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Peptide dendrimer-conjugates of ketoprofen: Synthesis and *ex vivo* and *in vivo* evaluations of passive diffusion, sonophoresis and iontophoresis for skin delivery

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ABSTRACT

The aim of this study was to evaluate skin delivery of ketoprofen when covalently tethered to mildly cationic (2^+ or 4^+) peptide dendrimers prepared wholly by solid phase peptide synthesis. The amino acids glycine, arginine and lysine formed the dendrimer with ketoprofen tethered either to the lysine side-arm (N_ε) or periphery of dendrimeric branches. Passive diffusion, sonophoresis- and iontophoresis-assisted permeation of each peptide dendrimer-drug conjugate (D1–D4) was studied across mouse skin, both *in vitro* and *in vivo*. In addition, skin toxicity of dendrimeric conjugates when trialed with iontophoresis or sonophoresis was also evaluated. All dendrimeric conjugates improved aqueous solubility at least 5-fold, compared to ketoprofen alone, while also exhibiting appreciable lipophilicity. *In vitro* passive diffusion studies revealed that ketoprofen in its native form was delivered to a greater extent, compared with a dendrimer-conjugated form at the end of 24 h ($Q_{24\ h}$ (μg/cm^2): ketoprofen (68.06 ± 3.62) > D2 (49.62 ± 2.92) > D4 (19.20 ± 0.89) > D1 (6.45 ± 0.40) > D3 (2.21 ± 0.19). However, sonophoresis substantially increased the skin permeation of ketoprofen-dendrimer conjugates in 30 min ($Q_{30\ min}$ (μg/cm^2): D4 (122.19 ± 7.14) > D2 (66.74 ± 3.86) > D1 (52.10 ± 3.22) > D3 (41.66 ± 3.22)) although ketoprofen alone again proved superior ($Q_{30\ min}$: 167.99 ± 9.11 μg/cm^2). Next, application of iontophoresis was trialed and shown to considerably increase permeation of dendrimeric ketoprofen in 6 h ($Q_{6\ h}$ (μg/cm^2): D2 (711.49 ± 39.14) > D4 (341.23 ± 16.43) > D3 (89.50 ± 4.99) > D1 (50.91 ± 2.98), with a $Q_{6\ h}$ value of 96.60 ± 5.12 μg/cm^2 for ketoprofen alone). *In vivo* studies indicated that therapeutically relevant concentrations of ketoprofen could be delivered transdermally when iontophoresis was paired with D2 (985.49 ± 43.25 ng/mL). Further, histopathological analysis showed that the dendrimeric approach was a safe mode as ketoprofen alone. The present study successfully demonstrates that peptide dendrimer conjugates of ketoprofen, when combined with non-invasive modalities, such as iontophoresis can enhance skin permeation with clinically relevant concentrations achieved transdermally.

1. Introduction

Historically, the skin was thought to be totally impervious to exogenous chemicals (Scheuplein and Blank, 1971). However, once it was implicit that the skin was a semi-permeable membrane rather than a totally impervious barrier; there arose new possibilities for the use of this route as a portal for systemic drug absorption. In general, the epidermis (more specifically the stratum corneum – the top-most skin layer) limits the delivery of drug molecules. Only low molecular weight drugs (generally <500 Da) with adequate physicochemical properties can be passively transported through skin (Prausnitz et al., 2004). Various approaches have evolved to expand the number of drugs delivered through skin using formulation manipulation like supersaturated solution, microemulsion, liposomal systems, and chemical enhancement using permeation enhancers such as polyalcohols, pyrrolidones, amines, amides, fatty acids, sulphoxides, esters, terpenes, alkanes, surfactants and phospholipids (William and Barry, 2004). Physical penetration enhancement methods like iontophoresis, sonophoresis, electroporation and magnetophoresis have also been studied with promising outcomes achieved (Patel et al., 2011).

In recent years, dendrimers have been identified as permeation enhancers for skin delivery of drugs. The development of dendrimers as potential drug vehicles or scaffolds now is one of the most active areas of biomedical and pharmaceutical sciences (Cheng and Xu, 2008). Dendrimers are hyperbranched macromolecules having a tree-like

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structure, consisting of a core from which alternating layers of monomers extend. They possess several features such as regular branching, multivalency, small-size, high density of surface-functional groups, extremely low polydispersity, host-guest entrapment properties and precise molecular weight. There are reports on the use of dendrimers, specifically poly(amidoamine) (PAMAM) systems, in transdermal drug delivery prepared as PAMAM-drug conjugates (Najlah et al., 2006; Kumar et al., 2010). Although PAMAM dendrimers exhibited promise in the delivery of drugs like 5-fluorouracil, tamsulosin, indomethacin, ketoprofen, riboflavin and diflunisal, their biodegradation and inherent cytotoxicity remains an issue (Duncan and Izzo, 2005; Venuganti and Perumal, 2008).

Peptide dendrimers have a wedge-like macromolecular structure. They are composed of amino acids connected *via* peptide/amide bonds which are present within the branching core as well as on their outer surface. They have many advantages including lower toxicity, biodegradation as well as cost effectiveness to prepare in bulk. Encapsulation and conjugation of drugs with peptide dendrimers have been studied for delivery of hydrophobic drugs (Gajbhiye et al., 2008), and this study explores the application of peptide dendrimers for drug delivery, and is an extension of our earlier work (Mutalik et al., 2009a, 2009b, 2012; Mutalik et al., 2013; Mutalik et al., 2014; Shetty et al., 2017). In this study, we have synthesized dendrimeric conjugates of ketoprofen using different natural amino acids. The release of drug from a conjugate occurs *via* chemical or enzymatic cleavage of a hydrolytically labile bond.

Ketoprofen was chosen as a model drug in this study given is available in topical form, serving as a non-steroidal anti-inflammatory agent (NSAID) with analgesic and antipyretic properties. The importance of ketoprofen in the therapeutic field has stimulated the development of topical dosage forms to improve its percutaneous absorption (Moretti et al., 2000).

Pertaining to application of peptide dendrimers in skin/transdermal delivery of bioactive molecules, we have previously studied the passive diffusion and the effect of sonophoresis and iontophoresis on the penetration of peptide dendrimers across human skin (Mutalik et al., 2012; Mutalik et al., 2013). Additionally, enhancement in deposition and permeation of 5-fluorouracil across human epidermis assisted by peptide dendrimers was also investigated (Mutalik et al., 2014). There are few reports available on the conjugation of drugs such as paclitaxel and methotrexate with PAMAM dendrimers. However these reports revealed the possible risks associated with these PAMAM dendrimeric conjugates in therapeutic use (Myc et al., 2008; Cline et al., 2013; Satsangi et al., 2014). Having understood this, we have now transitioned to assess chemically conjugated ketoprofen-peptide dendrimers for transdermal drug delivery on which no reports are available in the literature. In addition, the effect of sonophoresis and iontophoresis on the permeation of these dendrimeric conjugates, along with histological evaluation was studied.

2. Materials and Methods

2.1. Materials

Ketoprofen was kindly gifted by T & T Pharma Care Pvt. Ltd., Mumbai, India. Fmoc-Gly-OH, Fmoc-Lys(Fmoc)-OH, Fmoc-Arg(Pbf)-OH, *O*-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate (HBTU) and Rink amide resin were procured from Merck Biosciences AG, Darmstadt, Germany. Dichloromethane (DCM), acetonitrile and *N*,*N*-dimethylformamide (DMF) were purchased from RCI Labscan, Samutsakorn, Thailand. Trifluoroacetic acid (TFA), *N*,*N*-diisopropylethylamine (DIEA), triisopropylsilane (TIPS), *N*-methyl pyrrolidine (NMP), HEPES, piperidine, diethyl ether, and acetaminophen were purchased from Sigma-Aldrich, St. Louis, Mo., USA. Heptafluorobutyric acid (HFBA) was obtained from Fluka Chemie GmbH, Buchs, Switzerland.

Male Swiss Albino mice (6–8 weeks old; 20–25 g) were used in this study. The animal experimental protocol was approved by Institutional Animal Ethical Committee, Kasturba Medical College, Manipal University, Manipal (Approval No.: IAEC/KMC/12/2014).

2.2. Synthesis, Purification and Characterization of Dendrimeric Conjugates of Ketoprofen

In total, four peptide dendrimeric conjugates of ketoprofen, having either 2^+ or 4^+ charge were synthesized by Fmoc Solid Phase Peptide Synthesis (SPPS) (Mutalik et al., 2012). Details of the dendrimeric conjugates are given in Table 1 and the synthesis scheme is given in Fig. 1. Rink-amide resin was treated with DMF and Fmoc removal achieved with 20% v/v piperidine in DMF. Firstly, Fmoc-Gly-OH activated with HBTU and DIEA was coupled to the resin. The resin was then washed with DMF, and treated with 20% v/v piperidine in DMF prior to coupling of the next amino acid. This successive process was continued until the required dendrimeric conjugates of ketoprofen were formed. At each step of amino acid coupling, the coupling efficiency was established by the Ninhydrin test. Next amino acid was coupled only after achieving at least 99% coupling of the preceding amino acid. Once the desired peptide dendrimeric conjugate of ketoprofen was synthesized, terminal Fmoc groups were detached. This step was followed by flow washing with DMF, then DCM and vacuum-drying of the resin. The peptide dendrimer-ketoprofen conjugates were then separated off-resin by stirring the product in a mixture of TFA, DCM, water and TIPS (90:5:2.5:2.5) for 3–4 h. The resin mixture was then filtered, and TFA eluent removed *in vacuo*. The subsequent residue was treated with toluene (3 × 50 mL), mixed vigorously with ice-cold diethyl ether (3 × 20 mL) and lyophilized.

The dendrimeric conjugates of ketoprofen were purified using a preparative HPLC (Waters, Milford, MA, USA) and subjected to mass spectrometry (MS) analysis, differential scanning calorimetry (DSC), Fourier transform infrared spectroscopy (FTIR) and zeta potential measurements. In MS analysis, all the synthesized dendrimeric conjugates of ketoprofen (D1, D2, D3 and D4) were analyzed using ESI^+-MS (2000 QTRAP Nano spray™, MDS Sciex, a division of MDS Inc., Ont., Canada) for their molecular ion $[M + H]^+$.

For DSC analysis, the sample (plain ketoprofen and D1, D2, D3 or D4 conjugates) was sealed in an aluminium pan and scanned using a Differential scanning calorimeter (DSC-60; Shimadzu, Kyoto, Japan) between 25 and 300 °C, under nitrogen flow (30 mL/min), at a heating rate of 5 °C/min. The reference used was an empty aluminium pan. Temperature calibration was done using indium as the standard (Devarakonda et al., 2005).

Infrared spectroscopy was performed using IR Spectrophotometer (FTIR 8300 Spectrophotometer, Shimadzu, Kyoto, Japan). The samples were mixed with KBr (200–400 mg) and compressed into discs by applying a pressure in a hydraulic press. The pellet was placed in the light path and the spectrum was recorded in the region of 4000 to 400 cm^{-1}.

Zeta potential was measured using a Zetasizer (Nano ZS, Malvern Instruments, UK). Ketoprofen, D1, D2, D3 or D4 were prepared in HEPES buffer pH 7.4 to get a concentration of 1 mg/mL each. Ten measurements were taken for each sample and the mean was reported.

2.3. Solubility Studies

Ketoprofen alone or dendrimeric conjugates (D1, D2, D3 and D4) were added to Ria tubes containing 3 mL of water. Excess quantity

Table 1
Details of different peptide dendrimeric conjugates of ketoprofen synthesized.

Type of dendrimeric conjugate	Dendrimer sequence (C-to-N terminus)	$[M + H]^+$ peak and MW (calculated)
D1 (arginine 4^+)	Gly-Lys(Keto)-Lys-$(Arg)_2$	879.53; 878.52
D2 (lysine 4^+)	Gly-Lys(Keto)-Lys-$(Lys)_2$	823.52; 822.51
D3 (arginine 2^+)	Gly-Lys-$(Arg\text{-}Keto)_2$	987.52; 986.51
D4 (lysine 2^+)	Gly-Lys-$(Lys\text{-}Keto)_2$	931.51; 930.50