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

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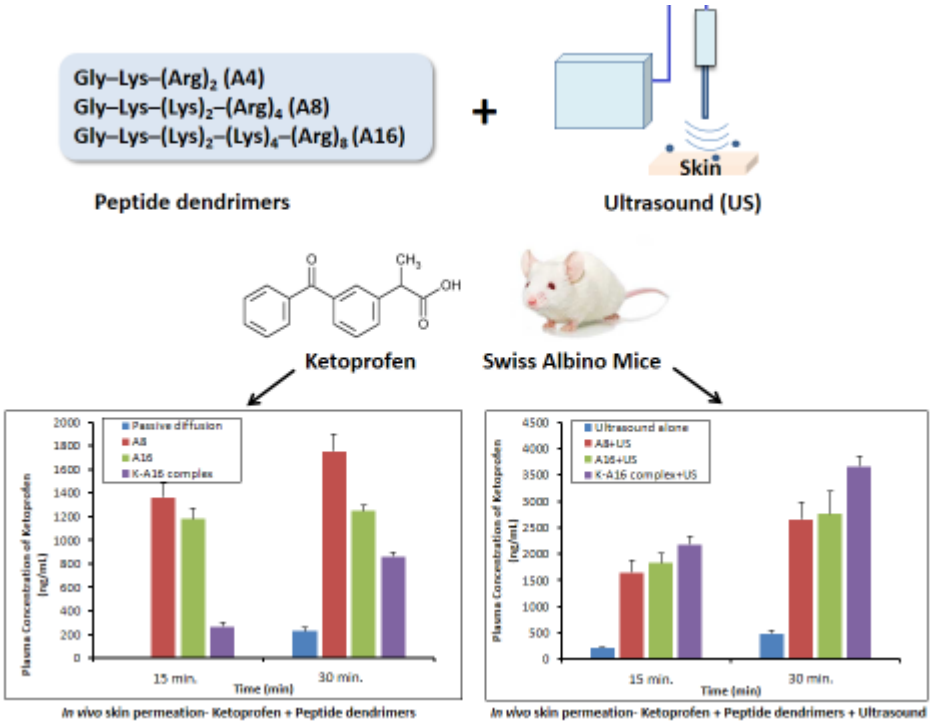
Graphical abstract



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.

Keywords: Transdermal, Peptide dendrimers, Sonophoresis, Ketoprofen, Ultrasound.

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 synthesised 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 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 2004, Mutalik et al., 2013a).

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, 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 (Jain et al., 2010; 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, 2013a and 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., 2002;, Mitragotri and Kost 2004; Mitragotri et al., 2005). 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., 2013b).

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 synthesised by Fmoc SPPS (Mutalik et al., 2013a, 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) was homogenized in 10 mL of buffer solution for 10 min in an ice bath, to prepare skin extract. The homogenate was centrifuged at 3000 rpm for 20 min and the supernatant was collected. 200 µL of drug solution in HEPES buffer (pH 7.4) was spiked with equal quantity of these extracts separately. The samples were stirred for 8 h (30 rpm) at room temperature. The concentration of drug in each solution was analyzed at different time intervals by HPLC. Drug solution in pH 7.4 HEPES buffer was used as the control (Morris et al., 2009).

2.5. HPLC Estimation of Ketoprofen:

Analysis was performed by reverse phase HPLC (Ahmed and Fatahalla 2007). The HPLC system (Shimadzu, Kyoto, Japan) consisted of a pump (LC-10 AD), variable-wavelength UV/Vis detector (SPD-10A), degasser (DGU 20As Prominence, Shimadzu), system controller (CBM-20A Prominence, Shimadzu), auto-injector (SIL-10AXL) and LC Solution software. The HPLC column was a reverse phase C18 column (particle size 5 µm;

250 x 4.6 mm), maintained at 25 °C. Acetonitrile and potassium dihydrogen phosphate buffer (pH 3, 10 mM) mixture (55:45) was used as the mobile phase at a flow rate of 0.8 mL/min. The injection volume was 100 µL and the wavelength of detection was 260 nm. The method was validated with respect to limit of quantification (5.21 ng/mL), limit of detection (1.72 ng/mL), calibration curve ($R^2 > 0.9994$), precision of area (RSD values ranging between 0.05 and 1.60%) and accuracy (between 99 and 101% at different concentrations).

2.5.1. HPLC Analysis of Ketoprofen in Plasma:

Extraction of drug from plasma: Protein precipitation using chilled acetonitrile was used for extracting the drug from plasma. Lercanidipine hydrochloride was used as the internal standard. Plasma (90 µL) was mixed with 10 µL of lercanidipine hydrochloride (IS) standard solution (50 µg/mL in acetonitrile: water 50:50) and vortexed for 2 min. Chilled acetonitrile (300 µL; precipitating agent) was added (extraction ratio was 1:3) and the mixture was again vortexed for 2 min. This was followed by centrifugation at 10000 rpm for 10 min in a cooling centrifuge (Sigma Laborzentrifugen GmbH, Germany) at 4 °C. The supernatant was then collected separately and injected into the HPLC. Analysis was performed as mentioned above, with the differences being the mobile phase ratio (50:50), flow rate (1.0 mL/min) and injection volume (50 µL). This bioanalytical method was validated with respect to precision, accuracy and recovery.

2.6. Drug Dendrimer Complexation and Characterization:

Ketoprofen (25.43 mg) was dissolved in 20 mL of methanol. A16 dendrimer (55.55 mg) was dissolved in 20 mL of Milli-Q water, added to the dendrimer solution and stirred overnight on a magnetic stirrer at 600 rpm (molar ratio of drug and dendrimer was 1:0.25). The solvent was evaporated under vacuum in a rotary flash evaporator. Milli-Q water (15 mL) was added and the mixture was stirred for 12 h to extract the drug-dendrimer complex. The solution was filtered through a 0.45 µm syringe filter. The filtrate was dialyzed and lyophilized to get

the complex in powder form (Devarakonda et al., 2005 and 2007).The prepared drug-polymer complexes were characterized with respect to Differential Scanning Calorimetry, FTIR spectroscopy and zeta potential (Caminade et al., 2005, Mutalik et al., 2014**).**

2.7. *In vitro* Skin Permeation Studies:

2.7.1. Animals:

Male Swiss Albino mice, 6-8 weeks old, weighing 20-25 g were used for the experiments. The animals were housed at 24-26°C, exposed to a daily 12:12 h light: dark cycle and had unrestricted access to standard mice chow and water. The mice were handled daily for 1 week prior to experimentation to reduce the stress associated with the experimental procedure. The animal ethical protocol was approved by the Institutional Animal Ethical Committee, KMC, Manipal (Approval No.: IAEC/KMC/25/2012).

2.7.2. Preparation of skin for permeation studies:

Skin was obtained from the abdominal portion of the mice. The mice were sacrificed using excess of ketamine (i.p.). Abdominal fur was removed using an electric clipper and the skin was excised. Subcutaneous fat and adhering tissue were removed and the skin was washed under running water. Membrane integrity was determined using a digital multimeter. Epidermal membranes that showed a resistance of >20 kΩ only were used in the study. At the end of each permeation study, the resistance of the sample skin was measured again to ascertain that its integrity had been maintained throughout the experiments.

2.7.3. Passive diffusion studies:

Vertical type Franz diffusion cells (diffusion area of 1 cm^2 and receptor compartment capacity of 3.5 mL) were used for the *in vitro* skin permeation studies. Freshly excised skin was sandwiched between the donor and receptor compartments, with the stratum corneum facing the donor compartment. HEPES buffer of pH 7.4 were used in both the donor and receptor compartments. The selection of pH 7.4 was to avoid irritation or damage to the skin

at lower and higher pH values. The receptor phase was stirred using a magnetic bead at 600 rpm. After hydrating the skin for one hour, 2 mL of drug suspension (5 mg/mL of ketoprofen in HEPES buffer pH 7.4; excess of ketoprofen was added based on solubility study) was added to the donor compartment and plain HEPES buffer pH 7.4 was added to the receptor compartment. Samples (1 mL) were collected from the receptor compartment at regular time intervals up to 24 h and analyzed for ketoprofen using HPLC. Samples from the receptor compartment were replaced with fresh receptor solution with each sampling to maintain appropriate volume (Mutalik et al., 2012; Panus et al., 1997).

2.7.4. Permeation studies with dendrimer treatment:

The permeation studies were conducted in a manner similar to the above, with the addition of dendrimer. Experiments using dendrimers were conducted either by (a) simultaneous application of both drug and dendrimer or (b) pretreatment of skin with dendrimers 2 h before application of drug solution (Mutalik et al., 2014). For the simultaneous mode of application, saturated drug solution was initially prepared in HEPES buffer solution of pH 7.4 and dendrimer was stirred (for 3 h) and dissolved in this solution. Then, the drug was again added to make it a suspension (5 mg/mL) and this was used as the donor solution. In studies involving pretreatment, the skin was treated with 25 mg/mL (1 mL) of dendrimer in pH 7.4 HEPES buffer for a time of 2h. Then the dendrimer was completely removed by washing and permeation studies were conducted as mentioned earlier.

Permeation studies were also conducted with drug-dendrimer complex using both simultaneous application and pretreatment techniques. In simultaneous application, an amount of the complex equivalent to give 5 mg/mL of ketoprofen was dispersed in HEPES buffer pH 7.4. The dispersion (2 mL) was added to the diffusion cell and the permeation study was conducted as mentioned above. In pretreatment method, the skin was treated with the respective dendrimer solution on the SC (stratum corneum) side for a specified period of time (2 h). After

washing the skin, permeation study was carried out in a similar manner, dispersing an amount of complex equivalent to give 5 mg/mL of ketoprofen.

2.7.5. Application of ultrasound:

2.7.5.1. In the absence of dendrimer:

These experiments were conducted in a manner similar to the previous, with low frequency ultrasound applied to the donor compartment with a probe sonicator (VibraCell, VC 130, Sonics and Materials, Newton, CT, USA). The tip of the probe was maintained at a distance of 3-5 mm from the skin.

Sonication Parameters: Following are the sonication parameters (Mutalik et al., 2012) -Time: 30 min; Amplitude: ~30; Output wattage: 7-8/ cm^2; On/off cycle: 1 s 'on', followed by 1 s 'off'.

The on/off cycle was maintained to prevent drastic increase in temperature during sonication. Sample collection was performed at 10^{th} min up to 30 min. Replacement of donor solution at every 2 min was also done to prevent increase in temperature and ultrasound was not applied during this changing time. Samples of 1 mL were collected from the receptor compartment and an equal volume of buffer was replaced. The drug content in the samples was determined by HPLC.

2.7.5.2. In the presence of dendrimers:

Permeation studies with ultrasound in the presence of dendrimers were carried out by (a) simultaneous application of both drug and dendrimer or (b) pretreatment of skin with dendrimers 2 h before application of drug solution (c) complexation of drug and dendrimer, followed by simultaneous application or pretreatment. Ultrasound was applied as explained in the previous section (2.7.5.1).

2.8. *In vivo* Permeation Studies:

The abdominal fur of the mice was removed using an electric clipper, 24 h prior to experimentation. The mice were anesthetized by intraperitoneal administration of ketamine hydrochloride. Additional doses, each comprising 1/3rd of the initial dose, were given every 30 min, if required. After about 10 min into anesthesia, the mice were fixed on their backs and the donor compartment of the Franz diffusion cell was positioned on the animal's abdomen. Adhesive tape was also used to prevent leakage of the drug solution from the diffusion cell.

2.8.1. Passive diffusion study:

The donor compartment was filled with ketoprofen solution (5 mg/mL) in HEPES buffer pH 7.4. At different time intervals, blood (about 300 µL) from the mice was withdrawn by retro orbital puncture, into heparinized tubes. The blood samples were immediately centrifuged to separate the plasma and the latter was stored at -20°C. The drug content in the samples was then determined by HPLC.

2.8.2. Permeation studies in the presence of dendrimers:

The dendrimer that showed highest permeation enhancement in the *in vitro* experiments was selected for this study. The *in vivo* experiments were conducted in a manner similar to the *in vitro* experiments. Ketoprofen dispersions (5 mg/mL) were prepared by stirring (30 rpm, 24 h) in HEPES buffer pH 7.4. The dendrimer was added in a concentration similar to the *in vitro* studies and stirred for 3 h. This was taken in the donor compartment and the permeation study was continued. Only simultaneous application was performed.

2.8.3. Permeation study in the presence of sonophoresis alone:

The experiment was conducted in a manner similar to the above method. Ultrasound was applied by immersing the transducer in the donor solution. The location of the centre of the diffusion cell was chosen to avoid damage to blood capillaries. The tip of the probe was maintained at a distance of 3-5 mm from the skin. Ultrasound was applied for of 30 min. at an

amplitude of 30, to achieve output wattage of 7-8/ cm^2 (Mutalik et al 2012). Sample collection was at the 15^{th} and 30^{th} min. The ultrasound application procedure was similar to the *in vitro* experiments. At the end of 15 min and 30 min, blood (about 300 µL) was withdrawn by retro orbital puncture, into heparinized tubes. The blood samples were immediately centrifuged to separate the plasma and the latter was stored at -20°C. The drug content in the samples was then determined by HPLC.

2.8.4. Permeation studies in the presence of both dendrimers and sonophoresis:

The experiment conducted in a manner similar to the previous, adding the respective concentrations of dendrimer/ drug-dendrimer complex in the ketoprofen dispersion. Only simultaneous application was performed. The animals were divided into 6 groups of 6 animals each. *In vivo* transdermal permeation was also compared with oral absorption of ketoprofen in mice. The oral dose of ketoprofen was 20 mg/ kg (Rhee et al., 1999).

2.8.5. Histopathological studies of the treated skin for toxic reactions:

Following the *in vivo* permeation studies, the treated skin was excised out and subjected for histopathological evaluation after staining with hemotoxilin and eosin. The skin sections were observed under optical microscope for skin reactions such as necrosis, inflammation, cavitation, cellular atypia and degeneration.

2.9. Statistical analysis:

One-way analysis of variance (ANOVA) followed by Dunnet's post hoc-test (in case of comparison of results with control) and Student's t-test (to compare two groups) were used to analyze the results (GraphPad Prism). A '*p*' value less than 0.05 was considered statistically significant.

3. RESULTS AND DISCUSSION:

3.1. Dendrimer Synthesis and Characterization:

Arginine terminated peptide dendrimers having varying positive charge and molecular weights (4^+, 8^+ and 16^+; 515.8, 1084.4 & 2222.5 Da) were synthesized by SPPS. The purity of each dendrimer subjected to skin permeation experiments was >95%. All the synthesized peptide dendrimers were purified by preparative RP-HPLC (semi-automated) and were collected within the first 10 min of the total run time. All the dendrimers showed the desired molecular ion ($[M+H]^+$) in ESI-MS, which was in agreement with the theoretically calculated molecular weights of each dendrimer. All the peptide dendrimers also presented single peak profiles in analytical RP-HPLC. Therefore, the formation of the target dendrimers and chromatographic purity were confirmed.

3.2. Solubility Studies:

The solubility of ketoprofen in water and HEPES buffers (pH 4.5, 7.4 and 9.2) was determined. Ketoprofen showed pH-dependent solubility; solubility increased with increase in pH and was highest in pH 9.2 (2764.87±18.99 µg/mL), followed by pH 7.4 (2273.10±131.36 µg/mL). Solubility was lower in pH 4.5 (160.80±1.10 µg/mL) and Milli-Q water (66.13±0.21 µg/mL). Based on the results of the solubility study and favourable physiological conditions to avoid skin irritation, pH 7.4 was selected for further permeability studies (Singh and Jayaswal, 2008).

Results of solubility with peptide dendrimers at pH 7.4 showed that the solubility of ketoprofen decreased in the presence of dendrimers. Further, ketoprofen solubility decreased with increase in molecular weight of the dendrimer (1491.80±20.36 µg/mL and 1074.62±14.68 µg/mL with A4 and A16 dendrimers respectively). Solubility of ketoprofen varied when the concentration of dendrimer in solution was increased (381.43±9.23 µg/mL, 1491.80±20.36 µg/mL and 1461.20±26.42 µg/mL with 5 mg/mL, 10 mg/mL and 20 mg/mL of A4 dendrimer).

3.3. Determination of partition coefficient:

The partition coefficient value of ketorpfen in n-octanol/ water system was determined to be 12.89 (log *P*= 1.11). This value is in close agreement with previous reports (Schmitt and Guentert, 1990; www.accessdata.fda.gov).The corresponding value in the presence of A8 dendrimer was found to be much higher (52.50; log *P*= 1.72). This increased value of partition coefficient indicates that the peptide dendrimers could increase the partitioning of ketoprofen into the lipid rich stratum corneum (Venuganti and Perumal, 2008).

3.4. Stability of ketoprofen in skin:

The skin has metabolic activity, so stability study of ketoprofen in different skin extracts was determined (Banga, 2006). The stability of ketoprofen was determined in epidermal, dermal and whole skin extracts up to 8 h. Ketoprofen displayed reasonable stability in all tested media, with values of 83.64±1.27%, 91.2±2.13% and 80.99±1.04% (mean±SD; n=3), in the epidermal, dermal and skin extracts respectively, compared to 87.25±2.56% in HEPES buffer pH 7.4 (control). These results indicate that ketoprofen has reasonable stability in the skin and is suitable for transdermal administration.

3.5. Characterization of drug-dendrimer complex:

Ketoprofen-A16 complex was prepared by coprecipitation method. Formation of complex between the drug and dendrimer was confirmed by Differential Scanning Calorimetry (DSC), Fourier Transform Infrared Spectroscopy (FTIR) and zeta potential measurements.

Differential Scanning Calorimetry: If a complex of drug is formed with the dendrimer, melting point, enthalpy and entropy of fusion of the complex may be different from that of the plain drug (Devarakonda et al., 2005). If interaction occurs, there could be a shift in the value of melting endotherm of the plain drug or complete masking of the endothermic peak of drug. If interactions are absent, the thermograms of the mixtures would show endothermic peak with intensity corresponding to that of plain drug. If interaction occurs, this is indicated in the

thermogram of the mixture by a shift in the value of melting endotherm of the pure drug or masking of the endothermic peak of drug. The DSC thermograms of ketoprofen, A16 dendrimer, ketoprofen-A16 complex and the mixture of ketoprofen+A16 from the donor solution with 3 h of stirring are given in Fig. 1A-1D. Thermograms of ketoprofen (Fig. 1A) and A16 dendrimer (Fig. 1B) exhibited endothermic peaks at 94 °C and 264 °C respectively corresponding to the melting point of ketoprofen and terminal amino acid (arginine) in the dendrimer. However, ketoprofen-A16 dendrimer complex (Fig. 1C) did not exhibit any prominent peaks. This ascertains association between drug and dendrimer. Also, the mixture of ketoprofen+A16 from the donor solution did not exhibit any prominent peak (Fig. 1D). This could imply that during the 3 h of stirring, the drug could be getting complexed to the dendrimer in the donor solution.

Fourier Transform Infrared Spectroscopy: The samples that were analyzed by FTIR were ketoprofen, A16 dendrimer, ketoprofen-A16 complex, ketoprofen-A16 mixture in the donor solution with 3 h stirring and their corresponding FTIR spectra are given in Fig. 2A-2D. The FTIR spectrum of ketoprofen (Fig. 2A) exhibited characteristic peaks at 2982 cm^{-1} (CH alkane stretch), 2935 cm^{-1} (CH_3 group), 1697 cm^{-1} (C=O group), 1651.12 cm^{-1} (alkenyl C=C stretch), 3740 cm^{-1} (OH group) and 2359 cm^{-1} (carboxylic acid group). In Fig. 2C and Fig. 2D, the C=O group (1750- 1690 cm^{-1}) is not evident. Therefore, probably, the C=O group of ketoprofen is involved in linkage with $–NH_2$ of peptide dendrimer which results in formation of CONH linkage with a prominent peak at 3732 cm^{-1}. This along with the DSC results supports the formation of complex between ketoprofen and A16 dendrimer.

Zeta potential measurement: Zeta potential was measured to measure the change in the charge upon complexation. The zeta potential of A16 dendrimer in HEPES buffer pH 7.4 was 4.73±0.71 mV. On the other hand, the zeta potential of ketoprofen-A16 complex in HEPES buffer pH 7.4 was much higher (6.73±0.89 mV). This difference in zeta potential of the

complex and the plain dendrimer clearly suggest the formation of complexes. The high positive zeta potential of the complex (discussed in later sections) could be one of the reasons for increased permeation, as at pH above the isoelectric point (pI), skin shows cation permselectivity (Banga, 2006).

3.6. *In vitro* permeation studies:

The *in vitro* skin permeation of ketoprofen was studied under different conditions of dendrimer and ultrasound application *viz.*, differing charge of dendrimer, different modes of dendrimer application (simultaneous application, pretreatment and complexation), ultrasound application alone, ultrasound application with dendrimer treatment (with simultaneous application and pretreatment and complexation).

Permeation studies of ketoprofen with dendrimers alone: The effect of differing peptide dendrimer charge on the permeation of ketoprofen with simultaneous application was investigated and the results are shown in Fig. 3 and Table 1. All the dendrimers significantly ($p<0.05$) increased the cumulative amount of drug permeated in 24 h (Q_{24}). Accordingly flux values were also observed in the similar manner. As the charge increased from 4^+ to 8^+, drug permeation also increased, but further increase in charge (16^+) decreased both the flux of the drug and Q_{24}. The permeation parameters observed were highest with A8 dendrimer (Q_{24}: 174.81±11.01 µg; Jss 9.68±1.23 µg/cm^2/h; enhancement ratio (ER): 2.93, compared to Q_{24}: 51.88±5.07 µg; Jss: 3.30±0.24 µg/cm^2/h observed with passive diffusion).

Permeation studies were also conducted by pretreating the skin with dendrimer for 2 h before application of ketoprofen dispersion in pH 7.4 buffer solution. The pretreatment studies were carried out to understand the interaction of dendrimers with the skin. All the tested dendrimers considerably increased the skin permeation of ketoprofen in the pretreatment studies (Table 1 and Fig. 4). The highest permeation enhancement was found with A16 dendrimer (Q_{24}: 212.12±12.15 µg; J_{ss}: 10.75±0.40 µg/cm^2/h and enhancement ratio: 3.25).

The pretreatment studies indicate that the dendrimers penetrate and alter the skin barrier. The skin is negatively charged at physiological pH; therefore cations have greater affinity for the skin. Interaction of dendrimers having amine termination with lipid bilayers and consequent enhancement of transport of molecules has been shown in model lipid bilayers and cell cultures. Reports are available on the interaction of dendrimers with the negatively charged phosphate head groups of model phospholipids and consequent fluidization of the lipid bilayers. Also, dendrimers can interact with skin ceramides and free fatty acids. Dendrimers have also reported to cause alterations in lipid stretching peaks in FTIR spectrum of skin (Venuganti and Perumal, 2008). Cationic dendrimers (like full generation PAMAM dendrimers) have been found to induce hole-formation in model lipid bilayers and cell membranes (Mecke et al., 2004).

The partition coefficient experiments with the peptide dendrimers (presented above) also indicated that they improve partitioning of ketoprofen into the stratum corneum. Apart from increasing the oil/water partition coefficient of the drug, peptide dendrimers may also interact with proteins and lipids in the viable cells of skin, thus, enhancing the transdermal transport of drug molecules (Mutalik et al., 2014).

In both simultaneous as well as pretreatment modes, ketoprofen-A16 dendrimer complex did not show higher permeation for ketoprofen. This could be due to larger size of the complex which hinders the permeation across the skin. When simultaneous application and pretreatment modes were compared with respect to ketoprofen-A16 complex, pretreatment mode showed higher Q_{24} value. This could be due to the interaction between A16 dendrimer and skin during pretreatment period that disrupts the skin integrity (ketoprofen-A16 complex alone: Q_{24}, Jss and ER were 98.71±9.77 µg, 7.07±0.87 µg/cm^2/h and 2.14, respectively; ketoprofen-A16 complex after pretreatment with A16: Q_{24}, Jss and ER were182.81±6.67 µg, 10.55±0.72 µg/cm^2/h and 3.19, respectively).

Permeation studies of ketoprofen with dendrimers + ultrasound:

When the skin permeation experiments were conducted with ultrasound application, a host of surprising results were derived. With ultrasound application alone, the amount of ketoprofen that permeated in just 30 min ($Q_{0.5}$: 118.67±15.28 µg) was more than that permeated in 24 h in passive diffusion (51.88±5.07 µg).

In the second set of experiments, when ultrasound was combined with simultaneous application of dendrimers, again it was found that the amount of ketoprofen permeated in 30 min using sonophoresis and dendrimer application, was significantly ($p<0.05$) higher than that permeated in 24 h without sonophoresis, for all dendrimers tested (Table 1 and Fig. 5). This clearly demonstrates the permeation enhancing effect produced by the combination of dendrimer and ultrasound, which is not achievable by either of them alone. The results indicated that, with simultaneous mode of application, ketoprofen-A16 dendrimer complex showed the highest cumulative drug permeated in 30 min (829.44±73.34 µg). A4, A8 and A16 dendrimers combined with ultrasound produced permeation of 182.95±15.81, 650.05±34.76 and 593.46±67.80 µg of ketoprofen in 30 min, respectively.

With pretreatment mode of dendrimer application, combining ultrasound with dendrimers produced very high permeation rates of ketoprofen (Table 1 and Fig. 6). Like the earlier experiments, ketoprofen-A16 dendrimer complex produced highest permeation (1922.88±240.55 µg) followed by A16 dendrimer (761.47±89.34 µg), A8 dendrimer (673.09±73.20 µg) and A4 dendrimer (364.47±28.70 µg) in 30 min.

Low frequency ultrasound (LFU) has been shown to improve the transdermal permeation of drugs and macromolecules (Boucaud et al., 2002; Han and Das, 2013; Mitragotri and Kost, 2000, Mitragotri and Kost, 2004; Polat et al., 2011; Tezel et. al., 2002a). LFU causes acoustic cavitation, or creation of microbubbles in water and tissue, which forms water channels in lipid bilayers. On the one hand, while sonophoresis has been found to enhance

transdermal permeation, it has been found that this enhancement depends on the physicochemical properties of the drug, including the n-octanol/water partition coefficient (Mitragotri 1997) expressed in the equation below:

$$e \sim \frac{K_{o/w}^{0.75}}{(4 \times 10^4) P_P}$$

where 'e' is the relative sonophoretic transdermal transport, $K_{o/w}$ is the drug octanol-water partition coefficient and P_P is the passive skin permeability of the drug (cm/h). If the permeability coefficient is unchanged, higher partition coefficient would result in higher permeation enhancement with ultrasound. Although the above equation was developed for studies utilizing therapeutic frequency ultrasound (1-3 MHz), perhaps the same can be extended to low frequency ultrasound also. Peptide dendrimers were found to considerably increase the n-octanol/water partition coefficient of ketoprofen (mentioned in section 3.3). Therefore, this improvement in partition coefficient could also contribute greatly to the observed improvement in permeation when peptide dendrimers are combined with ultrasound treatment. This can also be confirmed by the observation that if both sonophoresis and dendrimer treatment were acting alone, the permeation enhancement observed should be equal to that produced by the enhancing treatment, which in this case is sonophoresis.

Synergistic enhancement in transdermal drug delivery has been reported when LFU was combined with chemical enhancers like citral in ethanol (Mutalik et al., 2009), Tween-20, dimethyl formamide, propylene glycol, polyethylene glycol and ethanol (Johnson et al., 1996, Shetty et al., 2013), porous resins (Terahara et al., 2002) and a host of surfactants (Tezel, 2002b). But this is the first report of synergistic effects on permeation produced by LFU and peptide dendrimers.

Tezel et al (2002b) reported that the synergistic effect of surfactants and LFU could be due to i) increase in rate of surfactant entry into the skin and ii) dispersion of surfactant within

the skin, both induced by ultrasound. Mutalik et al (2012) also reported the increase in uptake of peptide dendrimers across skin by LFU. Therefore, the observed synergy between LFU and peptide dendrimers in transdermal permeation of ketoprofen with simultaneous application of drug and dendrimer could be mediated by these mechanisms. On the other hand, with pretreatment of dendrimer on skin, the permeation enhancement of drug could be due to fluidization of skin lipid bilayers further accentuated by ultrasound.

When the permeation studies were conducted without the aid of ultrasound, the permeation produced by the drug-dendrimer complex was lesser than that produced by drug and dendrimer individually. However, with the introduction of ultrasound, drug-dendrimer complex clearly gave much higher permeation enhancement than with the components used individually. A reason for this could be that transient microbubbles formed in the lipid bilayers upon ultrasound application (Tang et al., 2002) could be large enough to allow the transport of the entire drug and dendrimer complex. Also, the greater positive charge of the drug-dendrimer complex compared to plain dendrimer (section 3.5) could be bringing about the increased permeation. However, the underlying mechanism for this phenomenon needs to be explored in further detail.

The target skin permeation rate for ketoprofen was calculated using the following equation from the available pharmacokinetic data (Mutalik & Udupa, 2002; Shetty et al., 2013; www.accessdata.fda.gov):

$$Jss.A = Cl.Cp.W$$

where, Jss is the flux (μg/cm^2/h), A is the area of application (cm^2), Cl is the clearance rate (0.08 L/h/kg), Cp is plasma concentration (0.4 μg/mL) and W is the weight of subject (65 kg). The target permeation rate for ketoprofen as calculated from the above equation was found to be 2.08 mg/h. The flux values obtained with the aid of ultrasound and dendrimers indicate

that the target permeation rate for ketoprofen can be achieved within an appreciable range of application area.

3.7.*In vivo* skin permeation experiments:

In vivo skin permeation studies were performed with dendrimer application and sonophoresis. With passive diffusion, the concentration of ketoprofen in plasma was found to be 226.05±31.19 ng/ mL at the end of 30 min. Sonophoresis alone produced permeation of 481.45±63.38 ng/mL of ketoprofen in plasma. Skin permeation studies of ketoprofen performed with A8 dendrimer, A16 dendrimer and ketoprofen-A16 dendrimer complex produced 1753.31±142.61, 1245.79±54.61 and 859.45±38.05 ng/ mL of ketoprofen in plasma in 30 min. respectively, which were significantly different ($p<0.05$) from the passive diffusion value at the respective time intervals (Table 2). The same combinations of ultrasound and dendrimer application when tested for *in vivo* permeation resulted in 3660.86±199.79, 2760.52±103.47 and 2650.20±223.79 ng/mL of ketoprofen in plasma respectively (Table 2). These results revealed a clear correlation between the *in vitro* and *in vivo* experiments, with respect to dendrimer treatment (± ultrasound application). In both *in vitro* and *in vivo* experiments without ultrasound, A8 dendrimer resulted in highest increase in permeation. In the studies involving the use of ultrasound, ketoprofen-A16 complex produced the greatest permeation enhancement.

3.8. Comparison of transdermal and oral administration:

Swiss albino mice were administered with 20 mg/kg of ketoprofen. Oral administration of drug suspension produced 3626.56±143.55 ng/mL and 8406.56±265.37 ng/mL of ketoprofen in the respective time intervals. Nevertheless, combination of ultrasound and A8 dendrimer with ketoprofen produced 2176.32±166.81 ng/mL and 3660.86±199.79 ng/mL of ketoprofen concentration in plasma in 15 and 30 min. respectively. This indicates that although oral absorption of ketoprofen is more rapid, the above transdermal administration produced

about ≈ 44% of plasma levels compared to oral route. The results of this study are encouraging because with little increase in the skin application area, the drug concentration as obtained with oral route can easily be achieved.

3.9. Histopathological studies of the treated skin for toxic reactions:

After *in vivo* permeation studies, the excised mouse skin was subjected to histopathological evaluation to observe if the synergy between ultrasound and peptide dendrimers also translated to increased skin irritation. Ultrasound alone showed slight necrosis, inflammation and cavitation in treated skin, when compared to control. Similar skin reactions were observed in previous studies when ultrasound was applied to skin (Barnett et al., 1997; Nyborg 2001). Skin of the animals treated with A16 dendrimer (with and without ultrasound) showed slight/ negligible cellular atypia, degeneration, necrosis, inflammation and cavitation. This shows the appreciable dermal safety and non-toxicity of the tested peptide dendrimers.

4. CONCLUSIONS

All the tested peptide dendrimers considerably increased the transdermal permeation of ketoprofen. Application of ultrasound alone for 30 min was found to produce a similar skin permeation of ketoprofen as obtained with passive diffusion in 24 h. Remarkable enhancement in skin permeation of ketoprofen was observed when ultrasound was applied along with dendrimer treatment. Dendrimer and sonophoresis-assisted transdermal delivery was found to produce plasma levels of drug comparable to oral administration. Histopathological evaluation of skin upon different treatments did not reveal considerable dermal toxicity. The study reveals that combined approach of dendrimer treatment (chemical enhancement) along with low frequency ultrasound application (physical permeation enhancement technique) is an attractive approach for achieving high transdermal permeation rates for ketoprofen and this approach could be further extended to other drugs.

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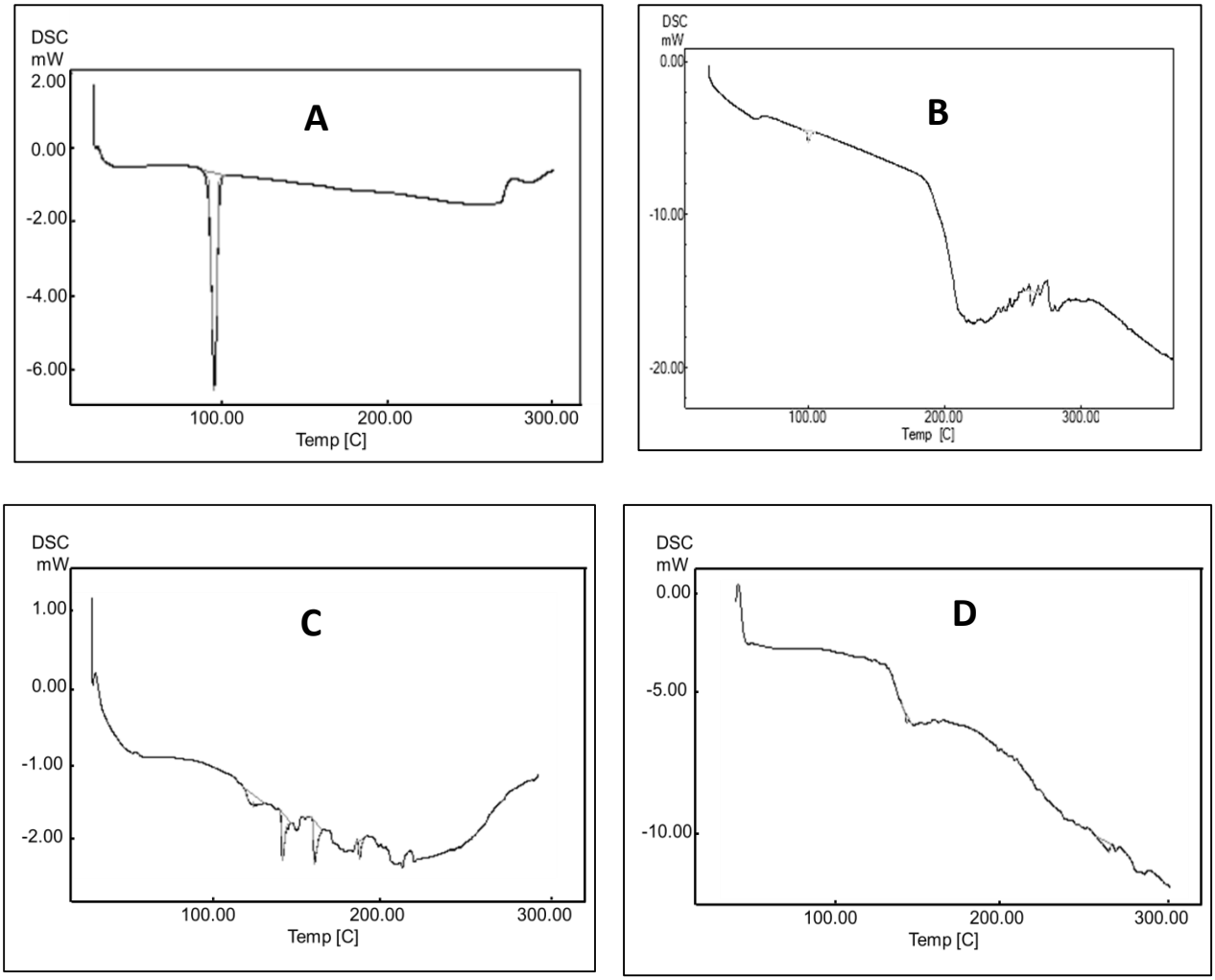

Fig.1. DSC thermograms

A) Ketoprofen B) A16 peptide dendrimer C) ketoprofen-A16 dendrimer complex (1:0.25 molar ratio) and D) ketoprofen-A16 mixture after 3 h of stirring from donor solution.

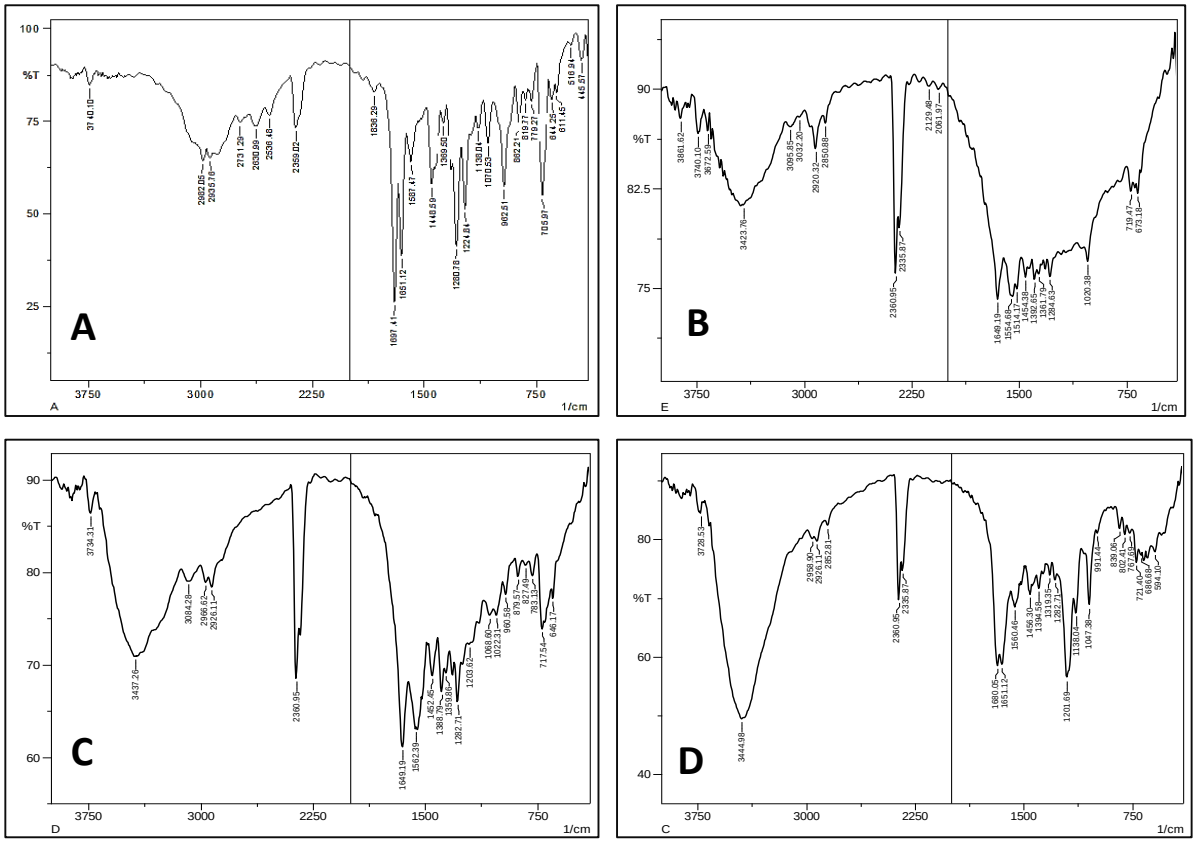

Fig. 2. FTIR spectra

A) ketoprofen B) A16 peptide dendrimer C) ketoprofen-A16 dendrimer complex (1:0.25 molar ratio) and D) ketoprofen-A16 mixture after 3 h of stirring from donor solution.

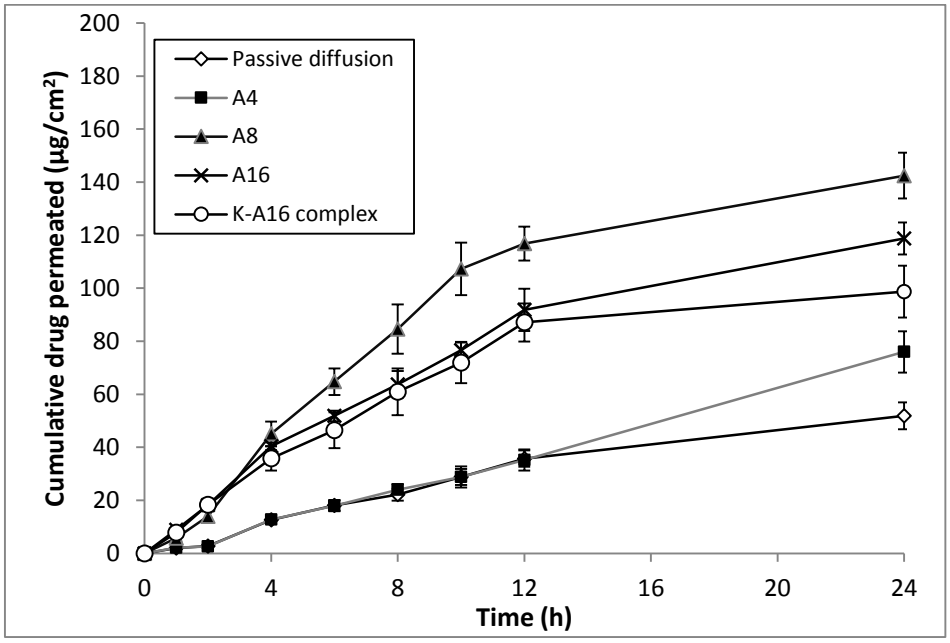

Fig. 3. *In vitro* skin permeation profiles of ketoprofen after simultaneous application of peptide dendrimers

K-A16= Ketoprofen-A16 peptide dendrimer complex

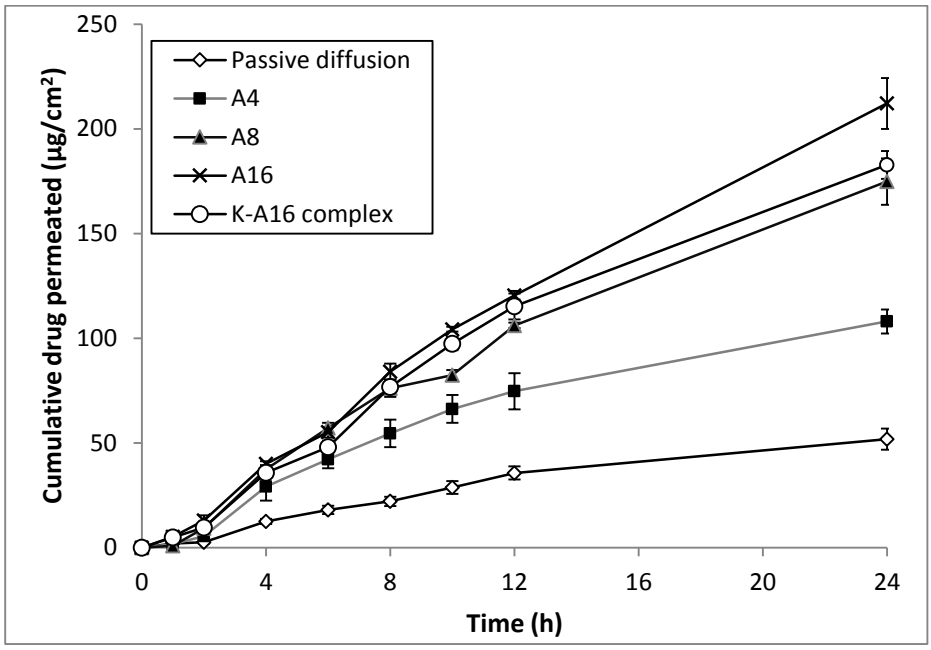

Fig. 4. *In vitro* skin permeation profiles of ketoprofen after pretreatment with peptide dendrimers

K-A16= Ketoprofen-A16 peptide dendrimer complex

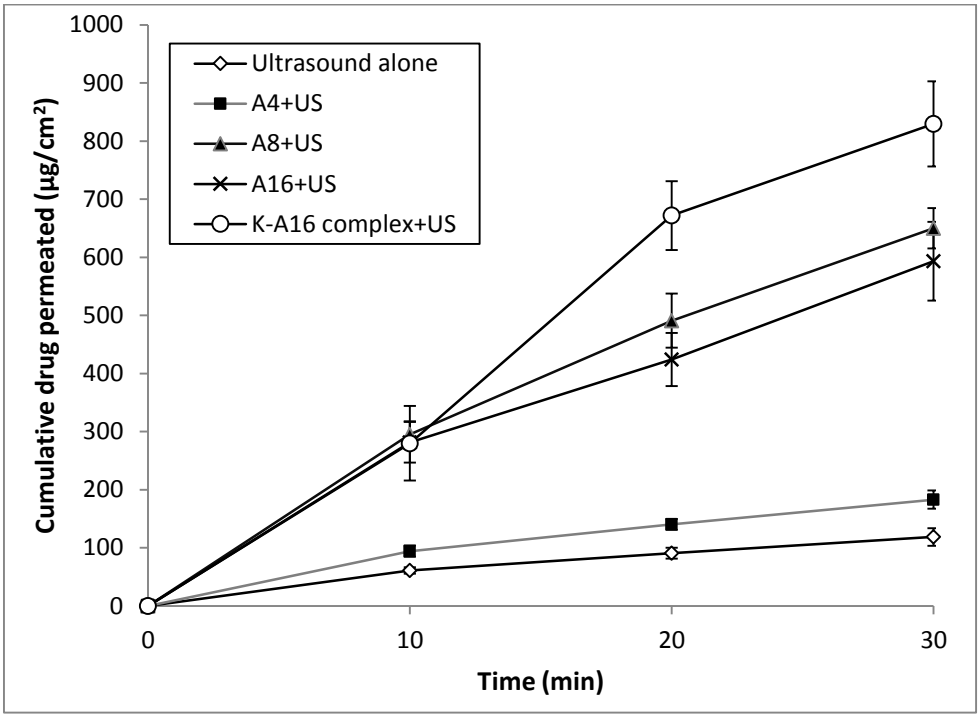

Fig. 5. *In vitro* skin permeation profiles of ketoprofen with simultaneous application of peptide dendrimers and sonophoresis

US=Ultrasound; K-A16= Ketoprofen-A16 peptide dendrimer complex

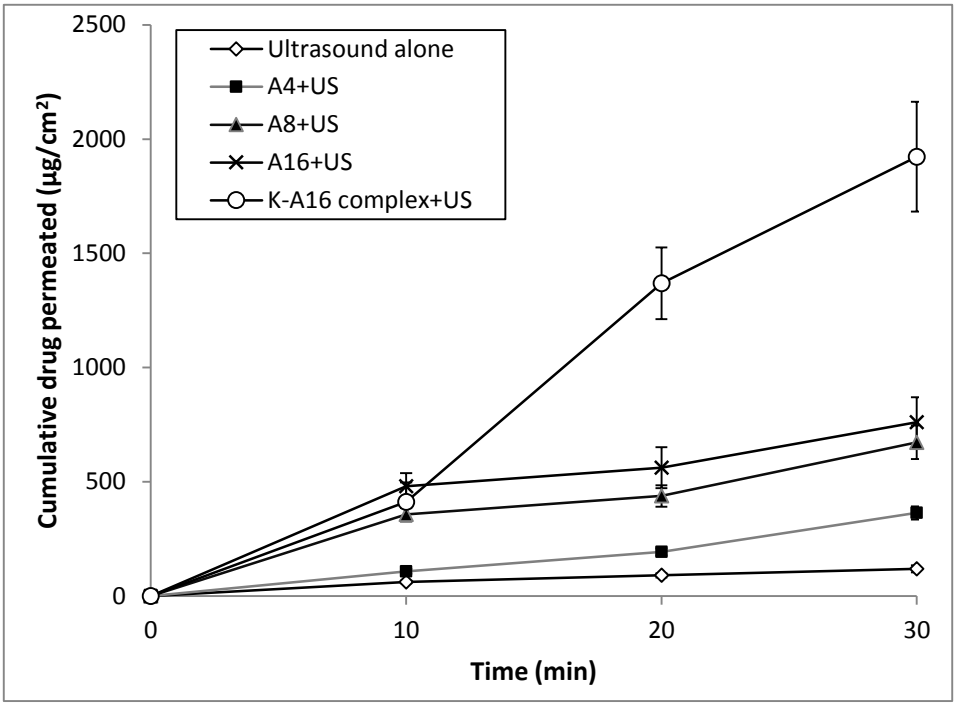

Fig. 6. *In vitro* skin permeation profiles of ketoprofen with pretreatment with peptide dendrimers followed by sonophoresis

US= Ultrasound; K-A16= Ketoprofen-A16 peptide dendrimer complex

Table 1. *In vitro* skin permeation studies of ketoprofen with different treatments and modes of application

Treatments	**Mode of application**	**$Q_{24}^{\#}$ OR $Q_{0.5}^{\$}$ (µg/cm^2)**	**J_{ss} (µg/cm^2/h)**	**ER**
Passive diffusion (drug alone)	NA	51.88±5.07$^{\#}$	3.30±0.24	NA
Ketoprofen + A4 dendrimer	Simultaneous application	75.97±7.79$^{\#*}$	3.66±0.21	1.01
Ketoprofen + A8 dendrimer		142.43 ±8.61$^{\#*}$	10.29±0.79*	3.12
Ketoprofen + A16 dendrimer		118.71±6.03$^{\#*}$	7.35±0.75*	2.23
Ketoprofen-A16 complex		98.71±9.77$^{\#*}$	7.07±0.87*	2.14
Ketoprofen + A4 dendrimer	Pretreatment	108.04±5.77$^{\#*}$	6.92±0.82*	2.10
Ketoprofen + A8 dendrimer		174.81±11.01$^{\#*}$	9.68±1.23*	2.93
Ketoprofen + A16 dendrimer		212.12±12.15$^{\#*}$	10.75±0.40*	3.25
Ketoprofen-A16 complex		182.81±6.67$^{\#*}$	10.55±0.72*	3.19
US+ Ketoprofen	NA	118.67±15.28$^{\$}$	172.00±19.76*	52.08
US+ Ketoprofen + A4 dendrimer	Simultaneous application	182.95±15.81$^{\$*}$	265.44±18.41*	80.37
US+ Ketoprofen + A8 dendrimer		650.05±34.76$^{\$*£}$	1063.11±36.42$^{*£}$	321.90
US+ Ketoprofen + A16 dendrimer		593.46±67.80$^{\$*£}$	933.38±98.74$^{*£}$	282.62
US+ Ketoprofen-A16 complex		829.44±73.34$^{\$*£}$	1644.94±26.79$^{*£}$	498.06
US+ Ketoprofen + A4 dendrimer	Pretreatment	364.37±28.70$^{\$*£}$	766.23±55.30$^{*£}$	232.00
US+ Ketoprofen + A8 dendrimer		673.09±73.20$^{\$*£}$	944.97±79.64$^{*£}$	286.12
US+ Ketoprofen + A16 dendrimer		761.47±89.34$^{\$*£}$	840.90±83.11$^{*£}$	254.61
US+ Ketoprofen-A16 complex		1922.88±240.55$^{\$*£}$	4521.86±363.92$^{*£}$	1369.15

US= Ultrasound application; Jss=Steady state flux; ER= Enhancement ratio;
Values are represented as Mean ± SD; n=3.
* Significantly different ($p<0.05$) compared to Passive diffusion (drug alone); $^{£}$ Significantly different ($p<0.05$) compared to US+ Ketoprofen.

Table 2. *In vivo* permeation studies of ketoprofen with different treatments

Treatment	Plasma concentration of drug (ng/mL)*	
	15 min	30 min
Passive diffusion (drug alone)	4.47±1.01	226.05±31.19
Ketoprofen + A8 dendrimer	1364.33±117.56*	1753.31±142.61*
Ketoprofen + A16 dendrimer	1177.67±95.63*	1245.79±54.61*
Ketoprofen-A16 complex	267.67±31.13*	859.45±38.05*
US + Ketoprofen	216.59±14.39	481.45±63.38
US + Ketoprofen + A8 dendrimer	1634.92±244.23*#	2650.20±223.79*#
US + Ketoprofen + A16 dendrimer	1826.78±87.91*#	2760.52±103.47*#
US + Ketoprofen-A16 complex	2176.32±166.81*#	3660.86±199.79*#

US= Ultrasound application; All the values are represented as Mean ± SD, n=3.
* Significantly ($p<0.05$) different compared to passive diffusion (drug alone); # Significantly ($p<0.05$) different compared to US+ Ketoprofen.