International Journal of Pharmaceutics 499 (2016) 301–320

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

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

Nanostructured lipid carrier mediates effective delivery of methotrexate to induce apoptosis of rheumatoid arthritis *via* NF-κB and FOXO1

Neeraj K. Garg[a,1], Rajeev K. Tyagi[b,c,1], Bhupinder Singh[a,d], Gajanand Sharma[a], Pradip Nirbhavane[a], Varun Kushwah[e], Sanyog Jain[e], Om Prakash Katare[a,*]

[a] *Drug Delivery Research Group, University Institute of Pharmaceutical Sciences, UGC Centre of Advanced Studies, Panjab University, Chandigarh 160014, India*
[b] *Department of Periodontics, College of Dental Medicine Georgia Regents University, 1120, 15th Street, Augusta, GA 30912, USA*
[c] *Institute of Science, Nirma University, Sarkhej-Gandhinagar Highway, Ahmedabad, 382 481 Gujarat, India*
[d] *UGC-Centre of Excellence in Applications of Nanomaterials, Nanoparticles & Nanocomposites (Biomedical Sciences), Panjab University, Chandigarh 160 014, India*
[e] *Centre for Pharmaceutical Nanotechnology, Department of Pharmaceutics, National Institute of Pharmaceutical Education and Research (NIPER), Sector 67, SAS Nagar (Mohali), Punjab 160062, India*

ARTICLE INFO

Article history:
Received 26 October 2015
Received in revised form 24 November 2015
Accepted 21 December 2015
Available online 6 January 2016

Keywords:
Nanostructured lipid carriers (NLCs)
Methotrexate
Rheumatoid Arthritis
Apoptosis
NF-κB-IkB
FOXO1
RT-PCR
α-Terpineol
Phospholipids S-100
Gelucire 50/13
Transcutol P
Transdermal delivery

ABSTRACT

Present study was designed to develop novel nano-structured lipid carriers (NLCs) formulated by lipid mixture and chemical permeation enhancer-based hydrogel for an effective transdermal delivery of methotrexate (MTX). The prepared NLCs were optimized with different preparative variables such as particle size $<$200 nm, poly-dispersity index (PDI) $<$0.2, and entrapment efficiency ~85%. The drug incorporated into NLCs-gel base showed excellent spread ability without any grittiness during rheological behavior and texture profile analysis. The *in vitro* release showed biphasic release pattern with initial fast release of drug (>50%) in 8 h followed by sustained release (up to 85%) by the end of 48th h. NLCs showed greater uptake in human hyper-proliferative keratinocyte cell line (HaCaT). NLCs showed increased expression of inflammatory mediators as well asapoptosis in U937 monocytic cells. The greater expression of pro-apoptotic gene Bim regulated by NF-κB-IkB and FOXO1 is supported by fold regulations calculated for various apoptotic and pro-inflammatory biomarkers carried out by RT-PCR. The immunocytochemistry to detect IL-6 expression and immunofluorescence assay suggested that induced apoptosis occurs in experimentally induced *in vitro* arthritis model treated with NLCs-MTX. We saw reduced inflammation and triggered apoptosis through NF-κB & FOXO1 pathways induced by MTX loaded NLCs in rheumatoid arthritic cells. In addition, formulated NLCs exhibit better skin permeation with higher permeation flux & enhancement ratio as shown by confocal laser scanning microscopy (CLSM). Moreover, histopathological examinations of skin are suggestive of safety potential of NLCs.
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1. Introduction

Methotrexate (MTX) is a folate anti-metabolite widely used for anti-neoplastic activity, and known for anti-inflammatory effect. MTX has been used for treating psoriasis, cancer and rheumatoid arthritis (RA) (Amarji et al., 2015; Jain et al., 2015a; Paulus, 1990). Among various disease-modifying anti-rheumatic drugs (DMARDs), oral administration of MTX has been commonly used for treating RA (Paulus, 1990; Weinblatt et al., 1991). MTX has reportedly shown to slowdown the rate of joint destruction by inhibiting pro-inflammatory cytokines such as interferon (IFN-γ), tumor necrosis factor (TNF-α), interleukin (IL)-1β, IL-6, IL-15, and IL-18 (Choy and Panayi, 2001; Cutolo et al., 2001; Feldmann et al., 1996; Zwerina et al., 2005), and joint-destructive enzymes *viz.* inducible nitric oxide synthase (iNOS), NOS_2 and cyclooxygenase-2 (COX-2) during chronic inflammation in joints (Choy and Panayi, 2001; Feldmann et al., 1996; Lee et al., 2012; Zwerina et al., 2005).

Although MTX tablets and injections have been licensed to the market, long term usage results into adverse effects including mucosal ulceration, stomatitis, bone marrow suppression, loss of

* Correspondence author at: Division of Pharmaceutics, University Institute of Pharmaceutical Sciences, UGC-Centre of Advanced Study, Panjab University, Chandigarh 160 014, India. Fax: +91 172 2543101.
E-mail address: drkatare@yahoo.com (O.P. Katare).
[1] Co- first author (contributed equally to this work).

http://dx.doi.org/10.1016/j.ijpharm.2015.12.061
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appetite, drug induced hepatic fibrosis and cirrhosis (Frank and Alan, 2004). The drug-related adverse effectsprompted nearly 30% patients for not to receive RA treatment (van Rossum et al. 1998). Transdermal delivery of MTX has shown encouraging results to overcome the above-stated limitations by avoiding the gastrointestinal tract,and improved MTX therapeutic potentialthrough accumulation of drug at inflamed sites (Prausnitz and Langer, 2008; Prausnitz et al., 2004).

Transdermal delivery of MTX (hydrophilic molecule, log $P = -1.85$) faced challenges from stratum corneum (SC), the outermost hardest layer of skin, and main physical barrier to most of the substances (Bos and Meinardi, 2000; Steele et al., 1979). Variety of novel drug delivery systems *viz* .liposomes for transepidermal (Ali et al., 2008), deformable liposomes for dermal (Trotta et al., 2004), niosomes (Abdelbary and AbouGhaly, 2015), ethanolic liposome(Dubey et al., 2007), solid-in-oil nanocarriers (Yang et al., 2012) and temperature-responsive nanogels (Samah et al., 2010; Singka et al., 2010) have been engineered to increase permeation of the drug *via* skin. Also, physical penetration ability of MTX using transdermal application such as iontophoresis (Alvarez-Figueroa et al., 2001; Prasad et al., 2007) & electroporation (Wong et al., 2006) was attempted.

Nanostructured lipid carriers (NLCs), are promising new generation colloidal lipid carriers for improved penetration and permeation of drugs throughskin (Müller et al., 2007; Souto and Müller, 2005; Souto et al., 2004). The limitations of solid lipid nanoparticles (SLNs) including limited drug loading, gelation, drug leakage during storage due to lipid polymorphism, have been shown to overcome by NLCs (Müller et al., 2002). Moreover, better skin permeation efficiency of NLCs as compared to SLNs has been reported (Andrade et al., 2014). NLCs prepared by physiological and biodegradable lipids showed comparatively low systemic cytotoxicity (Müller et al., 2007). There are some recent studies claiming NLCs and their engineered versions as efficiently transdermal delivery systems (Fan et al., 2013), and co-administration of drugs (Vitorino et al., 2013) to see the synergistic effect.

The skin permeation of novel NLCs, prepared by lipid mixture was determined by cell uptake in HaCaT cells. The therapeutic efficacy and sustained delivery potential to induce apoptosis in RA cells of developed formulation was estimated by measuring different gene expression profile through PCR micro-array in inflammation mimicked human monocytic cells. The immunocytochemistry (ICC) and immunofluorescenceassay (IFA) were employed to induce apoptosis regulated by NF-κB and FOXO1, which in turn regulated the expression of Bim, the pro-apoptotic gene. Therefore, we proposed amodel indicating a mechanism whereby cell-cell interaction inhibits apoptosis of RA cells. We believe without cell-cell interaction, transcription factor NF-κB, associated with its inhibitor IkB, and remains in the cytoplasm. On the contrary, pro-apoptotic factor FOXO1 from nucleus regulates the expression of pro-apoptotic family member Bim. We have estimated the greaterrateof apoptosis in RA cells regulated by nuclear factors, NF-κB & FOXO1. Present study is an attempt to develop a simple micro-emulsion (ME) based method to prepare MTX loaded lipid-based nano-formulation to achieve greater loading efficiency for an effective transdermal delivery of MTX in order to induce apoptosis in RA cells. We discovered a novel apoptotic pathway *via* NF-κB & FOXO1 induced by MTX to subside inflammation in RA cells. To our knowledge, we are the first group to report NLCs prepared by novel lipid mixture (SA + Gelucire and transcutol P) for transdermal delivery of MTX playing an instrumental role in inducing selective apoptosis in RA cells.

2. Materials and methods

2.1. Materials

MTX was procured as a generous gift from IPCA Laboratories, Mumbai, India. Phospolipon S 100 (PL-S100) was provided as a gift from Lipoid GmbH, Ludwigshafen, Germany. Transcutol®P (diethylene glycol monoethyl ether) and Gelucire® 50/13 (Stearoyl polyoxyl-32 glycerides) were procured by Gatefosse, Saint Priest Cedex/France, as gift samples. Poloxamers (Kolliphor® P 188, Kolliphor® P 338 and Kolliphor® P 407) were obtained as gift samples from BASF, Mumbai, India. Stearic acid (SA) was purchased from Central Drug House Pvt., Ltd., New Delhi, India. Tween 80 was purchased by Fischer Scientific Pvt., Ltd., India. Annexin V-FITC and propidium iodide (PI) staining kit were procured from eBioscience, USA. α-Tubulin (PA5-29135), and anti-Bim polyclonal antibody (PA5-20089). Pierce ECL Western blotting substrate was obtained from Pierce/Thermo Scientific, USA. Dialysis membrane (MWCO-12 kDa) was purchased from Himedia Labs, Mumbai, India. α-terpineol and other chemicals & reagents were of analytical grade, while the HPLC solvents were of HPLC grade procured from local vendors.

2.2. Pseudo ternary phase diagram

Pseudoternary phase diagram (PPD) was prepared as per our previous report (Amarji et al., 2015). Lipid phase was constituted of solid lipid (SA: Gelucire® 50/13) and liquid lipid (Transcutol® P) in the ratio of 4:1, while internal ratio of solid lipid was 1:2 for SA and Gelucire. S_{mix} was the mixture of surfactant (Tween 80) and co-surfactants (PL-S100 in ethanol in different ratios), whileaqueous phase was distilled water. The required quantities of surfactant phase (S_{mix}) and the lipid phase were heated to the same temperature. To find out the ME-region, at 60 °C, a mixture of known amount of S_{mix} and lipid phase (*i.e.*, 10:0 – 0:10 w/w) was taken and subsequently titrated with aqueous phase (*i.e.*, aqueous titration where aqueous phase acts as titrant) with the help of microsyringe. Titration was continued till the appearance of turbidity, if initially samples were clear or *vice versa*. Similarly in back titration, Smix + aqueous phase was titrated with the lipid phase, and the above procedure was followed. After getting the final weight of titrant for particular $S_{mix,}$ lipid and/or aqueous ratio, weight percent was calculated and plotted in Gibbs phase triangle as boundary points (Amarji et al., 2015).

2.3. Preparation of NLCs

Hot micro-emulsion (ME) method was used to prepare MTX loaded NLCs by using High shear homogenizer (Heidolph, Silent Crusher M, Germany). Briefly MTX and lipid mixture (SA + Gelucire®50/13 + Transcutol® P) were heated to melt, followed by the addition of ethanolic solution of phospholipid and Tween 80. Calculated amount of water was then added to form primary ME and heated at about 60–70° with stirring and sonicated for 15 s at 3 W by using probe sonicator (Sonicator 3000, Misonix) to form hot primary ME. Hot primary ME was then added to surfactant solution (0.5% w/v poloxamer) by micro syringe under homogenization. Homogenization was done at 10,000 rpm for 10 min by using high shear homogenizer, followed by magnetic stirring (Remi, Mumbai, India) for 2–3 h at 500 rpm. The dispersion of NLCs was dialyzed by cellulose dialysis bag (MWCO 10 kDa) against a dialyzing medium containing double distilled water and dimethyl formamide (DMF) mixture (2:1) in order to remove any unentrapped drug.

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