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Raloxifene-/raloxifene-poly(ethylene glycol) conjugate-loaded microspheres: A novel strategy for drug delivery to bone forming cells

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ABSTRACT

Raloxifene (Ral)- or Ral-poly(ethylene glycol) (PEG) conjugate-loaded microspheres were prepared with poly(ε-caprolactone) (PCL) alone or with the blend of PCL and poly(D,L-lactide-co-glycolide) (PLGA) to provide controlled and sustained Ral release systems. Benefits of these formulations were evaluated on bone regeneration. Ral-loaded PCL microspheres had the highest encapsulation efficiency (70.7 ± 5.0%) among all groups owing to high hydrophobic natures of both Ral and PCL. Cumulative amount of Ral released from Ral-PEG (1:2) conjugate-loaded PCL:PLGA (1:1) microspheres (26.9 ± 8.8%) after 60 days was significantly higher relative to other microsphere groups. This finding can be ascribed to two factors: i) Ral-PEG conjugation, resulting in increased water-solubility of Ral and increased degradation rates of PCL and PLGA with enhanced water penetration into the polymer matrix, and ii) usage of PLGA besides PCL in the carrier composition to benefit from less hydrophobic and faster degradable nature of PLGA in comparison to PCL. *In vitro* cytotoxicity studies performed using adipose-derived mesenchymal stem cells (ASCs) demonstrated that all microspheres were non-toxic. Evaluation of intensities of Alizarin red S staining conducted after 7 and 14 days of incubation of ASCs in the release media of the different microsphere groups was performed with Image J analysis software. At day 7, it was observed that the matrix deposited by the cells cultivated in the release medium of Ral-PEG (1:2) conjugate-loaded PCL:PLGA (1:1) microspheres had significantly higher mineral content (26.78 ± 6.23%) than that of the matrix deposited by the cells cultivated in the release media of the other microsphere groups except Ral-loaded PCL:PLGA (1:1) microsphere group. At day 14, Ral release from Ral-PEG (1:2) conjugate-loaded PCL:PLGA (1:1) microsphere group resulted with significantly higher mineralization of the matrix (32.31 ± 1.85%) deposited by ASCs in comparison to all other microsphere groups. Alizarin red S staining results eventuated in parallel with the release results. Thus, it can be suggested that Ral-PEG (1:2) conjugate-loaded PCL:PLGA (1:1) microsphere formulation has a potential as an effective controlled drug delivery system for bone regeneration.

1. Introduction

Raloxifene (Ral), a nonsteroidal benzothiophen derivative, is a second-generation selective estrogen receptor modulator (SERM). It was approved for the treatment and prevention of osteoporosis, and for reduction of breast cancer risks by Food and Drug Administration (FDA) (Maximov et al., 2013). It is currently in clinical use as oral formulations. Although about 60% of Ral is absorbed after oral administration, absolute bioavailability of the drug is only 2%. Besides its low water solubility and dissolution, high presystemic clearance is the other reason for the poor bioavailability of the drug (Elsheikh et al., 2012). Consequently, when Ral is administrated orally, patients have to take the drug daily and at a high dose of 60 mg. Administration of high dose of Ral systemically for long time periods would cause to increase risk of Ral side effects. Possible side effects of Ral include venous thromboembolism, pulmonary embolism, hot flushes and leg cramps (Goodman and Gilman, 2001; Maximov et al., 2013). The adverse effects and the administration frequency of Ral can be lowered provided that long-term, controlled and sustained release of Ral from a drug delivery system is accomplished. Furthermore, total amount of Ral consumed can be decreased since lower dose of

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the drug will be sufficient in comparison to the free drug. In recent years, researches on Ral delivery systems have been increasing (Bikiaris et al., 2009; Babanejad et al., 2014; Prakash et al., 2014; Park et al., 2009; Öcal et al., 2014).

Biodegradable polymers of which degradation products are non-toxic have also been used widely in drug delivery systems. In this study, Ral- or Ral-poly(ethylene glycol) (PEG) conjugate-loaded microspheres were prepared by using poly(ε-caprolactone) (PCL) or blend of PCL and poly(D,L-lactide-co-glycolide) (PLGA). PCL and PLGA, FDA-approved polyesters, are biocompatible and biodegradable polymers having the degradation products which are easily eliminated from the body (Singh et al., 2006). In this study, in addition to PCL, a blend of PCL and PLGA was used for microsphere preparation in order to benefit from the advantages of both polymers. In literature, it was indicated that particles/spheres prepared from only PLGA has some disadvantages. For example, Murillo et al. (2002) prepared microspheres of PLGA (100%) or PLGA:PCL (at 75:25 and 50:50 ratios) and loaded with antigenic extract *Hot Saline* from Brucella ovis. Their results showed that the pH of the medium during release dropped from 7.4 to 3.5 in the formulation based on PLGA whereas the presence of PCL declined the pH drop. Moreover, Cao and Schoichet (1999) indicated that the microspheres prepared from a blend of PCL and PLGA (50/50) had a degradation profile intermediate between those of PCL and PLGA (50/50), therefore providing a method to further control degradation rate.

Another important point in this study is that profile of Ral release from the microspheres of PCL or the blend of PCL:PLGA is highly dependent on low water solubility and dissolution characteristics of Ral. In order to enhance the solubility and dissolution characteristics of poorly water-soluble drugs, many methods have been documented in literature, such as co-grinding (Friedrich et al., 2005; Garg et al., 2009; Patil et al., 2013), spray drying (Rogers et al., 2002), micronization (Chaumeil, 1998), solid dispersion (Khan et al., 2011; Ahuja et al., 2007), super critical fluid technology (Van Nijlen et al., 2003), complexation (Patil et al., 2013; Bandela and Anupama, 2009) and lipid-based drug delivery (Attama and Mpamaugo, 2006). In the present study, Ral-PEG (1:2) conjugation approach was used to increase Ral water solubility and degradation rate of the polymers (PCL and PLGA), in turn to further enhance rate of Ral release from the microspheres. PEG presents many advantages such as high solubility in water and in many organic solvents, lack of immunogenicity, antigenicity and toxicity, and elimination by a combination of renal and hepatic pathways. Additionally, PEG has been approved by FDA for human intravenous, oral and dermal applications (Greenwald et al., 2003). However, it is also worth noting that PEG has some limitations, the main of which is its non-biodegradability (Larson and Ghandehari, 2012). As a solution, low-molar-mass PEGs might be used. Nevertheless, by virtue of oxidation into diacid and hydroxy acid metabolites, oligomers with a molar mass less than 400 Da were observed to be toxic in humans. Since the oxidative degradation markedly decreases as molar mass increases, a molar mass higher than 400 Da is required (Herold et al., 1989; Hinds, 2005; Knop et al., 2010). Besides, in order to have prolonged blood circulation time, molar mass should be above the renal clearance limit, which is the range of 20–60 kDa for nondegradable polymers (equivalent to a hydrodynamic radius of approximately 3.5 nm) (Pasut and Veronese, 2007; Parveen and Sahoo, 2006; Veronese and Pasut, 2005; Torchilin, 2006; Takakura et al., 1987; Moghimi et al., 2001). According to some researchers, although the renal clearance limit is not easy to detect (Veronese and Pasut, 2005), over the limit of 20 kDa clearance through the liver becomes dominated and PEGs with a molar mass exceeding 40–60 kDa accumulate in the liver causing toxic effects (Pasut and Veronese, 2007). Another disadvantage of PEG is its low drug conjugation capacity because conjugation occurs at only one or two end chains of PEG (Rowinsky et al., 2003; Larson and Ghandehari, 2012). Regarding these limitations and uncertainties, branched (Nojima et al., 2009; Prencipe et al., 2009; Ramon et al., 2005) and multiarm (Zhao et al., 2008; Lee et al., 2011) biodegradable PEGs, which can be excreted more readily subsequent to cleavage in the body, have been studied.

The present study aimed to develop controlled and sustained Ral delivery system for bone regeneration. In order to enhance the delivery rate of Ral, two approaches were applied; Ral-PEG (1:2) conjugate was loaded into microspheres instead of Ral and the blend of PCL:PLGA (1:1) was used as the polymer carrier. Considering these, different microsphere formulation groups (unloaded PCL, unloaded PCL:PLGA (1:1), Ral-loaded PCL, Ral-loaded PCL:PLGA (1:1), Ral-PEG (1:2) conjugate-loaded PCL and Ral-PEG (1:2) conjugate-loaded PCL:PLGA (1:1) microspheres) were prepared and characterized for morphology, particle size, drug encapsulation efficiency, loading and cumulative release profiles. Moreover, because the main goal of the microspheres was bone regeneration, all groups were also evaluated in terms of their effects on viability and osteogenic differentiation of adipose-derived mesenchymal stem cells (ASCs) of female origin. To our knowledge, there is not a Ral delivery system utilizing both the blend of PCL:PLGA (1:1) as the carrier and Ral-PEG (1:2) conjugate as well as providing Ral release for a long period of 60 days. In this sense, this study presents means to increase the release rate of Ral from a polymeric matrix and makes contribution to the existing literature.

2. Materials and methods

2.1. Materials

Poly(D,L-lactide-co-glycolide) (PLGA, Mw: 8000–15000 g/mol, 75:25) was purchased from Boehringer Ingelheim (Germany). Poly (ε-caprolactone) (PCL, Mw: 14000 g/mol) and poly(vinyl alcohol) (PVA, Mw: ~27000 g/mol) were obtained from Aldrich (Germany). Poly(ethylene glycol) (PEG, Mw: 3500–4500 g/mol) was purchased from Fluka (Germany). Dichloromethane (DCM) was the product of Merck (Germany). Raloxifene (Ral) hydrochloride, dimethyl sulfoxide (DMSO), β-glycerophosphate, dexamethasone, L-ascorbic acid (99%), methanol (MeOH), Alizarin Red S, paraformaldehyde, collagenase type II from *Clostridium histolyticum*, bicinchoninic acid (BCA) solution and cupric sulfate pentahydrate were purchased from Sigma-Aldrich (USA). Alpha-Minimal Essential Medium (α-MEM) was obtained from Lonza (Belgium). Dulbecco's Modified Eagle's Medium/Ham's F-12 Medium (DMEM/F-12) (1:1) was obtained from Thermo Scientific (Utah, USA). Trypsin-EDTA, fetal bovine serum (FBS) and penicillin/streptomycin were the products of Biochrom (Germany). Alkaline Phosphatase (ALP) assay kit was the product of Abcam Inc. (USA). PrestoBlue® cell viability reagent was purchased from Invitrogen (USA). Bovine serum albumin was obtained from PAA Laboratories GmbH (Austria). Seventy-μm mesh used in cell culture experiments was the product of Greiner.

2.2. Preparation of Ral-PEG conjugate

For preparation of Ral-PEG conjugate, solvent evaporation method described by Bandela and Anupama (2009) was modified and used. Briefly, Ral and PEG at a weight ratio of 1:2 were dissolved in MeOH and stirred for 6 h at room temperature by an orbital shaker (BIOSAN, OS-10, Turkey). The resulting product was then dried in a vacuum dryer (Nüve-EV060, Turkey) at room temperature and kept at 4 °C until use.