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Use of remote film loading methodology to entrap sirolimus into liposomes: Preparation, characterization and in vivo efficacy for treatment of restenosis

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ABSTRACT

The main objective of this study was to formulate an effective controlled-release liposomal drug delivery system for sirolimus (SIR), a potent antiproliferative and anti-inflammatory drug, to be used for the treatment of restenosis following local vascular delivery. Liposomes were prepared using remote film loading method and characterized with regard to entrapment efficiency (EE), size distribution and zeta potential. The effects of key formulation and proceeding variables on both EE and drug release were studied using a fractional factorial design. By means of this entrapment technique, 98% SIR incorporation was achieved. Nanoliposomes were found to have average size of 110 nm and zeta potential of −9 mV. Developed formulations were found to have prolonged drug release for up to 3 weeks in vitro; this was best fitted by the Higuchi model. Other scopes of this work were to determine the applicability of sirolimus-loaded nanoliposomes (SIR-L) as drug carriers for the treatment of restenosis and to evaluate the effect of the presence of rigid lipids on the in vivo efficacy of the liposomal carrier of SIR. In vivo studies in balloon injured rat carotid arteries revealed the potential of SIR-loaded liposomes as efficient local and controlled drug delivery systems to reduce restenosis.

1. Introduction

Coronary artery disease is the most common type of heart disease. Although surgical coronary interventions, such as percutaneous transluminal balloon angioplasty (with or without stent placement), effectively remove the atherosclerotic lesion and restore blood flow, a significant number of patients redevelop obstructions, a disease process known as restenosis (Burt and Hunter, 2006; Melikian and Wijns, 2008). Proliferation of smooth muscle cells from the arterial wall into the lumen of the artery is considered as a major cause of restenosis (Costa and Simon, 2005; Lee et al., 2004). Systemic administration of antiproliferative agents at acceptable doses suffer from subtherapeutic arterial drug levels and are not quite effective (Popma et al., 1991), whereas local drug delivery of these therapeutic agents via drug eluting stents (DESs) has significantly reduced restenosis because it provides higher localized arterial drug levels (Hunter, 2006; Salam et al., 2006).

However, despite excellent short-term and intermediate-term clinical outcomes of DESs, recent increasing concerns about late adverse events plague these devices. Stent thrombosis is a severe complication after stent implantation, owing to its high morbidity and mortality (Genereux and Mehran, 2009; Luscher et al., 2007). The cause of late stent thrombosis is postulated to be multifactorial. The questionable biocompatibility of the materials used for stent preparation seems to be one of the principal causal factors (Luscher et al., 2007; Steffel and Tanner, 2007). Both Cypher and Taxus stents use non-erodible polymers as platforms for retardation of drug delivery (Salam et al., 2006). Many of the synthetic polymers induce an exaggerated inflammatory response in vivo (Azarbal and Currier, 2006; van der Giessen et al., 1996). Furthermore, local infiltration of eosinophils suggests hypersensitivity reactions to the polymer carriers (Luscher et al., 2007; Nebeker et al., 2006). These complications can consequently result in late adverse cardiac events in humans, such as late stent thrombosis (Luscher et al., 2007; Steffel

Abbreviations: %DR, percent drug released; $\%DR_{10h}$, percent drug released after 10 h; $\%DR_{72h}$, percent drug released after 72 h; $\%DR_{10h\text{-}p}$, percent drug released after 10 h in the presence of plasma; $\%DR_{72h\text{-}p}$, percent drug released after 72 h in the presence of plasma; Chol, cholesterol; DES, drug-eluting stent; DSPC, distearoylphosphatidylcholine; DSPG, distearoyl-sn-glycerophosphoglycerol; EE, entrapment efficiency; EEL, external elastic lamina; EL, empty liposome; EPC, egg phosphatidylcholine; IEL, internal elastic lamina; PC, phosphatidylcholine; SIR, sirolimus; SIR-L, sirolimus entrapped liposome; T_m, transition temperature.

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and Tanner, 2007) or late in-stent restenosis (Wessely et al., 2005). The presence and type of polymeric coating are believed to contribute to the rate of in-stent restenosis and the thrombogenic potential of DES because polymers can lead to acute and chronic vascular inflammation, adverse tissue reactions, delayed vascular healing and a prothrombotic environment (Finn et al., 2005; Luscher et al., 2007; Steffel and Tanner, 2007). Other drawbacks have also been reported for DESs. Some recent reports indicate poor attachment between the DES and the arterial wall and, even worse, aneurysms (Ong et al., 2005).

On the other hand, 30–40% of critical lesions cannot be stented, mainly because they occur at branch sites or in small and tortuous vessels (Scheller et al., 2004); they also reocclude more easily following stent placement. Moreover, coronary bifurcations, which account for up to 15% of all current percutaneous coronary interventions, still represent a challenging lesions subset due to a high restenosis rate, especially at side branches (Melikian et al., 2004; Sukhija et al., 2008).

To eliminate the previously mentioned drawbacks and limitations of permanent implants, other methods for prevention of restenosis beyond the drug-eluting-stents strategy are in great demand, particularly in vessels less amenable to stent therapy. Non-stent-based local delivery of antiproliferative drugs by means of colloidal carrier systems may offer additional flexibility and efficacy in the entire range of applications. Among these systems, biodegradable polymeric nanoparticles have shown a certain degree of success (Banai et al., 2005; Reddy et al., 2008). Despite the progress of the knowledge in this field, present limitations of polymer-based nanoparticles include the following: (a) their acidic degradation products and low surface to content ratio, which may lead to toxic local acid concentrations (Hunter, 2006), and (b) during polymer biodegradation, by-products including initiators, catalysts and solvents that are crucial to polymer processing are released, which often reduces biocompatibility (Commandeur et al., 2006).

Offering the advantage of higher biocompatibility, nanoliposomes have been proposed as a promising alternative to polymeric nanoparticles for local vascular drug delivery allowing sustained drug release at the injured site over a prolonged period of time.

Sirolimus (also known as Rapamycin) is a macrolide lactone antibiotic with profound antiproliferative and anti-inflammatory effects (Gallo et al., 1999; Marx et al., 1995). Additionally, in vitro studies show that SIR inhibits platelet-derived growth factor-induced migration of human vascular smooth muscle cells from the media to the intimal region, without affecting their cytoskeletal components or their ability to bind collagen (Poon et al., 1996).

In light of these considerations, the aims of the present study were to develop SIR liposomal formulation, identify the predominant formulation parameters and explore the formula's application for the treatment of restenosis following local vascular delivery. To overcome the technical challenge of producing SIR-Ls by the conventional method, which involves the passage of lipid and drug mixture through the extruder apparatus at temperatures above T_m that could lead to decrease in EE and drug degradation, a novel method of drug entrapment, termed "remote film loading," was employed for the loading of SIR into liposomes. This method includes preparing empty liposomes of desired lipid composition and size, and then the trapping of drug occurs following a few minutes of sonication. This entrapment technique was reported by Sadzuka et al. (2005) for the first time and was used for the effective entrapment of SN-38, a lipophilic drug, into liposomes. To our knowledge, however, the applicability and usefulness of this method for other insoluble drugs has not been reported yet.

A 2^{4-1} fractional factorial design was applied to assess the effects of three formulation variables (lipid to drug molar ratio, mol% cholesterol content and bilayer lipid composition) and one technological factor (sonication time) on the EE and release profile as the key parameters that may affect the performance of nanoliposome formulation in this application. This design considerably reduces the number of preparations in such a way that the information required is obtained in the most effective and precise ways possible, carrying out the necessary experiments and identifying the key variables for a better understanding of the process (Braun et al., 2006; Hamoudeh et al., 2007; Loukas, 1998). Eight different liposomal formulations with duplicates of the center point were prepared and characterized in terms of EE, release profile, size distribution and zeta potential. We also present the antirestenotic efficacy of the SIR-loaded nanoliposomes in the rat carotid artery balloon injury model.

2. Materials and methods

2.1. Materials

Sirolimus was kindly provided by Wyeth Pharmaceuticals (New York, USA). Purified egg phosphatidylcholine (EPC), distearoylphosphatidylcholine (DSPC) and distearoyl-sn-glycerophosphoglycerol (DSPG) were obtained from Lipoid GmbH (Switzerland). Cholesterol (Chol, purity >99%) was purchased from Sigma Chemical Company (St. Louis, MO, USA). Chloroform, methanol, HPLC-grade acetonitrile, Tween 80 and sodium chloride were supplied by Merck (Darmstadt, Germany). Cellulose dialysis tubing (molecular weight cutoff 12,000 Da) was from BioGene (USA).

2.2. Liposome preparation

SIR-Ls were prepared by the remote film loading method developed by Sadzuka et al. (2005) as a novel method for liposomal entrapment of SN-38, a lipophilic drug. This method involves the production of drug film and the subsequent addition to empty liposomes (ELs).

2.2.1. Preparation of empty liposomes (ELs)

ELs were prepared by the lipid film hydration method (Hope et al., 1985). Briefly, the lipid mixture of the desired molar composition was dissolved in chloroform/methanol (4:1) and dried under reduced pressure in a rotary evaporator (90 rpm) at 65 °C to form a thin lipid film. Evaporation was continued for 2 h after the dry residue appeared, to completely remove all traces of the solvent. The film was then hydrated with 0.9% NaCl at 65 °C for 1 h. The obtained multivesicular suspensions were extruded (Northern Lipids, Vancouver, BC, Canada) five times through each of 200 and 100 nm pore size Nucleopore polycarbonate membranes (Whatman, UK) to produce samples with a narrow size distribution. The extrusion was carried out at 65 °C to maintain vesicles above phase transition temperature.

2.2.2. Remote film loading of liposomes

Standard solutions of SIR were prepared in methanol and then evaporated to form a thin layer film. Following the addition of ELs, the mixtures were sonicated for desired time at 60 °C using a high-energy bath-type sonicator (Starsonic, Liarre, Italy). The liposomal suspensions were allowed to stand at room temperature for 1 h. Unentrapped drug was separated by centrifugation at 14,000 rpm for 15 min (Immordino et al., 2003). For in vivo experiments, nanoliposomes were filtered through a 0.22 μm syringe filter to maintain their sterility.

2.3. Effect of variables

Influences of the different process parameters on both %EE and %DR were investigated by an experimental design methodology