Drug Development and Industrial Pharmacy

http://informahealthcare.com/ddi
ISSN: 0363-9045 (print), 1520-5762 (electronic)

Drug Dev Ind Pharm, Early Online: 1–8
© 2014 Informa Healthcare USA, Inc. DOI: 10.3109/03639045.2014.971032

informa healthcare

Drug Development and Industrial Pharmacy Downloaded from informahealthcare.com by Michigan University on 10/17/14
For personal use only.

RESEARCH ARTICLE

Fusogenic liposomal formulation of sirolimus: improvement of drug anti-proliferative effect on human T-cells

Hadi Valizadeh[1], Saeed Ghanbarzadeh[2], and Parvin Zakeri-Milani[3]

[1]*Drug Applied Research Center and Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran,* [2]*Research Center for Pharmaceutical Nanotechnology and Student Research Committee, Tabriz University of Medical Sciences, Tabriz, Iran, and* [3]*Faculty of Pharmacy and Liver and Gastrointestinal Diseases Research Center, Tabriz University of Medical Sciences, Tabriz, Iran*

Abstract

Context: Fusogenic liposomes are unique delivery vehicles capable of introducing their contents directly and efficiently into the cytoplasm.
Objective: The objective of this study was to evaluate the potential of fusogenic liposomes containing Sirolimus to improve its anti-proliferative effect on T-lymphocyte cells.
Materials and methods: Conventional liposomes containing Sirolimus were prepared from Dipalmitoylphosphatidylcholine (DPPC) and cholesterol using the modified ethanol injection method. To prepare fusogenic liposomes, dioleoylphosphatidylethanolamine (DOPE) was added to the conventional liposome formulation. The liposomes were characterized by their size, zeta potential, encapsulation efficiency percent (EE%) and chemical stability during 6 months. The *in vitro* release of liposomes, anti-proliferative effect and liposome uptake of both types of liposomes with optimized formulations were studied on human T-lymphocyte cells employing the MTT assay and fluorescein isothiocyanate-loaded liposomes.
Results and discussion: The particle size of the liposomes was evaluated between 138 and 650 nm and mean zeta potential was in the range of −32.95 to −45.60 mV. The average EE% of the prepared conventional and fusogenic liposomes were 76.9% and 80.5%, respectively. Liposomal formulations released only 10–20% of encapsulated drug without any burst effect. *In vitro* immunosuppressive evaluation on T-cells showed that fusogenic liposomes have the best anti-proliferative effects and uptake on T-lymphocyte cell compared to the conventional liposomes.
Conclusion: Our results indicated that fusogenic liposomes can be useful carriers for improving the inhibition of T-cell proliferation.

Keywords

Anti-proliferative effect, conventional liposome, fusogenic liposome, Sirolimus, T-lymphocyte cells

History

Received 23 June 2014
Revised 3 September 2014
Accepted 26 September 2014
Published online 14 October 2014

Introduction

T-cell proliferation is a critical step in developing an effective immune response. To allow transplantation, therapeutic strategies intended to suppress the immune system through T-cell targeting and prevention of their proliferation[1,2]. Sirolimus (SRL, also known as rapamycin, C51H79NO13, CAS no: 53123-88-9), a macrocyclic lactone, was first isolated from the soil bacteria *Streptomyces hygroscopicus* by Suren Sehgal, an Indian scientist, from the soil of the Vai Atari region of Rapa Nui in 1975 (Easter Island, Chile). Although initially used as an anti-fungal agent with potent anti-candidiasis activity, subsequent studies revealed remarkable anti-tumor and immunosuppressive activities[1–3]. Sirilimus binds to the immunophilin, FK Binding Protein-12 (FKBP-12), to generate an immunosuppressive complex. This complex binds to and inhibits the activation of the mammalian Target Of Rapamycin (mTOR), a key regulatory kinase. mTOR inhibition prevents cell cycle progression from G1 to S phase in T-cells and as a result, T-cell proliferation. Inhibition of mTOR is a different mechanism of action from that of calcineurin-inhibiting agents such as cyclosporine. T-cell proliferation is regulated by cell surface receptor molecules as stimulated by CD28 and cytokine. The immunosuppressive properties of SRL are due primarily to blockage of interleukin-2 (IL-2)-induced proliferation of T-cells. The IL-2 can provide the signals necessary for T-cell cycle progression and differentiation. The IL-2 receptor can be targeted by blocking antibodies in order to aid in transplantation and prevent rejection of the allograft. In addition to its effects on T cell activity, SRL inhibits IL-2 dependent and independent proliferation of purified normal human B lymphocytes stimulation[4–8]. However, the activity of SRL is not limited to the immune system cells. Likewise, it has been shown that it affects the proliferation of non-lymphoid tumor cells, and smooth muscle cells stimulated with basic fibroblast growth factor, or platelet derived growth factor. SRL also inhibits the proliferation of hepatocytes and fibroblasts at higher

Address for correspondence: Parvin Zakeri-Milani, Faculty of Pharmacy and Liver and Gastrointestinal Diseases Research Center, Tabriz University of Medical Sciences, Tabriz, Iran. Tel: +98 (411) 339-2593. Fax: +98 (411) 334-4798. E-mail: Pzakeri@tbzmed.ac.ir

RIGHTSLINK

concentrations than in lymphoid cells. Transplantation has become a standard treatment option in patients with end-stage renal disease as it enhances the life quality, and dramatically reduces cost associated with dialysis. Patients administrating clinically approved transplantation immunosuppressive drugs suffer from dose-limiting toxic side-effects, including nephrotoxicity and neurotoxicity. SRL provides acceptable patient survival, graft survival and acute rejection rates when used as a part of first line immunosuppressive therapy for renal transplantation. SRL is currently recommended to be used in combination with cyclosporine (and corticosteroids) to reduce or prevent graft rejection by the host. Anti-proliferative effect of SRL on lymphocytes, vascular endothelial cells and renal tubular cells and its inhibition of fibrosis result in the range of toxicities observed with SRL. Sirolimus dose-related side effects include increased serum levels of cholesterol, triglycerides, and creatinine as well as decreased glomerular filtration rate. Hypertension, rash, anemia, arthralgia, diarrhea, hypokalemia, leukopenia, and thrombocytopenia also may occur[1,4,9–14]. These adverse effects can be reduced or even avoided by using novel drug delivery systems including liposomes. Sirolimus is a strong hydrophobic drug and according to the difficulties in preparing injectable formulations sirolimus is only available as oral formulations. Sirolimus bioavailability and clearance are dependent on intestinal and hepatic metabolism by cytochrome P-450 (CYP) 3A4 enzyme and transport by the multidrug efflux pump p-glycoprotein in the intestine. Its oral bioavailability is only about 17%[15–20]. Therefore, it is restrictively suitable for low-dosage treatment, such as immunosuppression in renal and liver transplant recipients. These are the potential shortcomings of sirolimus delivery by conventional dosage formulations.

Since the early 1970s, liposomes (lipid-based vesicles) have been widely studied as drug delivery systems due to their relative safety, their structural flexibility in size, composition and bilayer fluidity, and their ability to incorporate almost any molecule regardless of its structure. Liposomes can be made entirely from naturally occurring substances and are therefore non-toxic, biodegradable and non-immunogenic. Many hundreds of drugs, including anti-tumor and anti-microbial agents, chelating agents, peptide hormones, enzymes, vaccines and genetic materials, have been incorporated into the aqueous or lipid phase of liposomes of various sizes and types[21–26]. Due to their structure, chemical composition and colloidal size, all of which can be well controlled by preparation methods, liposomes exhibit several properties which may be useful in various applications. These properties point to several possible applications of liposomes as the solubilizers for difficult-to-dissolve substances, dispersants, sustained release systems, delivery systems for the encapsulated substances, stabilizers, protective agents and microencapsulation systems. Liposomes composition and properties can be finely modulated to improve their interaction with and/or penetration through cell membranes. The phospholipids by which liposomes are conventionally made, lead to the formation of lipid bilayers that closely resemble the cell and biological membranes. This allows an easy and deep interaction of these drug carriers with the cells and an improved release of the drugs encapsulated in them[27–34]. The interactions between the liposomal vesicles and cells can occur via one or more processes, such as stable physical adsorption, endocytosis, lipid exchange and fusion. The fusion event is attractive because it opens the possibility of easy release of the encapsulated drugs into the cytoplasm or cell organelles, get away from the endocytotic pathways, which is potentially dangerous for the stability of drugs. An unusual class of phospholipid vesicles is known as ''fusogenic'' liposomes. In general, their bilayers show an enhanced ability of interacting in their liquid crystalline phase with cell membranes, leading to lipid mix and thus the release of the vesicle content inside the cytoplasm[35–39]. Fusogenic liposomes can be produced by incorporating special lipids such as DOPE, which can promote destabilization of the bilayer and increase the vesicles fluidity and able them to promote the destabilization of biological membranes. Using these liposomes, researchers have delivered intact macromolecules such as proteins and DNA into tissue cells as well as cultured cells for targeting anti-cancer agents, cell labeling, improving anti-bacterial effect and targeting specific intracellular organelles. Liposomes made of DOPE mixed with other lipid components, such as cholesterol, are able to release their load into the cytoplasm upon a brief contact with eukaryotic cells.

Finally, other phospholipids like DPPC, is necessarily required to produce stable vesicles, since DOPE alone would form an inverted hexagonal phase instead of a lamellar phase[35,40–43]. Sirolimus should be bound to FKBP-12 and made immunosuppressive complex to inhibit the activation of the mTOR. Then, it is well worth delivering SRL directly into the cells to initiate its therapeutic effect. Therefore, application of fusogenic liposome type to deliver SRL inside the cells seems to be an interesting idea. For that reason, in the present study we employed DOPE to enhance the anti-proliferative effect of SRL conventional liposome on the human T-cell Lymphocytes.

Materials and methods

Materials

Sirolimus was purchased from Poli Pharmaceuticals (Lazio, Italy). Cholesterol (Chol) was obtained from Merck Company (Darmstadt, Germany). Dipalmitoylphosphatidylcholine (DPPC) and dioleoylphosphoethanolamine (DOPE) were purchased from Lipoid GMBH Company (Ludwigshafen, Germany). Thiazolyl blue tetrazolium bromide (MTT), L-glutamine, penicillin, streptomycin, phytohaemagglutinin (PHA) dimethyl sulfoxide and Sorenson buffer were purchased from Sigma-Aldrich (Bedford, MA). RPMI medium and fetal calf serum (FCS) were purchased from Gibco Company (NY). Ready-to-use dialyzers and membranes were used for drug release study (Harvard Apparatus, Holliston, MA). All solvents and reagents were HPLC and analytical grades and were purchased from Merck Company (Darmstadt, Germany).

Methods

Quantitative determination of sirolimus by high-performance liquid chromatography (HPLC)

An HPLC system (Beckman, FL) with a variable wavelength ultraviolet spectrophotometric detector (166 gold) set at 278 nm was used. System Gold software was used for data acquisition and system Gold nouveau software was used for data reporting and analysis. The separation was achieved using a Knauer column (C18, 5 μm, 4.6 × 150 mm). Mobile phase consisted of acetonitrile and ammonium acetate buffer (70:30, v/v %), at flow rate of 1.5 ml/min. The column temperature was kept at 54 °C. Response was linear in the range of 125–2000 ng/ml ($r^2 > 0.991$)[44].

Preparation of liposomes

A modified ethanol injection method was used for liposome preparation. After dissolving different proportion of lipids mixture and SRL in absolute ethanol, 5 ml of ethanolic solution of lipids and SRL was continually injected into 95 ml of distilled water under high-speed homogenization (10 000 rpm) at 45 °C (Heidolph, Germany). Liposomes were formed after extra 30 min stirring.

Drug Development and Industrial Pharmacy Downloaded from informahealthcare.com by Michigan University on 10/17/14
For personal use only.

RIGHTSLINK

Table 1. Formulation and characteristics of drug-loaded conventional (C1, C2 and C3) and fusogenic (F1, F2 and F3) liposomes.

Formulation code	DPPC:Chol:DOPE	Particle size (nm) mean ± SD	Zeta potential (mV) mean ± SD	Entrapment efficiency (%) mean ± SD	$D_{24\,h}$* (%)
C1	1:1:0	138.55 ± 0.07	−32.95 ± 1.34	91.1 ± 2.2	14.45 ± 2.51
C2	1:0.33:0	389.65 ± 4.45	−45.60 ± 1.35	77.9 ± 3.6	15.41 ± 2.62
C3	1:0.2:0	653.05 ± 23.33	−35.90 ± 0.28	61.8 ± 2.6	18.41 ± 2.95
F1	1:1:0.5	222.51 ± 0.28	−35.50 ± 2.40	92.0 ± 2.1	14.26 ± 3.12
F2	1:0.33:0.5	337.92 ± 18.67	−37.85 ± 0.85	82.4 ± 3.8	15.45 ± 3.64
F3	1:0.2:0.5	306.61 ± 7.64	−38.80 ± 0.07	67.1 ± 1.7	19.55 ± 2.32

*(D 24 h stands for cumulative percentage of drug release over 24 h).

Drug Development and Industrial Pharmacy Downloaded from informahealthcare.com by Michigan University on 10/17/14
For personal use only.

A 3^2 full factorial design was applied to examine the individual and combined effects of two formulation variables, each at three levels. Experimental factors and factor levels were determined based on preliminary studies. The molar ratios of DPPC/Chol and DOPE/DPPC were taken as independent variables. The encapsulation efficiency percent, particle size and cumulative release percent in 24 h were considered as dependent variables or responses. All other parameters (temperature, homogenizer speed, amount of ethanol, lipid concentration, etc.) were kept constant to minimize fluctuations. Following these preliminary screening experiments, using Minitab 15 software and response surface methodology (RSM) approach, optimization of formulation was carried out and suggested formulations based on optimization plots was prepared. Compositions of different formulations are illustrated in Table 1.

Characterization of liposomes

Particle size and zeta potential measurements

Particle size and zeta potential were measured by the dynamic light-scattering method (Zetasizer Nano ZS 90; Malvern Instruments, Worcestershire, UK) at room temperature in replicate of three by the necessary dilution of liposome suspension with distilled water.

Encapsulation efficiency

Encapsulation efficiency was measured using dynamic dialysis diffusion technique at less than glass transition temperature (Tg) under 100 rpm agitation in a water bath for 24 h to separate un-encapsulated drug. After the removal of unbound drug, liposomes were disrupted by ethanol and the remaining drug in liposome was considered as encapsulated drug. The amount of SRL was measured by our previously developed and validated HPLC method[44].

Encapsulation efficiency percent was calculated using the following equation[45,46]:

$$EE(\%) = [(C_{total} - C_{free})]/C_{total} \times 100.$$

Preparation of fluorescein isothiocyanate (FITC)-encapsulated liposomes

Fluorescein is a synthetic organic compound available as a dark orange/red powder slightly soluble in water and alcohol. It is widely used as a fluorescent tracer for many applications. Fluorescein has an absorption maximum at 494 nm and emission maximum at 521 nm (in water). One of the most used derivatives of fluorescein is FITC. FITC-labeled liposomes were prepared by the method of ethanol injection. Briefly, lipid solution in absolute ethanol was injected gently under vigorous vortexing into FITC solution (1 mM). Liposomes were then dialyzed to separate unencapsulated FITC.

In vitro release study

One milliliter of liposome suspension was placed into the dialysis chamber containing polycarbonate membranes (cut-off: 100 nm), which was then emerged in distilled water in a covered beaker with a stirring rate of 100 rpm. Preliminary studies demonstrated the adsorption of Sirolimus to cellulose acetate membrane; however, there was not any interaction between Sirolimus and polycarbonate membrane. At various time intervals (1, 2, 4. 6, 8, 12, 24 h), 2 ml of medium was withdrawn from the receiver compartment of the beaker and drug content was analyzed by the HPLC method[44]. Two milliliters of fresh prewarmed medium was replaced to maintain the fixed volume.

Physicochemical stability of liposomes

Particle size, encapsulation efficiency, zeta potential and drug concentration were determined monthly over 6 months.

Anti-proliferative studies with T-lymphocyte Cells

The MTT (3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) test was employed to evaluate lymphocyte proliferation. Human lymphocytes were isolated by Ficoll density centrifugation and seeded in triplicate in 96-well plates to make a final concentration of 2×10^4 cells/well. The T-lymphocytes cell line was cultured in RPMI-1640 media supplemented with 10% FCS, 1% glutamine, antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin), stimulated with phytohaemagglutinin and was incubated at 37 °C in a 5% CO_2-humidified incubator for 48 h. After the incubation period, the culture medium was replaced with fresh culture medium, vehicle of liposomes, blank conventional and fusogenic liposomes, SRL solution in liposome vehicles, as well as SRL conventional and fusogenic liposomes containing 25, 50 and 100 nM of SRL. Consequently, 50 µl of MTT staining solution (2 mg/ml) was added and after 4 h incubation, the purple formazan product was dissolved in 200 µl/well dimethyl sulfoxide and 25 µl/well Sorenson buffer. To evaluate the viability of the cells, the absorbance of the samples was determined at 570 nm using a plate reader (Bio Teck, Germany). The results of cell proliferation were expressed as a percentage of the T-cells inhibition (%) in the control condition without additives[43].

Uptake of FITC-encapsulated liposomes by T-cells

T-cells were incubated *in vitro* for 3 h, at 37 °C, with 200 µl FITC-loaded conventional and fusogenic liposomes. Blank liposomes were used as positive control. There were four examination solutions as below: the first solution was FITC in water, the second one was unencapsulated liposome solution where the third and the forth solutions were FITC-labeled conversional and fusogenic liposomes, respectively, all solutions were made in 1 mM. After incubation, cells were washed two times with PBS,

RIGHTSLINK

Drug Development and Industrial Pharmacy Downloaded from informahealthcare.com by Michigan University on 10/17/14
For personal use only.

rinsed with ammonium chloride (50 mmol/l), washed again with PBS, and finally incubated at room temperature for 30 min with paraformaldehyde (4%). The composition of the prepared FITC-loaded liposomes were similar to the optimized conventional and fusogenic liposomal formulations which were used for cytotoxicity study (DPPC:Chol, 2.3:1 for conventional liposomes, and DOPE:DPPC:Chol, 0.6:2.3:1 for fusogenic liposomes). All cell uptake experiments were performed in triplicate. For microscopic examinations, cells were fixed, then washed with PBS and observed on glass slides using a Zeiss Model 410 microscope (488ex, 520em).

Statistical analysis

Data analysis was performed using Microsoft Excel software. Data were expressed as mean ± standard deviation. Student's paired *t*-test was used to evaluate significant differences of mean values. A p value <0.05 was considered to be statistically significant.

Results

Preparation and characterization of liposomes

Table 1 illustrates the composition of prepared formulations, particle size, entrapment efficiency (%), and zeta potential immediately after preparation as well as cumulative drug release after 24 h.

Results indicated that liposomes with high proportion of cholesterol had lower particle size, and decreasing the ratio of cholesterol resulted in increased particle size. Conversely liposomes with high ratio of cholesterol indicated higher EE%. Zeta potentials of conventional liposomes were negative and in the range of −32.95 to −45.60; however, the fusogenic liposomes showed a much narrower range in zeta potential than for the conventional liposomes (−35.50 to −38.80).

Physicochemical stability study of liposomes

Physical stability study of SRL liposomes was conducted for a period of 6 months. Tables 2, 3 and 4 present all the data related to liposome stability study including changes of liposome size, polydispersity index and zeta potential of liposomes.

Zeta potential values of all liposomes were in the range of −33 to −45.6. The negative charge of liposomes was predictable due to the negative charge of phospholipids used in liposome bilayer.

Drug encapsulation efficiency of liposomes was assessed monthly for 6 months. Mean initial EE% for prepared liposomes was 78.75% and average EE% of SRL in liposomal formulations after 6 months were 72.47%. The EE% was predictable due to the high lipophilicity and high solubility of SRL in phospholipids[45,47,48].

Drug-release studies from liposomes

The release study was performed over a period of 24 h. Figure 1 shows the percentage of total SRL in each formulation released during 24 h. As indicated in this illustration, not only there was no burst effect but also SRL was released from liposomes very slowly and after 24 h, the percentage of drug released was in the range of 10–20%.

Anti-proliferative study on T-lymphocytes

Anti-proliferative effect of both types of liposomal formulations, as compared to the SRL hydro ethanolic solution and corresponding blank liposomes, are shown in Figure 2. Cell viability was expressed as the ratio of the amount of formazan produced by cells treated with SRL containing formulations to those produced by control non-treated cells. Figure 2 shows the result of T-cell proliferation inhibition of SRL solution, blank liposomes of both type and liposomal formulations of two types, optimized using factorial design and response surface methodology, containing 25, 50 and 100 nM of SRL. The T-cell proliferation inhibition activity of SRL hydroethanolic solution was used as a positive control. The control empty liposomes did not show any significant T-cell proliferation inhibition activity ($p < 0.05$) compared to negative control group. T-cells treated with both types of SRL liposomes had an average ($n = 3$) two- to three-fold increase in non-viable cells at 96 h compared with hydroethanolic solution of SRL and non-viable cells increased up to 64.5% and 77.5% in the case of conventional and fusogenic liposomes containing 100 nM SRL, respectively. Among the two types of liposomal formulations, the fusogenic liposomes containing SRL had the strongest T-cell proliferation inhibition activity which was significantly ($p < 0.05$) more than SRL conventional liposomes. The capacity of phospholipid-based vesicles of fusing with cell membranes has been proposed as a way to improve the effect of sirolimus.

Uptake of FITC-encapsulated liposomes

To examine the cellular localization of the FITC-loaded conventional and fusogenic liposomes the liposomes were fluorescently labeled using water-soluble FITC[46]. FITC is very hydrophilic and is practically unable to enter cells unless via liposome endocytosis. In this study, higher penetration was achieved *in vitro* with FITC-labeled fusogenic liposomes compared with conventional liposomes as seen in Figure 3. Results showed that due to the fusogenicity of fusogenic liposomes, the intensity of fluorescence in cells treated with these liposomes was higher. On the other hand, because of the hydrophobicity of FITC, aqueous solution of FITC cannot penetrate into cells.

Table 3. Polydispersity index during 6 months storage period.

Formulation code	After preparation	1st month	3rd month	4th month	5th month	6th month
C1	0.12	0.12	0.12	0.14	0.09	0.12
C2	0.52	0.32	0.20	0.28	0.25	0.21
C3	0.55	0.52	0.92	0.54	1.00	1.00
F1	0.34	0.35	0.39	0.51	0.36	0.43
F2	0.48	0.28	0.36	0.35	0.40	0.49
F3	0.40	0.34	1.00	0.94	0.73	0.71

Table 2. Changes of liposomes size (nm) during 6 months storage period.

Formulation code	After preparation	1st month	3rd month	4th month	5th month	6th month
C1	138.5 ± 3.0	147.3 ± 1.6	147.3 ± 2.47	150.7 ± 1.4	147.7 ± 0.4	149.8 ± 1.2
C2	389.6 ± 4.4	275.8 ± 0.4	317.1 ± 0.92	329.9 ± 0.7	311.9 ± 3.9	308.1 ± 4.9
C3	653.2 ± 23.3	2838.5 ± 2274.9	2320.9 ± 2073.2	2100.0 ± 306.4	2119.5 ± 366.9	2136.5 ± 481.5
F1	222.5 ± 13.2	559.1 ± 333.7	832.5 ± 26.2	829.5 ± 85.0	1029.4 ± 91.2	881.05 ± 130.6
F2	337.9 ± 18.6	557.5 ± 632.8	490.3 ± 1441.0	1277.1 ± 161.2	701.6 ± 58.6	825.1 ± 30.1
F3	306.6 ± 7.6	631.5 ± 27.4	751.5 ± 61.6	968.2 ± 105.3	1670.6 ± 140.9	2140.2 ± 144.3

RIGHTSLINK

Table 4. Changes of liposomes zeta potential (mV) during 6 months storage period.

Formulation code	After preparation	1st month	3rd month	4th month	5th month	6th month
C1	−32.95 ± 1.34	−29.55 ± 3.18	−32.60 ± 2.83	−29.55 ± 10.11	−22.20 ± 0.71	−42.15 ± 0.64
C2	−45.60 ± 1.25	−43.95 ± 1.48	−45.95 ± 0.92	−45.00 ± 1.27	−32.45 ± 0.92	−40.05 ± 0.35
C3	−35.90 ± 0.28	−26.05 ± 0.78	−24.75 ± 1.34	−27.60 ± 0.35	−36.95 ± 3.54	−28.30 ± 4.10
F1	−35.50 ± 2.40	−40.55 ± 4.88	−38.85 ± 3.04	−40.15 ± 0.28	−27.10 ± 0.35	−33.00 ± 1.84
F2	−37.85 ± 0.85	−27.30 ± 0.21	−29.70 ± 1.34	−27.70 ± 0.28	−31.35 ± 1.34	−34.65 ± 2.83
F3	−38.80 ± 0.07	−32.55 ± 2.55	−35.55 ± 0.99	−36.30 ± 1.56	−27.35 ± 0.92	−30.70 ± 2.76

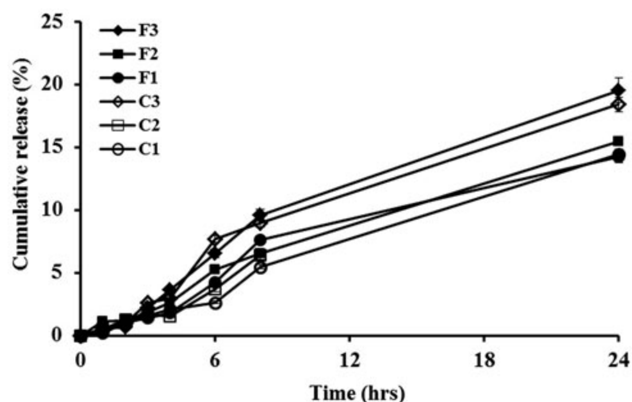

Figure 1. Cumulative percentage of drug released versus time from various liposomal SRL formulations ($n = 3$).

Discussion

Many methods are available to improve the solubility of poorly water soluble drugs such as salt formation, micronization, micellization, solid dispersion, and crystallization. For hydrophobic drugs such as Sirolimus, (Log P = 5), which can be entrapped in the hydrophobic environment of phospholipid bilayers, the liposomal formulation could be suitable as drug delivery system[49–53]. The drug to lipid ratio is very important for drug loading in liposomes for lipophilic drugs like SRL due to the restriction of drug loading into the hydrophobic region of liposomes. After preliminary experiments it was found that 5 μmol of sirolimus per 100 μmol of total lipid is the best drug to lipid ratio in the preparation of SRL liposomes and higher concentrations lead to SRL crystallization, indicating that there are excess of drug for the lipid to load in higher concentrations. DPPC is a saturated phospholipid and liposomes made of DPPC have high chemical stability due to resistance to oxidation. In preliminary studies, the various ratios of phospholipids and cholesterol were used to prepare conventional and fusogenic liposomes and finally the DPPC/Chol ratios of 1, 3 and 5 were selected to be evaluated for the effect of cholesterol on particle size, EE% and released drug (%) over 24 h. According to the results of conducted factorial design, DPPC/Chol ratio has significant effect on particle size and EE%. In low concentration of cholesterol, particle size was increased and EE% was decreased. *In vitro* release study showed that the release of SRL from liposomes was very slow and incomplete after 24 h which is in accordance with other studies[47,48,54]. Due to the high-phase transition temperature (Tg) of DPPC (41 °C) which was used as the main phospholipid in both types of prepared formulations, at 4 °C and even at room temperature, it would be in a gel-like solid state stable liposome form. However, in the *in vivo* conditions, these liposomes can destabilize and release SRL slowly. The fluidity of liposome bilayers is considered as an important factor influencing the drug release rate where by decreasing the DPPC/Chol ratio and incorporation of high amount of cholesterol into liposome formulation, the rigidity of the bilayer was increased and the drug penetration into medium was reduced. Consequently, liposomes with low concentration of cholesterol released higher amounts of SRL in 24 h. Results of release study indicated that, although $D_{24\,h}$ of fusogenic liposomes was not significantly different from corresponded conventional liposomes with the same DPPC/Chol ratio, our previous unpublished study showed that the best ratio of DOPE/DPPC in optimized formulation was 0.6. Hence, for anti-proliferative study to compare the conventional and fusogenic liposome, the ratio of 0.6 for DOPE/DPPC was used. Stability of liposome is a serious problem and at higher temperatures liposome instability is a well-established fact. Size of C1 and C2 formulations were approximately constant with low polydispersity index (0.12 and 0.21) and did not change during 6 months which is in accordance with the high zeta potential (>−40 mV) of these formulations. However, formulations C3 and F3 showed larger size increasing after 6 months (3 folds) and wide size distributions, which was revealed in the polydispersity index value (0.71–1). This is in accordance with our previous studies and indicating that with decreasing the molar ratio of Chol, particle size was decreased[46]. The mean encapsulation efficiency percent of conventional and fusogenic liposomes was reduced from 79.1 and 80.5% to 57.75 and 60.1%, respectively, which is probably due to the leakage of drug through bilayer. Results indicated that mean SRL content of conventional and fusogenic liposomes were 92.3 and 90.9% of initial SRL content. Moreover in our pervious study in lyophilized form, SRL content at 4 °C was reduced to 92.9% and 91.6% in conventional and fusogenic liposomes[45]. In the present investigation, the MTT assay, a simple, fast and inexpensive method, was used for the *in vitro* T-cell proliferation inhibition activity of the different liposomal formulations of SRL. In this method, tetrazolium salt of MTT reduces to blue-colored product (formazan) by the mitochondrial enzyme succinate dehydrogenase in live cells. Results revealed that the fusogenic liposomes have much higher inhibitory effect compared to conventional liposomes and SRL hydroethanolic solution. The main reason of this significant difference could be the higher diffusion of SRL into the cytoplasm of the T-cells when encapsulated in fusogenic liposomes. As it was mentioned the mechanism of action of SRL is blockage of interleukin-2 (IL-2)-induced proliferation of T-cells by formation of FKBP-12 complex and inhibition of the activation of the mTOR. Therefore, one proposed mechanism for T-cell proliferation inhibition effect of fusogenic liposomes containing SRL *in vitro* could be the direct fusion of this type of liposomes with the cell membrane. During this process liposomal bilayers combine with cell membrane, and thereby the liposomal content is released into the cytoplasm. This type of liposomes are suitable carriers for delivery of macromolecules, such as peptides, proteins, DNA, antigens and polysaccharides to the cell cytoplasm[35–37,39,43,55–57]. Although the improvement of SRL efficacy employing liposomal formulations on other cell lines have been reported, different liposomal compositions were

Drug Development and Industrial Pharmacy Downloaded from informahealthcare.com by Michigan University on 10/17/14
For personal use only.

RIGHTSLINK

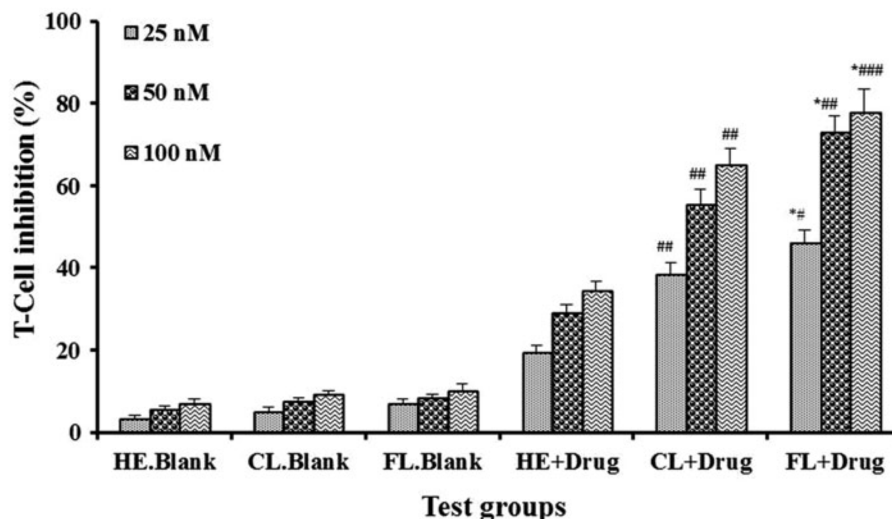

Figure 2. Anti-proliferative effects on T-lymphocyte cells of conventional and fusogenic liposomal SRL, Hydroethanol (HE) blank, blank conventional liposomes (CL.Blank), blank fusogenic liposome (FL.Blank), SRL in hydroethanol (HE + Drug) and SRL containing conventional liposome (CL + Drug) and SRL containing fusogenic liposomes (FL + Drug). (Data are the mean values of three replications, $*p < 0.05$ compared with CL + Drug and $\#p < 0.05$, $\#\#p < 0.01$ and $\#\#\#p < 0.001$ compared with HE + drug).

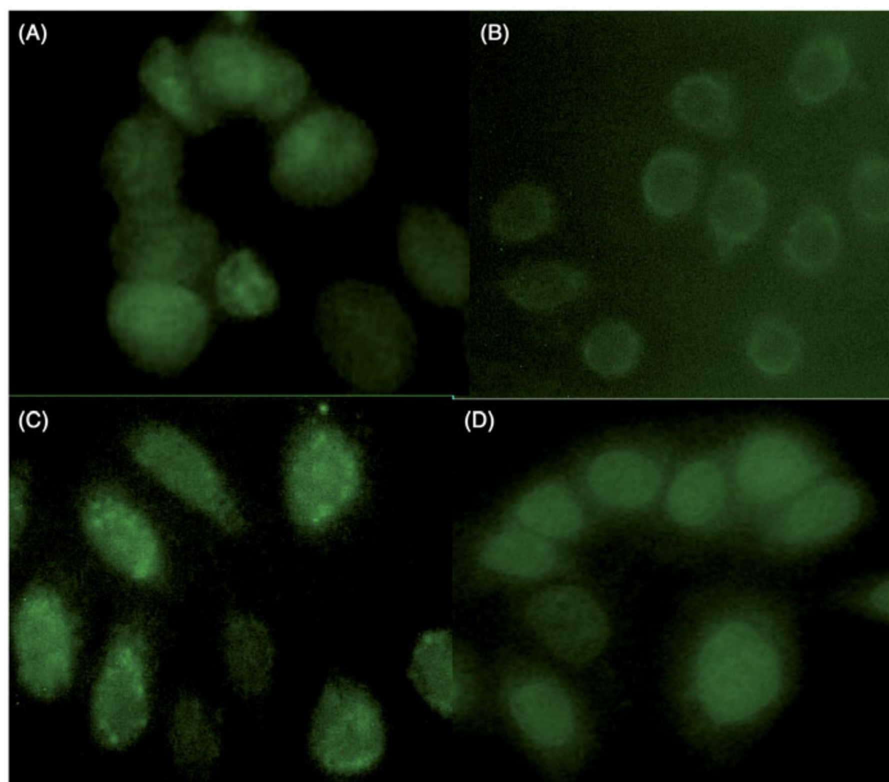

Figure 3. Uptake of aqueous solution of FITC (A) Blank (B), FITC-encapsulated conventional (C) and fusogenic (D) liposomes in T-cells.

Drug Development and Industrial Pharmacy Downloaded from informahealthcare.com by Michigan University on 10/17/14
For personal use only.

used in the previously published studies[47,48,58–63]. In the present study, significant improvement of immunosuppressive activity of SRL was achieved by the addition of DOPE, as fusogenic agent, to the formulation. Higher inhibition of T-cell proliferation was obtained in fusogenic SRL liposomal formulations compared to the conventional ones containing SRL.

Conclusions

In the present study, we used a modified ethanol injection method as a simple, rapid and reproducible method and statistical design to prepare SRL conventional and fusogenic liposomes. Prepared liposomes were characterized and were found to be stable in the case of physical parameters. *In vitro* anti-proliferative effect of optimized formulations of conventional and fusogenic liposomes were studied on human T-cell lymphocytes. It was shown that both types of liposomes had higher anti-proliferative effect on T-cells compared to sirolimus hydroethanolic solution. Our data suggest that fusogenic liposomes have the higher capacity for transmission of a lipophilic drug into the T-cells. This work recommends that the inhibition of T-cell proliferation using conventional and especially fusogenic liposomes of sirolimus could be advantageous as an immunosuppressive formulation.

Acknowledgements

The authors would like to thank the Research Center for Pharmaceutical Nanotechnology, Tabriz University of Medical Sciences for financial support. This article is based on a PhD thesis (No. 54) submitted in Faculty of Pharmacy, Tabriz University of Medical Sciences.

Declaration of interest

This article was supported by the Research Center for Pharmaceutical Nanotechnology, Tabriz University of Medical Sciences. The authors report no declarations of interest.

RIGHTSLINK

References

1. Rangan GK, Nguyen T, Mainra R, et al. Therapeutic role of sirolimus in non-transplant kidney disease. Pharmacol Ther 2009; 123:187–206.
2. Sehgal SN. Rapamune® (RAPA, rapamycin, sirolimus): mechanism of action immunosuppressive effect results from blockade of signal transduction and inhibition of cell cycle progression. Clin Biochem 1998;31:335–40.
3. Paghdal KV, Schwartz RA. Sirolimus (rapamycin): from the soil of Easter Island to a bright future. J Am Acad Dermatol 2007;57: 1046–50.
4. Costa LJ. Aspects of mTOR biology and the use of mTOR inhibitors in non-Hodgkin's lymphoma. Cancer Treat Rev 2007;33:78–84.
5. Deters M, Hütten H, Kaever V. Synergistic immunosuppressive effects of the mTOR inhibitor sirolimus and the phytochemical curcumin. Phytomedicine 2013;20:120–3.
6. Kirken RA, Wang YL. Molecular actions of sirolimus: sirolimus and mTor. Transplant Proc 2003;35:S227–30.
7. Patsenker E, Schneider V, Ledermann M, et al. Potent antifibrotic activity of mTOR inhibitors sirolimus and everolimus but not of cyclosporine A and tacrolimus in experimental liver fibrosis. J Hepatol 2011;55:388–98.
8. van Rossum HH, Romijn FPHTM, Smit NPM, et al. Everolimus and sirolimus antagonize tacrolimus based calcineurin inhibition via competition for FK-binding protein 12. Biochem Pharmacol 2009; 77:1206–12.
9. Abouelnasr A, Roy J, Cohen S, et al. Defining the role of sirolimus in the management of graft-versus-host disease: from prophylaxis to treatment. Biol Blood Marrow Transplant 2013;19:12–21.
10. Ahya VN, McShane PJ, Baz MA, et al. Increased risk of venous thromboembolism with a sirolimus-based immunosuppression regimen in lung transplantation. J Heart Lung Transpl 2011;30:175–81.
11. Meier F, Guenova E, Clasen S, et al. Significant response after treatment with the mTOR inhibitor sirolimus in combination with carboplatin and paclitaxel in metastatic melanoma patients. J Am Acad Dermatol 2009;60:863–8.
12. Mi R, Ma J, Zhang D, et al. Efficacy of combined inhibition of mTOR and ERK/MAPK pathways in treating a tuberous sclerosis complex cell model. J Genet Genom 2009;36:355–61.
13. Wagner D, Kniepeiss D, Schaffellner S, et al. Sirolimus has a potential to influent viral recurrence in HCV positive liver transplant candidates. Int Immunopharmacol 2010;10:990–3.
14. Wessely R, Schömig A, Kastrati A. Sirolimus and paclitaxel on polymer-based drug-eluting stents: similar but different. J Am Coll Cardiol 2006;47:708–14.
15. Aspeslet LJ, Yatscoff RW. Requirements for therapeutic Drug monitoring of sirolimus, an immunosuppressive agent used in renal transplantation. Clin Ther 2000;22:B86–92.
16. Hu X, Lin C, Chen D, et al. Sirolimus solid self-microemulsifying pellets: formulation development, characterization and bioavailability evaluation. Int J Pharm 2012;438:123–33.
17. Kim M-S, Kim J-S, Cho W, et al. Supersaturatable formulations for the enhanced oral absorption of sirolimus. Int J Pharm 2013;445: 108–16.
18. MacDonald A, Scarola J, Burke JT, et al. Clinical pharmacokinetics and therapeutic drug monitoring of sirolimus. Clin Ther 2000;22: B101–21.
19. Salm P, Taylor PJ, Pillans PI. The quantification of sirolimus by high-performance liquid chromatography-tandem mass spectrometry and microparticle enzyme immunoassay in renal transplant recipients. Clin Ther 2000;22:B71–85.
20. Sun M, Zhai X, Xue K, et al. Intestinal absorption and intestinal lymphatic transport of sirolimus from self-microemulsifying drug delivery systems assessed using the single-pass intestinal perfusion (SPIP) technique and a chylomicron flow blocking approach: linear correlation with oral bioavailabilities in rats. Eur J Pharm Sci 2011; 43:132–40.
21. Chang C-C, Liu D-Z, Lin S-Y, et al. Liposome encapsulation reduces cantharidin toxicity. Food Chem Toxicol 2008;46:3116–21.
22. Gücer N, Ebel J, Gröning R. Encapsulation of drugs and excipients in liposomes — measurements with drug-specific electrodes. Pharm Acta Helv 1993;68:129–33.
23. Piel G, Piette M, Barillaro V, et al. Betamethasone-in-cyclodextrin-in-liposome: the effect of cyclodextrins on encapsulation efficiency and release kinetics. Int J Pharm 2006;312:75–82.
24. Sant'Anna V, Malheiros PdS, Brandelli A. Liposome encapsulation protects bacteriocin-like substance P34 against inhibition by Maillard reaction products. Food Res Int 2011;44:326–30.
25. Shrivastava S, Lole KS, Tripathy AS, et al. Development of candidate combination vaccine for hepatitis E and hepatitis B: a liposome encapsulation approach. Vaccine 2009;27:6582–8.
26. Xu X, Khan MA, Burgess DJ. Predicting hydrophilic drug encapsulation inside unilamellar liposomes. Int J Pharm 2012;423:410–18.
27. Barenholz Y. Liposome application: problems and prospects. Curr Opin Colloid Interface Sci 2001;6:66–77.
28. Betz G, Imboden R, Imanidis G. Interaction of liposome formulations with human skin *in vitro*. Int J Pharm 2001;229:117–29.
29. Düzgüneş N, Nir S. Mechanisms and kinetics of liposome–cell interactions. Adv Drug Delivery Rev 1999;40:3–18.
30. Heath TD, Martin FJ. The development and application of protein-liposome conjugation techniques. Chem Phys Lipids 1986;40: 347–58.
31. Junzo S, Tetsurou N, Toshinori S, et al. Direct transfer of tumor surface antigenic protein (TSAP) from tumor cell to liposome for making liposomal vaccine. J Contr Rel 1992;20:143–53.
32. Meisner D, Mezei M. Liposome ocular delivery systems. Adv Drug Delivery Rev 1995;16:75–93.
33. Rooijen NV, Sanders A. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. J Immunol Meth 1994;174:83–93.
34. Van Rooijen N. The liposome-mediated macrophage 'suicide' technique. J Immunol Methods 1989;124:1–6.
35. Kono K, Iwamoto M, Nishikawa R, et al. Design of fusogenic liposomes using a poly(ethylene glycol) derivative having amino groups. J Contr Rel 2000;68:225–35.
36. Kunisawa J, Masuda T, Katayama K, et al. Fusogenic liposome delivers encapsulated nanoparticles for cytosolic controlled gene release. J Contr Rel 2005;105:344–53.
37. Kunisawa J, Nakagawa S, Mayumi T. Pharmacotherapy by intracellular delivery of drugs using fusogenic liposomes: application to vaccine development. Adv Drug Delivery Rev 2001;52:177–86.
38. Sakaguchi N, Kojima C, Harada A, et al. Enhancement of transfection activity of lipoplexes by complexation with transferrin-bearing fusogenic polymer-modified liposomes. Int J Pharm 2006;325:186–90.
39. Watabe A, Yamaguchi T, Kawanishi T, et al. Target-cell specificity of fusogenic liposomes: membrane fusion-mediated macromolecule delivery into human blood mononuclear cells. Biochim Biophys Acta 1999;1416:339–48.
40. Auguste DT, Prud'homme RK, Ahl PL, et al. Association of hydrophobically-modified poly(ethylene glycol) with fusogenic liposomes. Biochim Biophys Acta 2003;1616:184–95.
41. Hara T, Kuwasawa H, Aramaki Y, et al. Effects of fusogenic and DNA-binding amphiphilic compounds on the receptor-mediated gene transfer into hepatic cells by asialofetuin-labeled liposomes. Biochim Biophys Acta 1996;1278:51–8.
42. Koshkaryev A, Piroyan A, Torchilin VP. Bleomycin in octaarginine-modified fusogenic liposomes results in improved tumor growth inhibition. Cancer Lett 2013;334:293–301.
43. Malaekeh-Nikouei B, Jaafari MR, Tabassi SAS, et al. The enhancement of immunosuppressive effects of cyclosporine A on human T-cells using fusogenic liposomes. Colloids Surf B Biointerfaces 2008;67:238–44.
44. Islambulchilar Z, Ghanbarzadeh S, Emami S, et al. Development and validation of an HPLC method for the analysis of sirolimus in drug products. Adv Pharmaceut Bull 2012;2:135–9.
45. Ghanbarzadeh S, Valizadeh H, Zakeri-Milani P. The effects of lyophilization on the physico-chemical stability of sirolimus liposomes. Adv Pharmaceut Bull 2013;3:25–9.
46. Ghanbarzadeh S, Valizadeh H, Zakeri-Milani P. Sirolimus nano liposomes: optimization of sirolimus nano liposome prepared by modified ethanol injection method using responce suerface methodology. Pharmind 2013;75. (In press).
47. Haeri A, Sadeghian S, Rabbani S, et al. Use of remote film loading methodology to entrap sirolimus into liposomes: preparation, characterization and *in vivo* efficacy for treatment of restenosis. Int J Pharm 2011;414:16–27.
48. Rouf MA, Vural I, Renoir JM, et al. Development and characterization of liposomal formulations for rapamycin delivery and investigation of their antiproliferative effect on MCF7 cells. J Liposome Res 2009;19:322–31.

Drug Development and Industrial Pharmacy Downloaded from informahealthcare.com by Michigan University on 10/17/14
For personal use only.

RIGHTSLINK

49. Kawabata Y, Wada K, Nakatani M, et al. Formulation design for poorly water-soluble drugs based on biopharmaceutics classification system: basic approaches and practical applications. Int J Pharm 2011;420:1–10.
50. Lukyanov AN, Torchilin VP. Micelles from lipid derivatives of water-soluble polymers as delivery systems for poorly soluble drugs. Adv Drug Delivery Rev 2004;56:1273–89.
51. Mu H, Holm R, Müllertz A. Lipid-based formulations for oral administration of poorly water-soluble drugs. Int J Pharm 2013;453: 215–24.
52. Rasenack N, Hartenhauer H, Müller BW. Microcrystals for dissolution rate enhancement of poorly water-soluble drugs. Int J Pharm 2003;254:137–45.
53. Tan A, Simovic S, Davey AK, et al. Silica-lipid hybrid (SLH) microcapsules: A novel oral delivery system for poorly soluble drugs. J Contr Rel 2009;134:62–70.
54. Ghanbarzadeh S, Valizadeh H, Zakeri-Milani P. Application of response surface methodology in development of sirolimus liposomes prepared by thin film hydration technique. Bioimpact 2013;2: 75–81.
55. Dwivedi V, Vasco A, Vedi S, et al. Adjuvanticity and protective immunity of Plasmodium yoelii nigeriensis blood-stage soluble antigens encapsulated in fusogenic liposome. Vaccine 2009;27: 473–82.
56. Yoshikawa T, Imazu S, Gao J-Q, et al. Augmentation of antigen-specific immune responses using DNA-fusogenic liposome vaccine. Biochem Biophys Res Commun 2004;325:500–5.
57. Yuba E, Harada A, Sakanishi Y, et al. A liposome-based antigen delivery system using pH-sensitive fusogenic polymers for cancer immunotherapy. Biomaterials 2013;34:3042–52.
58. Phillips LC, Klibanov AL, Wamhoff BR, et al. Localized ultrasound enhances delivery of rapamycin from microbubbles to prevent smooth muscle proliferation. J Contr Rel 2011;154:42–9.
59. Alemdar AY, Sadi D, McAlister VC, et al. Liposomal formulations of tacrolimus and rapamycin increase graft survival and fiber outgrowth of dopaminergic grafts. Cell Transplant 2004;13: 263–71.
60. Fricker G, Kromp T, Wendel A, et al. Phospholipids and lipid-based formulations in oral drug delivery. Pharm Res 2010;27: 1469–86.
61. Kakegawa T, Nemoto A, Matsuda M, et al. Effect of rapamycin on NF-kappaB induction. Jpn J Antibiot 2003;56:97–9.
62. Wang XY, Miao ZL, Chen C, et al. Preparation and study on formulation of sirolimus liposomes. Chin J Antibiot 2010;35: 119–22.
63. Zhao GQ, Zhang ZH, Liang T, et al. The effect of rapamycin liposome gutta inhibiting rat corneal neovascularization. Chin J Ophthalmol 2009;45:146–52.

Drug Development and Industrial Pharmacy Downloaded from informahealthcare.com by Michigan University on 10/17/14
For personal use only.

RIGHTSLINK