaneously inoculated with colon-38 tumor fragments from the donor mice. After the tumor volume reached 50 mm^3, mice were administered an initial 10 mg/kg dose of CLB-Sol or LN via the tail vein. The control group was treated with vehicle (without any drug). The balance of the procedure (for plasma, tissue and tumor extraction and analysis) was as described above.

3.6.4. Pharmacokinetic and statistical analysis

CLB pharmacokinetic parameters in mice were estimated using non-compartmental analysis (NCA) with WinNonlin version 5.0. The data were fitted to NCA using a weighed least square algorithm with uniform weighing. The area under the concentration–time profiles (AUC), the mean residence time (MRT), the volume of distribution at steady state (V_{ss}), total body clearance (CL) and plasma half-lives for the distribution ($t_{1/2\alpha}$) and elimination phase ($t_{1/2\beta}$) were calculated by the log-linear trapezoidal rule with extrapolation of the terminal slope to infinity by log-linear regression. The pharmacokinetic results were analyzed statistically using the Student's independent sample *t*-test and expressed as one-way *p* value. The statistical differences between the groups were evaluated by SigmaStat 3.5. In all analysis, a *p* value < 0.01 or 0.05 was considered statistically significant.

4. Results and discussion

4.1. Solubility study for selection of core lipid

For SLN formulation, solubility of drug in the lipid is a determinant of the encapsulation efficiency of lipid nanoparticles. It is expected that high lipid solubility would result in high encapsulation efficiency of the final formulation (Muller et al., 2000). To study the lipid solubility of CLB, a range of lipids (fatty acids and triglycerides) was selected. For lipids with melting point more than room temperature, solubility has to be determined in the lipid melt. Fig. 2 shows the amounts of different lipids required to solubilize 10 mg of CLB. Stearic acid had the highest potential to solubilize CLB, and has been used previously for intravenous SLN formulation (Fundaro et al., 2000). Due to these reasons, stearic acid was used for further studies.

4.2. Preparation of lipid nanoparticles and their characterization

Controlled temperature probe sonication technique involves dispersing melted lipid:drug oil globules in the hot surfactant solution by ultrahigh sonication for an optimized time, resulting in the formation of a hot oil-in-water nanoemulsion. This is then cooled (below re-crystallization temperature of the lipid) to form SLN. A general disadvantage of SLN prepared using lipids with high melting point (melting point above room temperature) is poor physical stability or gel formation/formation of macroscopic particles upon cooling (Heurtault et al., 2003). In the present study, compositions of each of the lipid nanoparticles (SLN, PEG-SLN, NLC and LN) were optimized to give good physical stability. For preparation of final formulations an optimized ultrasonication frequency (60% amplitude ~120 W energy output) was used for 4 min.

When observed after 90 days, all the formulations except LN had separated into two layers; a clear top layer (surfactant in aqueous phase) and a white bottom layer (lipid phase). Despite this phase separation, an average particle size of 146.8 (±10.1) nm was obtained for all the formulations, upon particle sizing (Fig. 3).

Zeta potentials were measured for different compositions of LN formulation. In LN formulations without CLB, zeta potential values increased (towards positive scale) from -38 ± 1.7 to -31.29 ± 3.41 mV as the concentration of DDAB was increased from 1 to 100 μM. In LN formulations with CLB, a zeta potential value of -7.04 ± 3.5 mV was observed.

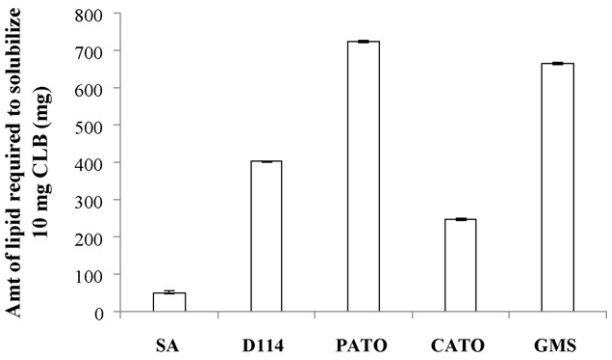

Fig. 2. Solubility of CLB in different solid lipids (SA, stearic acid; D114, Dynasan 114; PATO, Precirol ATO; CATO, Compritol ATO; GMS, glceryl monosterate). Data are mean ± SD, $n = 3$.

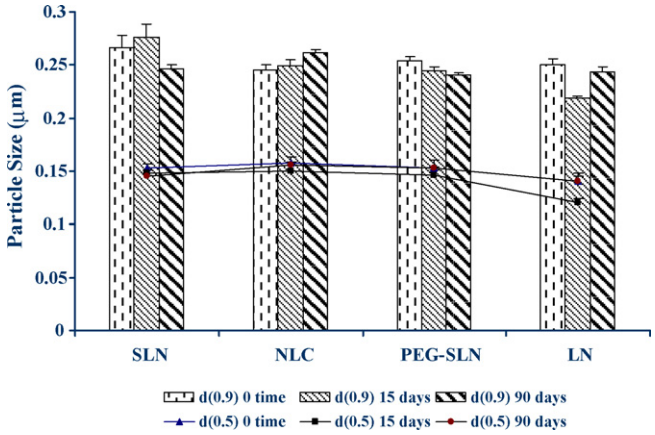

Fig. 3. Particle size of lipid nanoparticles (SLN, NLC, PEG-SLN and LN) over time. Average particle size is expressed as $d = 0.5$ (50% of the particle volume below this value) and $d = 0.9$ values indicate the 90% of particle volume below a particular size.

4.2.1. Physical stability

The physical stability of lipid nanoparticles was determined by measuring particle size of the formulations at 0, 15 and 90 days (Fig. 3). Statistically significant differences were obtained between particle sizes ($d = 0.9$) of different lipid nanoparticle formulations at each time point. The average particle size ($d = 0.5$) of the LN formulation was lower compared to other formulations. Despite the statistical difference, particle sizes in all the formulations were below 300 nm. Moreover, when individual formulations were compared, no significant difference was obtained between average particle sizes (two-way ANOVA, 95%CI) up to 90 days.

4.3. Drug encapsulation efficiency and chemical stability

As mentioned above, there is a tendency for SLN to expel encapsulated drug molecules with time, due to the re-crystallization of the lipid. During re-crystallization, the fatty acid chains of the lipid come close together (this process takes time) resulting in the formation of highly ordered state. Because the drug is present in between the fatty acid chains, the formation of a perfect crystal forces the drug molecule out of the lipid matrix resulting in less drug loading (Muller et al., 2000).

In the current study, to create separation between the fatty acid chains of stearic acid, three different excipients DDAB, PEG_{2000}DSPE and oleic acid were used. DDAB and PEG_{2000}DSPE have stearic acid group in their molecule. The hypothesis was that incorporation of DDAB and PEG_{2000}DSPE in stearic acid nanoparticles should increase the loading capacity by increasing the gaps between the fatty acid chains (Fig. 4A and B), thus making the stearic acid less crystalline. Oleic acid (*cis*-9-octadecenoic acid) is a monounsaturated fatty acid form of stearic acid. As shown in the Fig. 4C, the 'kink' present in the oleic acid structure may also create

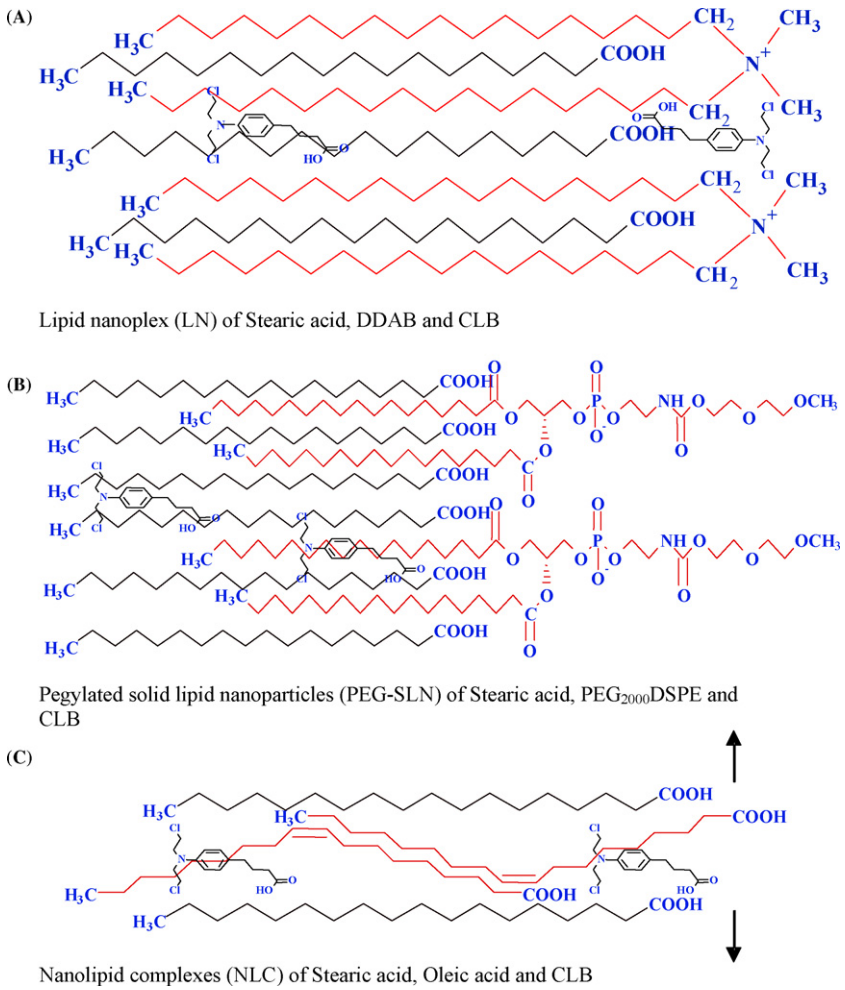

Fig. 4. Hypothetical structures of different lipid nanoparticles with stearic acid as core lipid (A) LN, (B) PEG-SLN and (C) NLC.

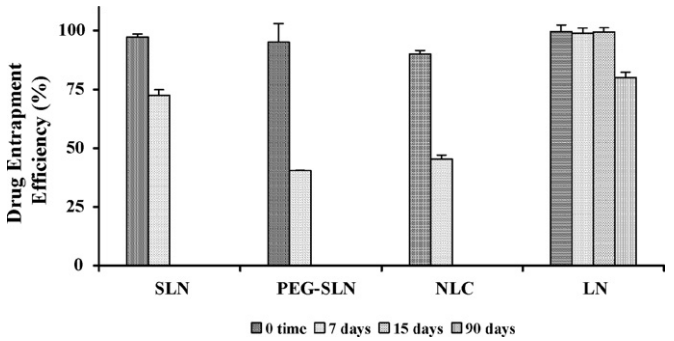

Fig. 5. Drug encapsulation efficiency (%) of CLB in lipid nanoparticles (SLN, NLC, PEG-SLN and LN). Here 0 time represent % assay content of CLB as determined by HPLC after 12 h of formulation preparation.

separation in the fatty acid chain of stearic acid, when mixed together.

The DEE values of the formulations were evaluated (using direct solubilization method) to determine the physical stability of the formulations with CLB (Fig. 5). The DEE of all the formulations except LN decreased significantly over time ($p < 0.05$). SLN, PEG-SLN and NLC showed a decrease of ~50% in CLB encapsulation within 7 days. This shows that CLB was expelled from the stearic acid core on re-crystallization, and then hydrolyzed in the outer aqueous environment. It should be noted that the ultrafiltration method showed >97% DEE, even after 90 days in all formulations, which is not acceptable. The formula used for the calculation of DEE after ultrafiltration utilizes the amount of drug present in the aqueous phase ($W_{obtained}$) in numerator. As CLB is not stable in aqueous medium, $W_{obtained}$ was always significantly less than $W_{initial}$, resulting in a higher DEE. Therefore, direct solubilization was utilized for estimation of DEE.

For PEG-SLN and NLC, the DEE did not improve upon addition of PEG_{2000}DSPE and oleic acid. This implies that either there is no separation (or an insufficient one) of the fatty acid chains of stearic acid on addition of PEG_{2000}DSPE and oleic acid, so that the latter remain separate from the stearic acid nanoparticles in aqueous surfactant solution.

There was no significant decrease ($p > 0.05$) in DEE ($99.13 \pm 1.88\%$) for LN for up to 15 days. For the same composition of the LN, removal of DDAB resulted in a significant decrease in DEE ($49.5 \pm 2.67\%$) after 15 days. Therefore, it appears that the presence of DDAB has a significant effect on the stabilization of CLB.

It should be noted that the methodology used to calculate DEE in the current study (direct solubilization) is based on the fact that CLB is a labile molecule and highly susceptible to hydrolysis in aqueous medium. As a result, CLB which is expelled out of the solid lipid and is present on the surface of the particles gets hydrolyzed by the surrounding aqueous medium in the absence of DDAB.

The pH of LN formulation is 3.6 ± 0.2. In addition, the LN formulation also contains 1% (w/v) Tween 60 in the aqueous phase which is a micelle forming surfactant. As the methodology used to calculate DEE does not differentiate between the drug that is encapsulated (by lipid) and the drug that is not encapsulated but not degraded in the aqueous medium (due to the low pH, micellization and complexation with DDAB), it is necessary to consider the effect of these factors on the rate of hydrolysis of CLB when present in the aqueous medium.

There are many studies where CLB stability has been systematically investigated as a function of pH, micellization, temperature and presence of macromolecules (Cullis et al., 1995; Ehrsson et al., 1980; Hopwood and Stock, 1971). These studies indicate that the rate of hydrolysis of CLB is dependent upon the presence of neutral aryl amino (I) group (Fig. 6). The hydrolysis proceeds through the formation of aziridinium ion (II), which has been reported to be the rate limiting step in hydrolysis. The pKa (dissociation constant) of CLB is 2.3 and in the pH range 8–3.5 the rate of hydrolysis is independent of pH. However, below pH 3.5 the rate drops due to the formation of protonated amine group (III) which inhibits the hydrolysis (Cullis et al., 1995). In addition, the rate of hydrolysis has also been reported to reduce with decrease in temperature and micelle formation (Cullis et al., 1995; Hopwood and Stock, 1971). As mentioned above, the LN formulation prepared without DDAB showed a decrease in DEE indicating that the increase in DEE upon addition of DDAB could be due to the formation of a stearic acid–CLB–DDAB complex such that the aryl amino group (present at pH 3.6) is protected from hydrolysis. This also rules out the possibility that stabilization of CLB in LN is mainly due to low temperature, pH and micellization.

DDAB is a synthetic cationic lipid that solubilizes in water above 40 °C and forms bilayer vesicles/bilayer fragments after sonication (Pacheco and Carmona-Ribeiro, 2003). This 'bilayer formation' characteristic of DDAB has been used to solubilize Amphotericin B and Miconazole, poorly water-soluble drugs (Lincopan et al., 2003; Pacheco and Carmona-Ribeiro, 2003). Being a cationic lipid, concentrations of more than 0.006% (w/v; ~100 μM) of DDAB have been shown to exert a cytotoxic effect on normal mammalian cells (Carmona-Ribeiro, 2003). Therefore in our study, the concentration of DDAB was kept at 0.0006% (w/v; ~10 μM). A stability study of LN vs. SLN indicated that addition of DDAB significantly increased the DEE. This shows that stearic acid–CLB nanoparticles are actually covered with the hydrophobic side chains of DDAB, with quaternary ammonium on the outer surface. The presence of DDAB on the surface of lipid nanoparticles containing CLB increased the zeta potential of LN to -7.04 ± 3.5 mV. In the previous studies, 1% (w/v; ~15.8 mM) of DDAB (bilayer fragments produced with tip sonication) was used to solubilize 50 mg of Amphotericin B (Lincopan et

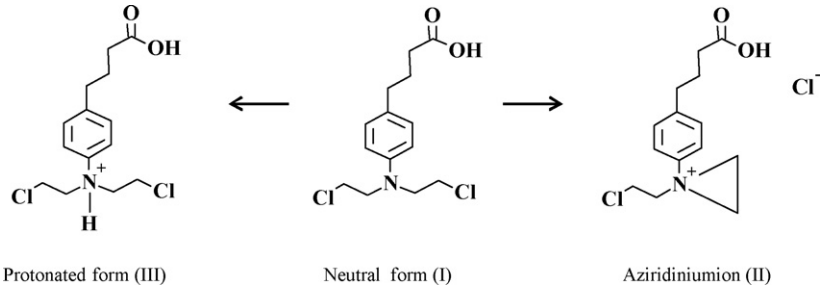

Fig. 6. Chlorambucil (CLB) has a pKa of 2.3 and its stability is dependent upon pH. In the pH range 3.5–8, CLB exists as neutral form (I) and is susceptible to hydrolysis which is mediated through the formation of aziridinium ion (II). At low pH (below 3), CLB exists in protonated form (III) which inhibits hydrolysis.

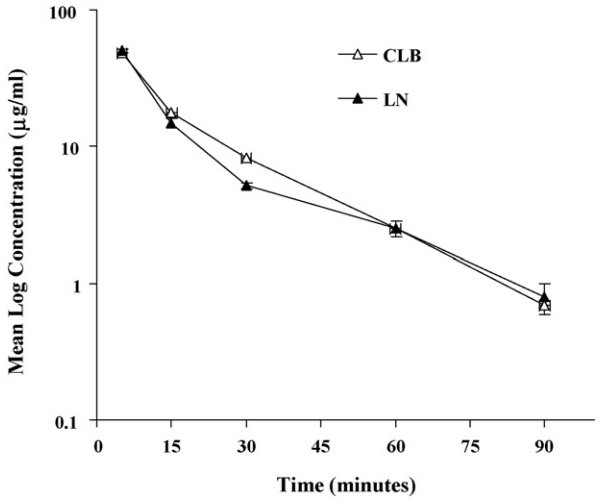

Fig. 7. Plasma log concentration–time curves for chlorambucil solution (CLB-Sol) (△) and chlorambucil loaded lipid nanocomplexes (LN) (▲) after 10 mg/kg chlorambucil i.v. in mice. Data are mean ± SD, $n = 3$ mice.

al., 2003). However in the current study, 0.0006% (w/v; ~10 μM) DDAB together with 5% (w/v) stearic acid was sufficient to stabilize 10 mg CLB.

The slow increase in zeta potential values for the LN formulations without CLB indicates that there is an adsorption of DDAB onto the surface of stearic acid nanoparticles. However, in the presence of CLB, the increase in zeta potential is more pronounced. This could be due to complexation of CLB (present on the outer surface of the particles) with DDAB side chains (solubilization effect as observed for Amphotericin B and Miconazole), such that the aryl amino group is protected from hydrolysis.

4.4. Pharmacokinetics and tissue distribution

Results of the validation showed that the HPLC method is linear in the range 0.1–20 μg/ml ($R^2 > 0.99$) with limit of quantification at 0.1 μg/ml (precision of 6.3% RSD). The CLB relative recovery values in plasma and tissues (liver, kidney, lungs, heart and tumor) were 102.39% and 84.2–97.7%, respectively, which are within the acceptable limits for biological samples.

The plasma pharmacokinetics profile of 10 mg/kg of CLB-Sol and LN containing CLB is shown in Fig. 7. Pharmacokinetic parameters as obtained from Winnonlin software are presented in Table 2. As shown in Fig. 7 and Table 2, plasma clearance kinetics are comparable for both CLB-Sol and LN. Also, the AUC value for LN is lower than that for CLB-Sol. This shows that LN is rapidly cleared from the plasma. This is in accordance with previous findings, where liposomes prepared from DDAB showed rapid plasma clearance as compared to free doxorubicin (Wu et al., 2007). The presence of DDAB in LN imparts electrostatic charge to the surface of particles (due to the presence of quaternary ammonium groups). This electrostatic charge plays a vital role in the interaction with plasma and tissue proteins, and it has been suggested to be exploitable for vascular targeting (Dass, 2003; Dass and Choong, 2006). Cationic liposomes [based on DDAB and 1,2-dioleoyl-3-trimethylammoniumpropane (DOTAP)] have previously been shown to accumulate selectively in activated tumor endothelium (Thurston et al., 1998; Wu et al., 2007). This feature of cationic liposomes makes them an attractive candidate for antivascular and antiangiogenic therapy.

Table 2
Pharmacokinetic parameters after i.v. administration of CLB-Sol and LN at a dose of 10 mg/kg of chlorambucil.

	CLB-Sol	LN
$AUC_{0-\infty}$ (μg ml^{-1} h^{-1})	16.9 ± 0.1	15.8 ± 0.1
MRT (h)	0.3 ± 0.01	0.29 ± 0.01
$t_{1/2\alpha}$ (h)	0.1 ± 0.03	0.13 ± 0.003
$t_{1/2\beta}$ (h)	0.3 ± 0.02	0.37 ± 0.05
Cl (ml h^{-} kg^{-1})	591 ± 3.5	634.2 ± 2.33
V_{ss} (ml/kg)	179.7 ± 7.1	181.42 ± 3.55

The values are shown as mean ± SD, $n = 3$.

Table 3
Comparison of normal tissue AUC of chlorambucil after i.v. administration of CLB-Sol and LN at a dose of 10 mg/kg of chlorambucil.

Tissues	$AUC_{0-\infty}$ (μg g^{-1} h^{-1})	
	CLB-Sol	LN
Liver	8.2 ± 0.2	4.6 ± 0.52
Kidney	11.1 ± 0.1	4.9 ± 0.45
Heart	10.8 ± 0.1	1.3 ± 0.13
Lungs	12.7 ± 0.2	2.2 ± 0.02
Tumor	0.49 ± 0.03	1.42 ± 0.46

Data are shown as mean ± SD, $n = 3$.
Statistically significant when AUC of LN compared with CLB-Sol in corresponding tissue at $p < 0.01$.

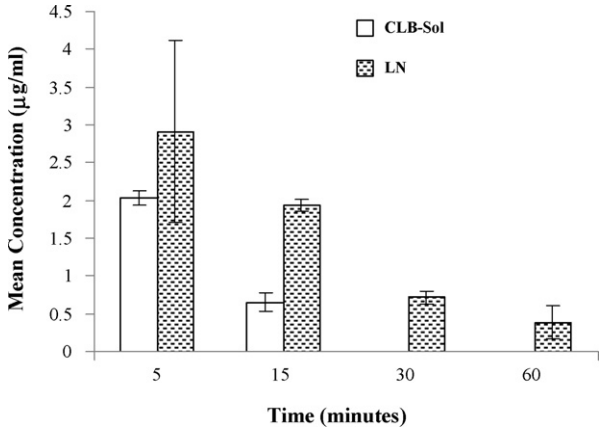

Fig. 8. Mean tumor concentration for chlorambucil solution (CLB-Sol) and chlorambucil loaded lipid nanocomplexes (LN) after 10 mg/kg chlorambucil i.v. in mice. Data are mean ± SD, $n = 3$ mice.

The CLB concentration in different tissues and tumor was determined to assess the distribution of CLB when administered as CLB-Sol (free CLB) and LN. Table 3 indicated that post administration LN was primarily accumulated in liver and kidney, similar to free CLB. However, normal tissue AUC values of LN were significantly ($p < 0.01$) reduced as compared to CLB-Sol in all the tissues. Of particular interest was the accumulation of LN in tumor (Fig. 8). After 15 min, no CLB was detected when administered as CLB-Sol. However, when administered as LN, CLB was detected up to 60 min. Moreover, AUC values for LN in tumor were significantly ($p < 0.01$) higher than CLB-Sol. This indicates a possible enhanced therapeutic activity of CLB when administered as LN formulation; however, this aspect needs further investigation.

5. Conclusion

Stearic acid–CLB–DDAB nanocomplexes (LN) showed enhanced stability (and encapsulation) over other formulations. The reason for stabilization appears to be the complexation of CLB with DDAB. This complexation is specific for some drugs (such as Amphotericin B, Miconazole and CLB) and is not applicable generally to

all drugs. Earlier studies showed that solubilization of poorly water soluble drugs such as Amphotericin B and Miconazole requires high amount of DDAB. In comparison, DDAB–CLB–stearic acid LN requires significantly less quantity of DDAB. This is probably due to the fact that some amount of CLB is already present in the stearic acid nanoparticle matrix and therefore the quantity of CLB requiring stabilization is less. Low drug encapsulation efficiency for SLN, PEG-SLN and NLC signifies the disadvantage of lipid nanoparticles based on solid lipids. The adsorption (and complexation) of DDAB on stearic acid–CLB nanoparticles also altered the pharmacokinetics and biodistribution of CLB, as indicated by rapid plasma clearance, low tissue toxicity and greater tumor accumulation of LN as compared to CLB solution.

Acknowledgements

The authors would like to thank Bruce C. Baguley, Elaine Marshall and Angela Ding, Auckland Cancer Society Research Centre, The University of Auckland, New Zealand for their support in animal experiments.

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