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Influence of peptide dendrimers and sonophoresis on the transdermal delivery of ketoprofen

Jyothsna Manikkath[a], Aswathi R. Hegde[a], Guruprasad Kalthur[b], Harendra S. Parekh[c], Srinivas Mutalik[a,*]

[a] Department of Pharmaceutics, Manipal College of Pharmaceutical Sciences, Manipal University, Manipal 576104, Karnataka State, India
[b] Department of Clinical Embryology, Kasturba Medical College, Manipal University, Manipal 576104, Karnataka State, India
[c] School of Pharmacy, Pharmacy Australia Centre of Excellence (PACE), The University of Queensland, Brisbane, QLD 4072, Australia

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ABSTRACT

The aim of this study was to determine the individual and combined effects of peptide dendrimers and low frequency ultrasound on the transdermal permeation of ketoprofen. Arginine terminated peptide dendrimers of varying charges (4^+, 8^+ and 16^+, named as A4. A8 and A16 respectively) were synthesized and characterized. Ketoprofen was subjected to passive, peptide dendrimer-assisted and sonophoretic permeation studies (with and without dendrimer application) across Swiss albino mouse skin, both *in vitro* and *in vivo*. The studies revealed that the synthesized peptide dendrimers considerably increased the transdermal permeation of ketoprofen and displayed enhancement ratios of up to 3.25 (with A16 dendrimer), compared to passive diffusion of drug alone *in vitro*. Moreover, the combination of peptide dendrimer treatment and ultrasound application worked in synergy and gave enhancement ratios of up to 1369.15 (with ketoprofen-A16 dendrimer complex). *In vivo* studies demonstrated that dendrimer and ultrasound-assisted permeation of drug achieved much higher plasma concentration of drug, compared to passive diffusion. Comparison of transdermal and oral absorption studies revealed that transdermal administration of ketoprofen with A8 dendrimer showed comparable absorption and plasma drug levels with oral route. The excised mouse skin after *in vivo* permeation study with dendrimers and ultrasound did not show major toxic reactions. This study demonstrates that arginine terminated peptide dendrimers combined with sonophoresis can effectively improve the transdermal permeation of ketoprofen.

1. Introduction

Peptide dendrimers are radially branched macromolecules that contain a peptidyl branching core and/or peripheral peptide chains (Sadler and Tam, 2002). Being dendrimers, they are nanostructures with precise architecture and low polydispersity. As they can be tailored to therapeutic needs, they are ideal carriers for drugs and biomolecules (Svenson, 2009). Compared to other polymers used in drug delivery, dendrimers offer a host of advantages including narrow polydispersity, nanometre size range (which makes them easily cross biological barriers) (Nanjwade et al., 2009), nanoscale container properties (i.e. encapsulation of drug) and nanoscaffolding properties (i.e. attachment or surface adsorption of drug) (Svenson, 2009). Peptide dendrimers have been used as protein mimetics, immunogens, in vaccine delivery, reaction catalysts, biomedical diagnostic reagents, anticancer and antiviral agents (Sadler and Tam, 2002) and therapeutic agents *per se* (Mignani et al., 2013). Peptide dendrimers have many advantages including formation of non-toxic metabolites, cost-effective bulk synthesis, easy purification by RP-HPLC and monodispersity when synthesized by solid phase peptide synthesis (SPPS) (Mutalik et al., 2009a).

Dendrimers have been tried in different routes of drug delivery including intravenous, intraperitoneal, transmucosal, oral, transdermal and ocular routes (Cheng et al., 2008). Transdermal delivery, which is one of the most important routes for chronic administration, is the non-invasive method of permeating drugs through the skin for systemic delivery. Transdermal delivery systems can avoid peaks and troughs of drug levels in plasma and provide consistent plasma drug concentrations. This simplifies the dosing regimen and improves compliance. Sustained/prolonged drug delivery and bypassing of hepatic first pass metabolism and

* Corresponding author.
E-mail addresses: ss.mutalik@manipal.edu, ssmutalik@yahoo.com (S. Mutalik).

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chemical degradation in the gastrointestinal tract are some of the major advantages of transdermal drug delivery. Transdermal delivery comes with its own limitations of low permeation especially for drug molecules that are large in size (>500 Da), have low partition coefficient and require high doses (Prausnitz et al., 2004; Mutalik et al., 2013).

Dendrimers have been successfully shown to improve transdermal drug delivery, but most of the reports available are on PAMAM dendrimers (Chauhan et al., 2003; Cheng et al., 2007; Chauhan, 2015). Some of the widely used dendrimers including PAMAM dendrimers have been reported to be toxic in many studies (Jevprasesphant et al., 2003; Duncan and Izzo, 2005; Kolhatkar et al., 2007). Although not completely devoid of toxicity, peptide dendrimers were synthesized as an alternative to their more toxic PAMAM and PPI (polypropylene imine) counterparts (Shah et al., 2014). Eggimann et al. (2014) reported negligible cytotoxicity of peptide dendrimers compared to linear peptides.

The reports on the use of peptide dendrimers for transdermal delivery are limited to our research group. These studies reported the absence of perceptible permeation of peptide dendrimers themselves across skin without aid of external physical agents like sonophoresis and iontophoresis (Mutalik et al., 2009a, 2012, 2013, 2014).

Sonophoresis is the use of ultrasound energy to transport molecules into and across skin (Mutoh et al., 2003; Polat et al., 2011). Although ultrasound can be classified into low frequency ultrasound or LFU (20–100 kHz) and therapeutic frequency ultrasound (1–3 MHz), it is the former that has been shown to improve transdermal permeation (Mitragotri and Kost, 2000; Tezel et al., 2002a; Boucaud et al., 2001; Mitragotri and Kost 2004; Mitragotri et al., 1995). The main mechanism behind ultrasound-assisted transdermal permeation enhancement is thought to be acoustic cavitation, i.e., formation and oscillation of microbubbles in the coupling medium (Tang et al., 2002; Tezel et al., 2002a; Ueda et al., 2009). Using LFU, the extent of skin perturbation and the resulting skin permeability enhancement can be controlled, by varying the ultrasound application parameters. For transdermal permeation enhancement, sonophoresis has been used both alone, and in combination with chemical enhancers. Synergistic skin permeability enhancement could occur with the combination of multiple skin penetration enhancers, both chemical and physical (Polat et al., 2010). Not only that, this combination is believed to reduce the severity of chemical enhancers required to achieve target permeation rate (Mutalik et al., 2013).

There are currently no reports on the combined use of peptide dendrimers and low frequency ultrasound in transdermal permeation enhancement. This work attempts to synergise the effects of both these enhancers to improve the transdermal permeation of a model drug, ketoprofen.

2. Materials and methods

Fmoc amino acids O-(1H benzotriazol-1-yl)-1,1,3,3- tetramethyluronium hexafluorophosphate (HBTU), (Fmoc-Gly-OH, Fmoc-Lys(Fmoc)-OH, Fmoc-Arg(Pbf)-OH and Rink amide resin (0.70 mmol/g) were purchased from Merck Biosciences, Darmstadt, Germany. Dichloromethane (DCM), acetonitrile and *N,N*-dimethylformamide (DMF) were obtained from RCI Labscan, Samutsakorn, Thailand. *N,N*-diisopropylethylamine (DIEA), trifluoroacetic acid (TFA), triisopropyl silane (TIPS), piperidine, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and diethyl ether were obtained from Sigma-Aldrich, St. Louis, MO, USA. Ketoprofen was purchased from T&T Pharma Care Pvt. Ltd. Thane, India. All other chemicals used were of analytical grade.

2.1. Synthesis of peptide dendrimers

Arginine terminated peptide dendrimers of varying charge (4^+, 8^+ and 16^+) were synthesized by Fmoc SPPS (Mutalik et al., 2013; Parekh et al., 2006). Initially, rink amide resin was swollen using DMF. Fmoc removal was brought about by piperidine (20% v/v) in DMF. Fmoc-Gly-OH, activated with HBTU and DIEA was coupled to the rink amide resin. The resultant product was treated with piperidine (20% v/v) in DMF and the next amino acid coupling was performed in a similar manner. This process was continued in sequence until the dendrimer of the desired generation was obtained. At every amino acid coupling step, the efficiency was established by the ninhydrin test and the next amino acid was coupled only after achieving at least 99% coupling of the previous amino acid. Once the required dendrimer was synthesized, its Fmoc groups were removed. This was followed by flow washing using DMF and DCM and drying of the resin *in vacuo*. Cleaving of the dendrimer from the resin was effected by stirring in a mixture of TFA, DCM, water and TIPS (90:5:2.5:2.5) for 3 h. TFA was removed *in vacuo* and the resulting product was azeotroped using toluene and then triturated in ice-cold diethyl ether. This was followed by lyophilization in deionised water. Purification of the dendimers was done using a preparative HPLC system (Waters, Milford, MA, USA). Characterization was done by ESI^+-MS (2000 QTRAP Nano spray, MDS Sciex, Ontario, Canada) for the molecular ion $[M+H]^+$. Analytical RP-HPLC was then performed to ensure single peak purity.

2.2. Solubility studies

Solubility of ketoprofen was determined in water and in HEPES buffer solutions of varying pH 4.5, 7.4 and 9.2, according to the reported method (Higuchi and Connors, 1965). Excess of ketoprofen was added to 10 mL vials with water/HEPES buffer solution (pH 4.5, 7.4 and 9.2) and kept on stirring for 24 h at room temperature. The dispersions were then filtered through 0.45 μm membrane filter and the amount of the drug dissolved was determined by HPLC. The solubility of ketoprofen was also determined in the presence of peptide dendrimers.

2.3. Determination of partition coefficient

The oil/water partition coefficient values of ketoprofen were determined in the presence and absence of dendrimers in n-octanol/water system. Oil phase (3 mL of n-octanol) was added to an equal volume of saturated drug solution (in Milli-Q water) and kept on a shaking water bath at 25 °C for 24 h. After the study, the aqueous layer was separated and clarified by centrifugation (10,000 rpm, 5 min). Both initial and final concentrations of drug were determined by HPLC. Partition coefficient (K or $K_{o/w}$) was calculated using following equation:

$$K = (\text{concentration of drug})_{oil} / (\text{concentration of drug})_{aqueous}$$

2.4. Degradation of ketoprofen in skin

Stability of ketoprofen in different extracts viz., epidermal, dermal and homogenized skin extracts was determined to rule out enzymatic degradation of drug in the skin. Freshly excised mouse skin was placed in the vertical Franz diffusion cell, with the stratum corneum facing the donor compartment and dermis facing the receptor compartment. Both compartments were filled with HEPES buffer solution (pH 7.4) and stirred for 8 h at 400 rpm. After 8 h, the donor (epidermal) and receptor (dermal) extracts were collected separately. Freshly excised mouse skin (area 1 cm^2)