International Journal of Pharmaceutics 360 (2008) 115–121

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

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

Pharmacokinetics and pharmacodynamics of chlorambucil delivered in parenteral emulsion

Srinivas Ganta[a], James W. Paxton[b], Bruce C. Baguley[c], Sanjay Garg[a,*]

[a] School of Pharmacy, The University of Auckland, Private Bag 92019, Auckland, New Zealand
[b] Department of Pharmacology, The University of Auckland, Private Bag 92019, Auckland, New Zealand
[c] Auckland Cancer Society Research Centre, The University of Auckland, Private Bag 92019, Auckland, New Zealand

ARTICLE INFO

Article history:
Received 19 December 2007
Received in revised form 14 April 2008
Accepted 15 April 2008
Available online 22 April 2008

Keywords:
Chlorambucil
Parenteral emulsion
Pharmacokinetics
Tissue distribution
Anticancer activity
Colon-38 adenocarcinoma

ABSTRACT

The aim was to assess the pharmacokinetics and anticancer activity of chlorambucil (CHL) incorporated in a parenteral emulsion (PE). A chlorambucil-loaded PE was prepared by a high energy ultrasonication method. Soybean oil was chosen as a triglyceride oil core and egg phosphatidylcholine as an emulsifier in the formulation. The particle size distribution and zeta potential were measured using Zetasizer. The results showed that the average encapsulation efficiency of chlorambucil-loaded parenteral emulsion (CHL-PE) was 98.6 ± 3.2% with a particle size of 182.7 ± 0.8 nm, and a zeta-potential of −37.2 ± 1.1 mV. Osmolality and pH of the formulation were 305.6 ± 2.3 mOsm/kg and 7.4, respectively. The chlorambucil was stable in the PE for at least 6 months stored at 4–8 °C. The pharmacokinetics, tissue distribution, and anticancer activity of CHL-PE and chlorambucil solution were studied after intravenous administration to C57 BL/6 male mice. CHL-PE exhibited a significantly greater $AUC_{0-\infty}$ (32.4 ± 0.1 μg/ml h vs. 16.9 ± 0.1 μg/ml h), mean residence time (MRT) (1.32 ± 0.01 h vs. 0.30 ± 0.01 h), volume of distribution (409 ± 15 ml/kg vs. 180 ± 7 ml/kg) and elimination half-life (1.83 ± 0.1 h vs. 0.27 ± 0.02 h) (all $P < 0.01$), and a significantly reduced plasma clearance (309 ± 16 ml/(h kg) vs. 591 ± 4 ml/(h kg), $P < 0.01$) compared to the CHL. In addition CHL-PE treatment caused significantly greater tumour growth suppression rate (%T/C) of the colon-38 adenocarcinoma in the mouse compared to CHL treatment (%T/C, 75 ± 3.4% vs. 49 ± 7.4%, $P < 0.01$). These results suggest that CHL-PE could be an effective parenteral carrier for chlorambucil delivery in cancer treatment.

1. Introduction

Much attention has focused on drug delivery systems for cancer therapy which aim at tumour-specific drug delivery, thus enhancing the efficacy and reducing the toxicity of antitumour agents (Lundberg et al., 1999; Takenaga, 1996; Wu et al., 2007). Liposomes (Schiffelers et al., 2005), lipid emulsions (Collins-Gold et al., 1990; Lundberg et al., 1999; Takenaga, 1996), nanoparticles (Couvreur et al., 1990) and solid lipid nanoparticles (Zara et al., 2002) have been investigated to find ways of delivering antitumour agents to target sites. A lipid emulsion (parenteral emulsion, PE) stabilized with amphipathic lipids is an appealing alternative for a drug carrier for anticancer drug delivery (Khandavilli and Panchagnula, 2007; Kurihara et al., 1996; Patlolla and Vobalaboina, 2005; Sarker, 2005; Takenaga, 1996). The potential pharmaceutical applications include use as carrier for lipophilic drugs (Khandavilli and Panchagnula, 2007; Kurihara et al., 1996; Patlolla and Vobalaboina, 2005; Sarker, 2005) and for site-specific drug delivery by binding of ligands for various cell surface receptors to the particle surface (Lundberg et al., 1999). PE are biodegradable, biocompatible, physically stable, easy to scale up and cost effective when compared to other drug carriers, especially liposomes (Igarashi et al., 1996). Therapeutic advantages of PE have been demonstrated by a decrease in drug toxicity in contrast to other dosage forms as reported for amphotericin B (Fukui et al., 2003). PE produced from soybean oil and lecithins have also been used clinically as parenteral nutrition (Intralipid® and Lipofundin®). A PE with a mean particle diameter of 200 nm can solubilise considerable amounts of lipophilic drugs in the high volume fraction of the oil phase (liquid triglyceride) and is believed appropriate for highly lipophilic anticancer drugs.

Chlorambucil, a lipophilic anticancer agent (log *P* 1.7), has been used clinically against chronic lymphocytic leukemia, lymphomas, and advanced ovarian and breast cancers (Armitage, 1993; Galton et al., 1965). Its mechanism of action is believed to be intercalation with DNA (Hall and Tilby, 1992). However, chlorambucil use is limited by its toxic side effects, including myelotoxicity, and neuro-

* Corresponding author. Tel.: +64 9 373 7599x82836; fax: +64 9 367 7192.
E-mail address: s.garg@auckland.ac.nz (S. Garg).

0378-5173/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2008.04.027

toxicity (Dorr and Fritz, 1982). A promising approach to circumvent the toxic effects is encapsulation of chlorambucil in a suitable nano-sized tumour-targeted carrier.

The aim of this study was to investigate parenteral emulsion as a carrier for chlorambucil. We prepared a chlorambucil-loaded parenteral emulsion (CHL-PE) which was characterized for particle size, zeta potential, drug encapsulation efficiency, and long-term stability. The pharmacokinetics, tissue distribution, and anti-cancer activity of CHL and CHL-PE were investigated in C57 BL/6 mice.

2. Materials and methods

2.1. Materials

Chlorambucil (CHL), praziquantel (internal standard, I.S.), cholesterol, soybean oil, olive oil, squalene, castor oil and glycerol were purchased from the Sigma Chemicals (St. Louis, USA). Egg phosphatidylcholine (Lipoid E 80) (ePC) was kindly provided by Lipoid GmbH (Ludwigshaffen, Germany). Water for injection BP (Pfizer, Australia) was used for preparation of all aqueous solutions. All the other chemicals and reagents were of an analytical grade and were used as received.

2.2. Solubility and partitioning behaviour of chlorambucil

The solubility of CHL in the oil phase was determined according to the method of Higuchi and Connors (1965). Saturated solutions of CHL in soybean oil, olive oil, castor oil and squalene were prepared by adding excess amounts of CHL to 1 ml of oil and shaken reciprocally on the shaking water bath at $25 \pm 0.5\,^{\circ}C$ for 24 h. After centrifugation (10,000 rpm, 15 min), the supernatant was diluted with acetonitrile and assayed by high-performance liquid chromatography (HPLC).

Partitioning behaviour of CHL was studied by dissolving 10 mg CHL in 1 ml oil and thoroughly mixed with equal amount of water in a glass vial. The samples were kept undisturbed for 24 h at room temperature, and then centrifuged (10,000 rpm, 15 min) to separate the oil layer. A 50-μl oil-phase aliquot, after appropriate dilution with acetonitrile was injected into the HPLC for analysis. An appropriate calibration curve was prepared to calculate the drug concentrations from the above samples.

2.3. Preparation of the parenteral emulsion

The PE was prepared by homogenization, followed by high energy ultrasonication method. Aliquots of chlorambucil (0.2%, w/v), soybean oil (10%, w/v), ePC (1.8%, w/v), and cholesterol (0.2%, w/v) in chloroform, were dispensed into a glass vial. After evaporation of the chloroform with nitrogen, an aqueous phase (3.5 ml) consisting of glycerol (2.21%) at $50\,^{\circ}C$ was added to the resulting lipid film. After formation of a coarse emulsion with a Ultra-Turrax Homogenizer (IKA T10 basic, IKA Werke GmbH and Co., Germany) for 2 min at 6000 rpm, the latter was subjected to high energy ultrasonication (Ultrasonic processor UP200S, Hielscher, GmbH, Germany) at amplitude of 30% in continuous cycles for 20 min to form the nano-sized PE. The temperature of the sample was controlled by keeping the sample in a cold bath. Finally, the volume of the PE was adjusted to 5 ml with water, passed through 0.22-μm Minisart filters (Sartorius, AG, Germany) for filter sterilization, and then dispensed into vials and sealed. The blank PE were prepared in a similar manner but without chlorambucil.

2.4. Characterization of PE

2.4.1. Osmolality and pH measurement

Measurement of PE 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 3000 mOsm/kg, Advanced Instruments), the osmolality was recorded with 0.25 ml of sample. The osmolality of the PE was adjusted to 305.6 ± 2.3 mOsm/kg with glycerine. The pH was measured using pH meter (Mettler Toledo, Mettler-Toledo GmbH, Switzerland).

2.4.2. Characterization of particle size and zeta potential

The PE numbered average particle size and zeta potential were determined by photon correlation spectroscopy (PCS) using a Zetasizer (Malvern Instruments, Malvern, UK). The PE for particle size analysis was diluted with double-distilled water before analysis, and the average diameter and the polydispersity index (PDI) determined. For the zeta potential, PE samples were diluted with double-distilled water and placed in the electrophoretic cell of the Zetasizer.

2.4.3. Characterization of morphology

For transmission electron microscopy (TEM), the samples were placed on specimen mesh copper grids, and negatively stained with 50 μl of 1% (w/v) phosphotungstic acid for 10 min at room temperature. After draining off excess liquid with a Whatman filter paper, the grid containing the PE sample as a dry film was placed on the TEM sample holder and observed with a transmission electron microscope (JEOL 100-X transmission electron microscope, Peabody, MA) at an accelerating voltage and equipped with 20 mm aperture.

2.4.4. Drug assay and entrapment efficiency of the PE

The PE formulation was assayed for chlorambucil by diluting to 1 ml with acetonitrile and then further dilutions (0.1 ml diluted to 1 ml; approximately 10 μg/ml) were made in mobile phase. A 50-μl aliquot was injected into the HPLC for chlorambucil measurement. The encapsulation efficiency of the CHL-PE formulations was determined by an ultrafiltration method (Dipali et al., 1996) using Centrisart tubes (molecular weight cut-off 10,000 Da; Sartorius, AG, Germany). The sample was placed in the outer chamber and the unit was centrifuged at 3500 rpm for 15 min. The PE along with encapsulated drug remained in the outer chamber and aqueous phase moved into the sample recovery chamber through the filter. The concentration of the chlorambucil in the aqueous phase was estimated using HPLC. Entrapment efficiency was calculated using the formula: entrapment efficiency $(\%) = [(W_{\text{initial}} - W_{\text{obtained}})/W_{\text{initial}}] \times 100$, where W_{initial} is the amount of drug present initially in the formulation and W_{obtained} is the estimated amount from the aqueous phase of the formulation.

2.4.5. In vitro release of chlorambucil

A dialysis membrane having pore size 2.4 nm (molecular weight cut-off between 12,000 and 14,000 Da) was used for *in vitro* chlorambucil release. The membrane was soaked in distilled water overnight before placing in Franz diffusion cells (Logan Instruments Corp., USA). Receptor compartment of the Franz diffusion cells filled with phosphate-buffered saline (PBS) (Na_2HPO_4, 0.017 M; KH_2PO_4, 0.0014 M; NaCl, 0.1370 M, pH 7.4) containing 0.5% Tween 80 and maintained at $37\,^{\circ}C$. Samples were collected at predetermined time intervals and an equal volume of media was added each time after

sampling to maintain constant volume in the recipient compartment. The amount of drug in the samples was measured using HPLC.

2.4.6. Stability assessment

The drug content and particle size distribution of the PE were monitored over 6 months at 4 and 25 °C. To evaluate physical stability, PE was subjected to diluting fluids as described previously (Benita and Levy, 1993; Collins-Gold et al., 1990). PE was diluted in PBS (Na_2HPO_4, 0.017 M; KH_2PO_4, 0.0014 M; NaCl, 0.1370 M, pH 7.4), distilled water, and glycerol (2.21%, w/w) and their particle size distribution was determined using the Mastersizer, 2000 (Malvern Instruments, UK) after 1, 4, 6 and 24 h.

2.5. Preparation of chlorambucil injection

Chlorambucil injection was prepared according to the reported method (Lee et al., 1986). Chlorambucil (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. This was injected immediately into the mouse tail vein in a volume of 5 ml/kg body weight.

2.6. Pharmacokinetics and tissue distribution

Male mice (C57 BL/6) weighing 25–30 g were obtained from the 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 water *ad libitum*. All animal experiments were evaluated and approved by the Animal Ethics Committee, The University of Auckland, New Zealand. Groups of mice received either chlorambucil solution or chlorambucil-loaded PE at 10 mg/kg, or the maximum tolerated dose via the tail vein with a 1CC Tuberculin syringe fitted with a 26-gauge needle. Control groups received the appropriate vehicles. At predetermined time points (5, 15, 30 min and 1, 2, and 4 h), three mice from each group were anaesthetized with isoflurane, blood collected from the retro-orbital sinus into heparin (10 μl, 500 IU/ml) treated tubes, and centrifuged at 3500 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 and Co., Germany) in 1 ml of PBS (pH 7.4). After collection, both plasma and tissue samples were stored at −20 °C until analysis.

2.7. Plasma and tissue sample analysis

To determine chlorambucil 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 3500 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 was validated for the estimation of chlorambucil 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, 5 μg/ml I.S.). Intra-day and inter-day 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 (% R.S.D.). Chlorambucil 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.

2.8. HPLC analysis

An Agilent series LC 1100 (Agilent Corporation, Germany) consisting of a quaternary pump, an autosampler, and photodiode array detector was used for chlorambucil analysis. The LC was interfaced to Chemstation software for instrument control, data acquisition and processing. The mobile phase consisting of acetonitrile and 0.2% (v/v) acetic acid solution (65:35 (v/v)) was pumped through the 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) at a 1-ml/min flow rate. The autosampler temperature was maintained at 10 °C, and samples were analyzed at a wavelength of 258 nm (Ahmed et al., 1982).

2.9. Pharmacokinetics and statistical analysis

Chlorambucil pharmacokinetic parameters in mice were estimated using non-compartmental analysis (NCA) with WinNonlin version 5.0. The area under the concentration–time profiles (AUC), the mean residence time (MRT), the volume of distribution at steady state (V_{ss}), and total body clearance (CL) 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 calculated by SigmaStat 3.5. In all analysis, a *P*-value <0.01 or 0.05 was considered statistically significant.

2.10. Anticancer activity

Anticancer activity was investigated in mice (C57 BL/6, male) subcutaneously inoculated with colon-38 tumour fragments from the donor mice. After the tumour volume reached 50 mm^3, mice were administered an initial 10 mg/kg dose of CHL or CHL-PE via the tail vein and a second similar dose 7 days later. The control group was treated with vehicle (without any drug). The tumour diameters were measured two times a week with vernier callipers in two dimensions until tumours reached the end point (4000 mm^3) or mice survived. Individual tumour volumes (V) were calculated using the formula: $V = [\text{length (mm)} \times (\text{width (mm)})^2]/2$, where length ($L$) is the longest diameter and width (W) is the shortest diameter perpendicular to length. Tumour growth curves are presented as the mean volume relative to the values on the first day of the treatment. Percent tumour growth suppression rate (% T/C) was calculated using the formula: $\%\ T/C = [(W_c - W_t) \times 100]/W_c$, where the W_c and W_t are the mean tumour volumes of the control and test group, respectively. The % T/C was calculated on 20th day post-treatment.

3. Results

3.1. Solubility and partitioning behaviour of chlorambucil

Lipid solubility and partition coefficients of chlorambucil were determined using the HPLC method and the following calibration curve, $y = 144.12x - 16.09$ ($r^2 = 0.9999$). Chlorambucil solubility was 44.4 ± 0.6, 36.1 ± 1.1, 4.9 ± 1.2 and 6.2 ± 0.9 mg/ml in soybean oil, olive oil, castor oil, and squalene, respectively. Partition coefficients (ratio of the amount of chlorambucil in oil to the amount of chlorambucil in aqueous phase) obtained were 2657 ± 72, 2326 ± 256, 541 ± 147, and 602 ± 4 for soybean oil, olive oil, castor oil, and squalene, respectively. Chlorambucil exhibited a higher solubility and greater partition coefficient in soybean oil, and thus the latter was selected for preparation of the PE.

3.2. Preparation of PE

Methods of PE preparation have been described in detail (Benita and Levy, 1993; Lundberg, 1994; Patlolla and Vobalaboina, 2005; Seki et al., 2004). We optimized the high energy ultrasonication conditions and found that a 20-min ultrasonication (energy 30%, continuous cycle) resulted in a PE with particle size less than 200 nm. A prolonged sonication or increased energy did not improve this result as noted previously (Lundberg, 1994). In a series of initial experiments, the optimal composition of PE was evaluated with respect to emulsifier and stabilizer concentration, particle size and drug loading. The optimized PE consisted of chlorambucil (0.2%, w/v), soybean oil (10%, w/v), ePC (1.8%, w/v), cholesterol (0.2%, w/v) and glycerol (2.21%).

3.3. pH and osmolality

The pH of the PE may decrease upon storage due to the hydrolysis of phospholipids and triglycerides, and thus adjusting the initial pH to a satisfactory value (pH 7.4) is important to minimize the fatty acid formation (Hansrani et al., 1983). The required osmolality for parenteral preparations is 285–310 mOsm/kg, and the osmolality of the PE was maintained at 305.7 ± 2.3 mOsm/kg using 2.21% glycerine.

3.4. Morphology, measurement of size and zeta potential

Transmission electron micrographs of negatively stained PE showed that the particles were spherical in shape (Fig. 1). The mean size and PDI of blank PE and CHL-PE were 185 ± 1.3 and 0.13 ± 0.0, and 182.7 ± 0.8 and 0.18 ± 0.0 nm, respectively. The surface charge (zeta potential) of blank PE and CHL-PE were -38 ± 0.6 and -37.2 ± 1.1 mV, respectively.

3.5. Assay, entrapment efficiency and in vitro release

Approximately, 2.0 ± 0.1 mg/ml of CHL was present in the PE formulation. The entrapment efficiency of PE was 98.6 ± 3.2%. Due to the relative hydrophobicity of the drug (i.e., log octanol–water partition coefficient of 1.7), CHL remained in the oil phase of the PE. The *in vitro* release obtained from the PE was 8.3 ± 0.7% in 4 h.

3.6. Stability study

The stability results show that chlorambucil was stable for >6 months at 4 °C, and that the chlorambucil remained in the oil core during this period. However, the mean particle size of PE increased from 182.7 ± 0.8 to 223.6 ± 20.8. Additional stability experiments showed that PE was unaffected by diluting with distilled water, glycerol or PBS pH 7.4. The mean diameter of PE did not change significantly ($P < 0.05$) in these diluting fluids.

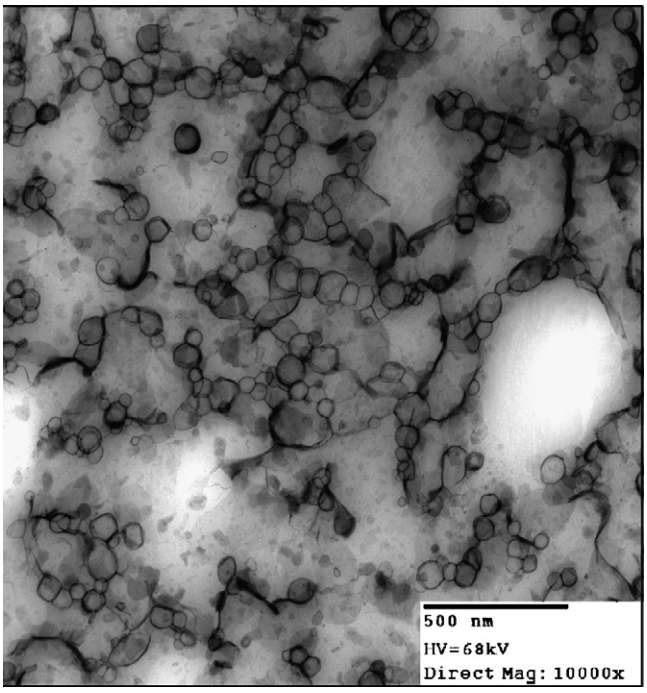

Fig. 1. The transmission electron microscopy image of phosphotungstic acid-stained parenteral emulsion.

3.7. Pharmacokinetics and tissue distribution

The validation of analytical method for chlorambucil in selected conditions shows that the chosen method is precise and accurate with linear response of 0.1–20 μg/ml. The limit of quantification was 0.1 μg/ml with a precision of 11.6%. Intra- and inter-day precision was 8.8% and 8.1%, respectively, and intra- and inter-day accuracy was ±3.7% and ±6.3% of the true values, respectively. These values were within the limits (<15%) specified for inter- and intra-day accuracy and precision. Chlorambucil relative recoveries from the plasma and tissue (liver, kidney, lungs and heart) were 96.8% and 89.8–96.9%, respectively.

The plasma log concentration–time curves for chlorambucil after the administration of CHL solution and CHL-PE are shown in Fig. 2 and calculated pharmacokinetic parameters in Table 1. The plasma $AUC_{0-\infty}$ for CHL-PE (32.4 ± 0.1 μg/ml h) was approximately twofold greater than that for CHL solution alone (16.9 ± 0.1 μg ml/h) with a significantly longer elimination half-life (1.83 ± 0.1 h after CHL-PE vs. 0.27 ± 0.02 h after CHL). Overall after CHL-PE, the total plasma chlorambucil clearance was reduced to approxi-

Table 1
Pharmacokinetic parameters after i.v. administration of CHL and CHL-PE at a dose of 10 mg/kg of chlorambucil

	CHL	CHL-PE
$AUC_{0-\infty}$ (μg/ml h)	16.9 ± 0.1	32.4 ± 0.1
MRT (h)	0.3 ± 0.01	1.3 ± 0.04
$t_{1/2\alpha}$ (h)	0.1 ± 0.03	0.3 ± 0.05
$t_{1/2\beta}$ (h)	0.3 ± 0.02	1.8 ± 0.1
Cl (ml/(h kg)	591 ± 3.5	309 ± 16
V_{ss} (ml/kg)	179.7 ± 7.1	409.1 ± 14.5

Data are shown as mean ± S.D., $n = 3$. Statistically significant when the pharmacokinetic parameters of CHL-PE compared with CHL at $P < 0.01$.

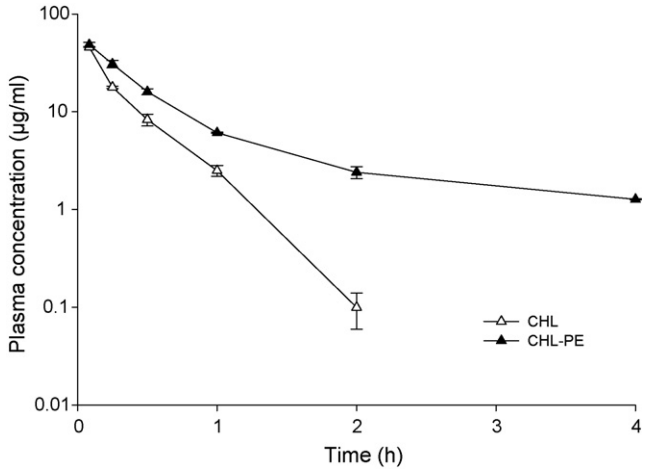

Fig. 2. Plasma log concentration–time curves for chlorambucil solution (CHL) (△) and chlorambucil-loaded parenteral emulsion (CHL-PE) (▲) after 10 mg/kg chlorambucil i.v. in mice. Data are mean ± S.D., $n = 3$ mice.

mately 50% of the value after CHL alone (309 ± 16 ml/(h kg) vs. 591 ± 4 ml/(h kg), $P < 0.01$), but with a significant increase in the volume of distribution (409 ± 15 ml/kg vs. 180 ± 7 ml/kg after CHL). Tissue (liver, kidney, heart, and lungs) concentrations of chlorambucil after the administration of CHL solution and CHL-PE are shown in Fig. 3, and AUCs reported in Table 2. Chlorambucil tissue concentrations were significantly ($P < 0.01$) lower after CHL-PE compared to after CHL solution at the initial time points (especially the 5 min time point), but by 30 min this difference had disappeared.

Table 2
Comparison of tissue AUCs of chlorambucil after i.v. administration of CHL and CHL-PE at a dose of 10 mg/kg of chlorambucil

Tissues	$AUC_{0-\infty}$ (μg/g h)	
	CHL	CHL-PE
Liver	8.2 ± 0.2	7.4 ± 0.02
Kidney	11.1 ± 0.1	7.7 ± 0.03
Heart	10.8 ± 0.1	8.2 ± 0.1
Lungs	12.7 ± 0.2	11.5 ± 0.2

Data are shown as mean ± S.D., $n = 3$. Statistically significant when AUC of CHL-PE compared with CHL in corresponding tissue at $P < 0.01$.

3.8. Antitumour activity

Evaluation of the antitumour activity of chlorambucil using a colon-38 adenocarcinoma in mice indicated a significantly ($P < 0.01$) greater tumour growth suppression rate (% T/C 74.7 ± 3.4%) with CHL-PE compared to CHL-treated mice (% T/C 48.5 ± 7.4%) (Fig. 4). In addition, the life span of tumour-bearing mice was significantly increased by CHL-PE treatment, with mice surviving over 40 days compared to 26 days with CHL treatment. Vehicle control (without any drug) did not show any effect on tumour weight and progression. Toxic effects in CHL-treated mice were evident, as three mice were removed from the study due to ulceration and severe dehydration before they reached the end point. These effects were not observed in any of the CHL-PE-treated mice. In addition, there was a 12% decrease in the body weight of CHL-treated mice over the treatment period. Tumour-bearing mice treated with CHL-PE did not show any appreciable loss in body weight over the period of this study. These results suggest that CHL-PE exhibited enhanced therapeutic efficacy and less toxicity over free CHL in this tumour model.

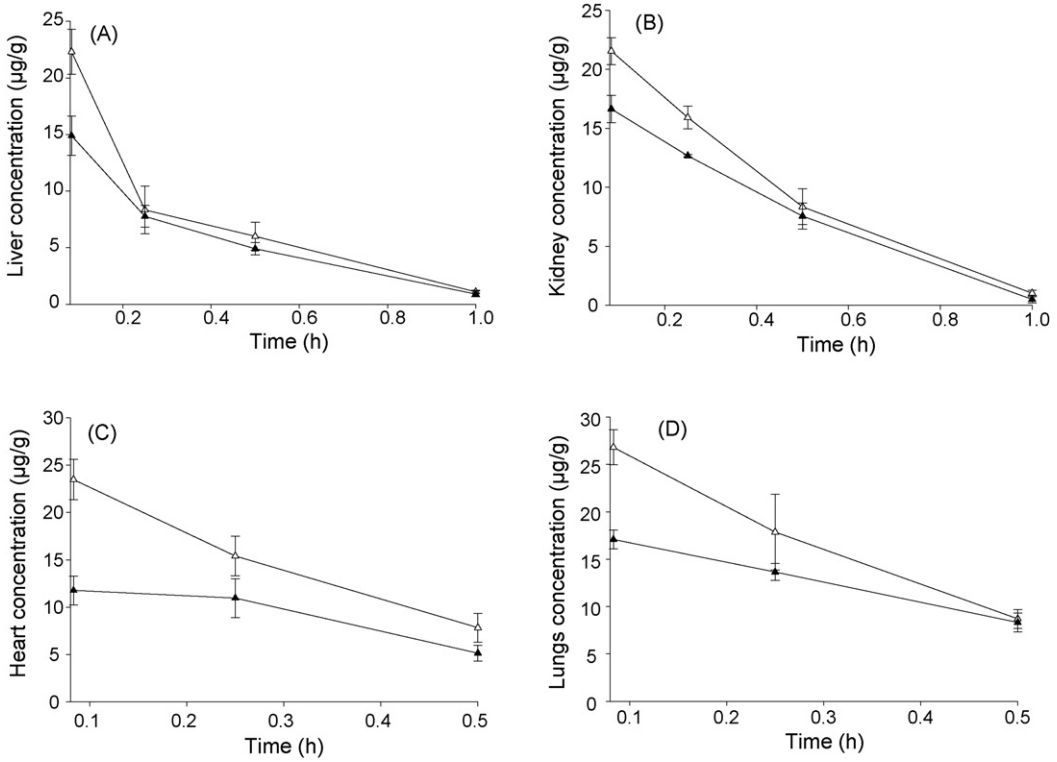

Fig. 3. Tissue distribution curves of chlorambucil solution (CHL) (△) and chlorambucil-loaded parenteral emulsion (CHL-PE) (▲) after 10 mg/kg chlorambucil i.v. in mice. (A) Liver, (B) kidney, (C) heart, and (D) lungs. Data are mean ± S.D., $n = 3$ mice.

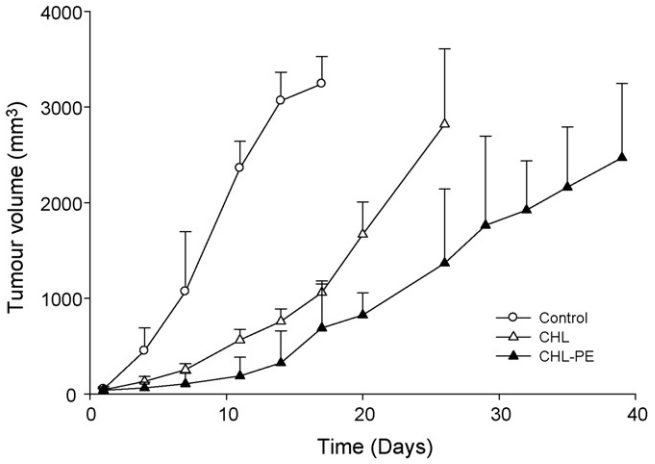

Fig. 4. Changes in tumour volume as a function of time in colon-38 adenocarcinoma-bearing mice after chlorambucil (10 mg/kg, i.v.) therapy. The legend depicts chlorambucil solution (CHL), chlorambucil-loaded parenteral emulsion (CHL-PE) and vehicle control (control). Data shown as mean ± S.D., $n = 6$.

4. Discussion

High solubility and partitioning of drug into lipid are essential for good encapsulation efficiency. Since, the rate of drug retention in lipid carriers is the function of partitioning behaviour of the drug between oil and aqueous phase. Chlorambucil exhibited a higher solubility and greater partition coefficient in soybean oil, and thus the latter was selected for preparation of the PE. The optimized PE consisted of 10% (w/v) soybean oil as an oil core, 1.8% (w/v) ePC as an amphiphilic phospholipid emulsifier and 0.2% (w/v) cholesterol as a stabilizer. Glycerol (2.21 %) was added to maintain the isotonicity of the formulation for parenteral administration. The amphiphilic phospholipids allow for stabilization of the PE due to adsorption of these agents at the oil–water interface and lowering the interfacial tension (Handa et al., 1990). At least 1.2% (w/w) of ePC is required to prepare an emulsion (Ishii et al., 1990). The use of 1.8% ePC produced a stable PE along with the cholesterol that imparts some rigidity to the monolayer. Phospholipids are weak emulsifiers (Lundberg, 1994), and therefore use of cholesterol in the formulation can enhance the stability of the PE due to formation of a more rigid monolayer with phospholipids (Patlolla and Vobalaboina, 2005). It has been reported that cholesterol can enhance the drug loading capacity of parenteral emulsions (Patlolla and Vobalaboina, 2005). However, a large amount of free cholesterol (22%) and phospholipids (23%) in the lipid emulsion can delay the lipolysis of the emulsion droplets, and may also cause rapid clearance from the blood (Maranhoo et al., 1986).

Drug encapsulation efficiency and stability are the important parameters in colloidal drug delivery systems. CHL-PE exhibited higher encapsulation efficiency (98.6 ± 3.2%) and good stability over 6 months. The size of the PE is important factors in determining its disposition. The larger colloidal particles given by an intravenous route are rapidly taken up by reticuloendothelial systems (Yamaguchi et al., 1984), whereas small particles (<400 nm) can significantly extravaste into tumour due to enhanced permeability of the tumour vasculature (Hultin et al., 1995).

The surface charge (zeta potential) of blank PE and CHL-PE were −38 ± 0.6 and −37.2 ± 1.1 mV, respectively, and is dependent on PE composition. The ePC used in the preparation of the PE contains 80% of phosphatidylcholine as the major component with other phospholipids such as phosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol and sphingomyelin as minor components. The phosphatidylcholine and phosphatidylethanolamine are neutral at the physiological pH range; whereas other phospholipids are ionized at pH 7, leading to a PE with high negative zeta potential which is responsible for the stability of PE by preventing coalescence of droplets upon random collisions (Yamaguchi et al., 1995).

Chlorambucil concentrations in the plasma declined biexponentially and were higher for CHL-PE than CHL-treated mice at all time points. Both profiles exhibited a rapid distribution phase ($t_{1/2\alpha}$) followed by a slower elimination phase ($t_{1/2\beta}$) (Table 1). The plasma $AUC_{0-\infty}$ for CHL-PE was greater than that of CHL. This was in accordance with previous findings, where lipophilic drugs given in lipid emulsions showed higher plasma concentration following i.v. injection than solution forms (Patlolla and Vobalaboina, 2005; Yamaguchi et al., 1994). The longer half-life of CHL-PE formulation was likely the result of increased volume of distribution and reduced plasma clearance. The initial lower tissue distribution of chlorambucil after CHL-PE administration was attributed to a lower uptake of CHL-PE into healthy tissues, and/or a slower drug release from this formulation in the blood. However, the leaky tumour microvasculature may allow the penetration of particles upto 400 nm in diameter (Hultin et al., 1995). It has been shown that macromolecules accumulate to a greater extent and for longer in tumour tissue compared to low molecular compounds due to enhanced permeation and retention (EPR) effect (Maeda and Matsumura, 1989). Parenteral emulsions with particle size <400 nm localize in the inflammatory area, injured vascular lesions, and tumour cells (Mizushima and Hoshi, 1993). This may be one of the reasons for improved anticancer activity of CHL-PE. Clinical use of chlorambucil is limited because of its toxicity (Dorr and Fritz, 1982). However, the improved antitumour activity and lesser toxicity obtained with CHL-PE suggest that chlorambucil can be used more effectively in this manner.

In conclusion, PE have been used as the parenteral delivery carriers for lipophilic anticancer drugs, and this has been found to be a suitable drug delivery system for tumour targeting. Chlorambucil is highly lipophilic anticancer drug and has limited clinical use due to its toxicities. A stable PE incorporating chlorambucil at high encapsulation efficiency made this formulation suitable for parenteral delivery of chlorambucil. PE has improved the pharmacokinetic profile of the chlorambucil resulting in reduced plasma clearance and a sixfold increase in the elimination half-life compared to CHL administered as a solution. Chlorambucil incorporated in PE also demonstrated increased therapeutic activity compared with CHL solution in colon-38 tumour-bearing mice. These results suggesting that the CHL-PE could be an effective parenteral carrier for chlorambucil delivery.

Acknowledgements

This work is supported by UniServices Drug Delivery Grant, The University of Auckland, New Zealand. We would like to thank Elaine Marshall, Auckland Cancer Society Research Centre, The University of Auckland, New Zealand, for her support in animal experiments.

References

Ahmed, A.E., Koenig, M., Farrish, J.H.H., 1982. Studies on the quantitation of chlorambucil in plasma by reversed-phase high-performance liquid chromatography. J. Chromatogr. B: Biomed. Appl. 233, 392–397.
Armitage, J.O., 1993. Leukaran® (Chlorambucil) in Current Clinical Guide, 2nd edition. Borroughs Wellcome Co., Oncology Products Wellcome Oncology NCM Publ., New York.
Benita, S., Levy, M.Y., 1993. Submicron emulsions as colloidal drug carriers for intravenous administration: comprehensive physicochemical characterization. J. Pharm. Sci. 82, 1069–1079.
Collins-Gold, L.C., Lyons, R.T., Bartholow, L.C., 1990. Parenteral emulsions for drug delivery. Adv. Drug Deliv. Rev. 5, 189–208.
Couvreur, P., Roblot-Treupel, L., Poupon, M.F., Brasseur, F., Puisieux, F., 1990. Nanoparticles as microcarriers for anticancer drugs. Adv. Drug Deliv. Rev. 5, 209–230.

Dipali, S.R., Kulkarni, S.B., Betageri, G.V., 1996. Comparative study of separation of non-encapsulated drug from unilamellar liposomes by various methods. J. Pharm. Pharmacol. 48, 1112–1115.
Dorr, R.T., Fritz, W.L., 1982. Cancer Chemotherapy Handbook. Elsevier Science, New York.
Fukui, H., Koike, T., Nakagawa, T., Saheki, A., Sonoke, S., Tomii, Y., Seki, J., 2003. Comparison of LNS-AmB, a novel low-dose formulation of amphotericin B with lipid nano-sphere (LNS (R)), with commercial lipid-based formulations. Int. J. Pharm. 267, 101–112.
Galton, D.A.G., Israels, L.G., Nabarro, J.D.N., Till, M., 1965. Clinical trials of *p*-di(2-chloroethylamine)–phenylbutyric acid (CB1348) in malignant lymphoma. BMJ II, 1172.
Hall, A.G., Tilby, M.J., 1992. Mechanisms of action of, and modes of resistance to, alkylating agents used in the treatment of haematological malignancies. Blood Rev. 6, 163–173.
Handa, T., Saito, H., Miyajima, K., 1990. Phospholipid monolayers at the trioleinsaline interface: production of microemulsion particles and conversion of monolayers to bilayers. Biochemistry 29, 2884–2890.
Hansrani, P.K., Davis, S.S., Groves, M.J., 1983. The preparation and properties of sterile intravenous emulsions. J. Parenter. Sci. Technol. 37, 145–150.
Higuchi, T., Connors, K.A., 1965. Phase solubility technique. Adv. Anal. Chem. Instrum. 4, 117–212.
Hultin, M., Carneheim, C., Rosenqvist, K., Olivecrona, T., 1995. Intravenous lipid emulsions: removal mechanisms as compared to chylomicrons. J. Lipid Res. 36, 2174–2184.
Igarashi, R., Takenaga, M., Matsuda, T., 1996. Distribution of lipid microsphere preparations. Adv. Drug Deliv. Rev. 20, 147–154.
Ishii, F., Sasaki, I., Ogata, H., 1990. Effect of phospholipid emulsifiers on physicochemical properties of intravenous fat emulsions and/or drug carrier emulsions. J. Pharm. Pharmacol. 42, 513–515.
Khandavilli, S., Panchagnula, R., 2007. Nanoemulsions as versatile formulations for paclitaxel delivery: peroral and dermal delivery studies in rats. J. Invest. Dermatol. 127, 154–162.
Kurihara, A., Shibayama, Y., Mizota, A., Yasuno, A., Ikeda, M., Sasagawa, K., Kobayashi, T., Hisaoka, M., 1996. Enhanced tumor delivery and antitumor activity of palmitoyl rhizoxin using stable lipid emulsions in mice. Pharm. Res. 13, 305–310.
Lee, F.Y.F., Coe, P., Workman, P., 1986. Pharmacokinetic basis for the comparative antitumour activity and toxicity of chlorambucil, phenylacetic acid mustard and β,β-difluorochlorambucil (CB 7103) in mice. Cancer Chemother. Pharmacol. 17, 21–29.
Lundberg, B., 1994. Preparation of drug–carrier emulsions stabilized with phosphatidylcholine–surfactant mixtures. J. Pharm. Sci. 83, 72–75.
Lundberg, B.B., Griffiths, G., Hansen, H.J., 1999. Conjugation of an anti-B-cell lymphoma monoclonal antibody, LL2, to long-circulating drug–carrier lipid emulsions. J. Pharm. Pharmacol. 51, 1099–1105.
Maeda, H., Matsumura, Y., 1989. Tumoritropic and lymphotropic principles of macromolecular drugs. Crit. Rev. Ther. Drug Carrier Syst. 6, 193–210.
Maranhoo, R.C., Tercyak, A.M., Redgrave, T.G., 1986. Effects of cholesterol content on the metabolism of protein free emulsion models of lipoproteins. Biochim. Biophys. Acta 875, 247–255.
Mizushima, Y., Hoshi, K., 1993. Recent advances in lipid microspheres technology for targeting prostaglandin delivery. J. Drug Target 1, 93–100.
Patlolla, R.R., Vobalaboina, V., 2005. Pharmacokinetics and tissue distribution of etoposide delivered in parenteral emulsion. J. Pharm. Sci. 94, 437–445.
Sarker, D.K., 2005. Engineering of nanoemulsions for drug delivery. Curr. Drug Deliv. 2, 297–310.
Schiffelers, R.M., Fens, M.H.A.M., Janssen, A.P.C.A., Molema, G., Storm, G., 2005. Liposomal targeting of angiogenic vasculature. Curr. Drug Deliv. 2, 363–368.
Seki, J., Sonoke, S., Saheki, A., Fukui, H., Sasaki, H., Mayumi, T., 2004. A nanometer lipid emulsion, lipid nano-sphere (LNS), as a parenteral drug carrier for passive drug targeting. Int. J. Pharm. 273, 75–83.
Takenaga, M., 1996. Application of lipid microspheres for the treatment of cancer. Adv. Drug Deliv. Rev. 20, 209–219.
Wu, J., Lee, A., Lu, Y., Lee, R.J., 2007. Vascular targeting of doxorubicin using cationic liposomes. Int. J. Pharm. 337, 329–335.
Yamaguchi, H., Watanabe, K., Hayashi, M., Awazu, S., 1984. Effect of egg yolk phospholipids plasma elimination and tissue distribution of coenzyme Q10 administered in an emulsion to rats. J. Pharm. Pharmacol. 36, 768–769.
Yamaguchi, T., Nishizaki, K., Itai, S., Hayashi, H., Ohshima, H., 1995. Physicochemical characterization of parenteral lipid emulsion: determination of haymaker constants and activation energy of coalescence. Pharm. Res. 12, 342–347.
Yamaguchi, T., Tanabe, N., Fukushima, Y., Nasu, T., Hayashi, H., 1994. Distribution of prostaglandin E1 in lipid emulsions in relation to release rate from lipid particles. Chem. Pharm. Bull. 42, 646–650.
Zara, G.P., Bargoni, A., Cavalli, R., Fundarò, A., Vighetto, D., Gasco, M.R., 2002. Pharmacokinetics and tissue distribution of idarubicin-loaded solid lipid nanoparticles after duodenal administration to rats. J. Pharm. Sci. 91, 1324–1333.