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Effect of poly(amidoamine) (PAMAM) dendrimer on skin permeation of 5-fluorouracil

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ABSTRACT

The aim of present study is to investigate the effect of poly(amidoamine) (PAMAM) dendrimer on skin permeation of 5-fluorouracil (5FU). Permeation studies were performed using excised porcine skin in a Franz diffusion cell and ^{14}C labeled 5FU samples were analyzed using liquid scintillation counter. Three different vehicles were used, including phosphate buffer (PB), mineral oil (MO) and isopropyl myristate (IPM). The studies were carried out by simultaneously applying the drug and dendrimer together or by pre-treating the skin with dendrimer before drug application. Simultaneous application of drug and dendrimer increased the flux of 5FU in IPM and MO, while there was no change in PB. The increased skin partitioning of dendrimer from lipophilic vehicles increased the drug solubility in skin. Pre-treatment with dendrimer increased permeability coefficient of 5FU by 4-fold in MO and 2.5-fold in IPM, while it decreased by half in PB. Skin partitioning of 5FU increased after dendrimer treatment from lipophilic vehicles. The flux increased linearly with increase in pre-treatment time. Dendrimer pre-treatment increased transepidermal water loss and decreased skin resistance. The decrease in skin resistance directly correlated to the enhancement in skin permeation of 5FU ($r^2 = 0.99$). Overall, the study showed that dendrimer increases the skin permeation of 5FU from lipophilic vehicles mainly by altering the skin barrier.

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1. Introduction

Drug delivery through skin is mainly limited by *stratum corneum* (SC), the top-most skin layer. The SC is composed of lipid-rich intercellular matrix and keratin filled corneocytes. The lipid bilayer in the intercellular matrix is the major transport barrier for drug molecules (Elias and Friend, 1975). Only small drug molecules (<500 Da) with optimal physicochemical properties (log P 1–3) can be passively transported through SC (Prausnitz et al., 2004). As a result, various chemical and physical enhancement strategies have evolved to expand the number of drugs delivered through skin (Prausnitz et al., 2004). In this regard, several chemical penetration enhancers have been widely investigated (Williams and Barry, 2004). A large number of these chemical enhancers are small molecules that penetrate the skin in significant amounts and cause skin irritation or irreversibly alter the skin barrier. On the other hand, polymeric enhancers due to their large molecular size cannot penetrate deep into the skin and hence do not cause skin irritation (Aoyagi et al., 1991; Akimoto et al., 1997). Akimoto et al. (1997) tested a series of linear polymeric enhancers and found them to be non-irritating to the skin. The present study focuses on evaluating branched dendritic polymers as skin penetration enhancers.

Dendrimers are monodisperse hyperbranched polymers with a core–shell architecture containing a high density of tunable surface functional groups. They are synthesized using a small organic molecule as core and stepwise addition of branches to the core (Esfand and Tomalia, 2001). Dendrimers are versatile carriers in which drugs can be loaded in multiple ways. Drugs can be encapsulated in the core and/or complexed or conjugated to the surface functional groups (D'Emanuele and Attwood, 2005). They offer distinct advantages over linear polymers because of the multivalency and the precision with which the number of surface functional groups can be altered by controlling the number of branching units. The unique architecture of dendrimers can be used to carry various types of molecules including drugs, diagnostic agents, targeting ligands or imaging agents (Esfand and Tomalia, 2001). Moreover their spherical architecture provides a compact structure with a small hydrodynamic radius (1–10 nm) for transport across biological membranes (Kitchens et al., 2005). Due to these unique features, dendrimers are widely investigated as drug and gene carriers by various routes of administration (Cheng et al., 2008). Studies in cell culture and intestinal membranes have shown that dendrimer increases the permeation by interacting with the membrane lipids (Kitchens et al., 2005; Cheng et al., 2008). Recent studies have shown that dendrimers increase the skin permeation of lipophilic

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drugs by increasing their water solubility (Chauhan et al., 2003; Yiyun et al., 2007). However, its ability to increase the skin permeation of hydrophilic drugs has not yet been demonstrated. Similarly, the mechanism of permeation enhancement and dendrimer–skin interactions are not known.

The present work deals with the influence of poly(amidoamine) (PAMAM) dendrimers on skin permeation of a model hydrophilic drug. Amine terminated generation 4 (G4-NH_2) PAMAM dendrimer is used in this study and has been widely used for drug delivery studies (Kitchens et al., 2005). 5-Fluorouracil (5FU) is used as a model hydrophilic drug ($\log P = -0.89$) and the drug is poorly permeable through skin (Cornwell and Barry, 1993). The drug is used in the treatment of psoriasis, premalignant and malignant skin conditions (Tsuji and Sugai, 1975; Goette, 1981). Various enhancement strategies including prodrugs, terpenes, fatty acids, iontophoresis, sonophoresis and laser ablation have been used to increase the skin permeation of 5FU (Beall and Sloan, 2002; Cornwell and Barry, 1993; Gao and Singh, 1998; Meidan et al., 1999; Merino et al., 1999; Lee et al., 2002). The main objective of this study is to investigate the *in vitro* skin permeation of 5FU from three different vehicles after pre-treatment and co-treatment with dendrimer. The goal is to delineate the mechanism of dendrimer–skin and dendrimer–drug interactions respectively.

2. Materials and methods

2.1. Materials

PAMAM dendrimer with amine surface groups were purchased from Dendritech, Inc., MI. ^{14}C-5FU was purchased from Moravek Pharmaceuticals, CO. Mineral oil and 5FU were purchased from Sigma–Aldrich Chemical Company, St. Louis, MO. Sodium azide, IPM and scintillation cocktail (ScintiVerse) were purchased from Fisher scientific, NJ. NCS-II Tissue solubilizer was purchased from Amersham Biosciences, NJ. All other chemicals were purchased from Sigma–Aldrich Chemical Company, St. Louis, MO.

2.2. Preparation of skin samples

Porcine ears were obtained from the slaughter house in the Department of Animal and Range Sciences at South Dakota State University. The ears were collected immediately after slaughtering and washed under tap water. Hair was removed using a hair clipper (Golden A5, Oster, Niles, IL) and the dorsal skin was excised from the underlying cartilage with scalpel and forceps. Fat adhering on the dermis side was carefully removed using a blunt scalpel and the skin was observed for any visible damage. Skin was stored at −20 °C and was used within 3 months.

Epidermis was removed using the method of Kligman and Christopher (1963). Briefly, full thickness skin was placed in a water bath at 60 °C for 90 s and then the epidermis was teased off using forceps. The intact epidermis was washed with de-ionized water and then dried using Kim wipe. Dried epidermal sheets were stored at −20 °C in a desiccator and used for partition studies.

2.3. Skin permeation studies

Skin was thawed at room temperature and was equilibrated in a Franz diffusion cell for 3 h. Micrometer was used to measure the skin thickness and the thickness was 1.1 ± 0.2 mm. Skin was cut into small pieces and was sandwiched between the donor and receptor chambers of the diffusion cell (PermeGear, Inc., PA) with the SC facing the donor chamber. Phosphate buffer (PB, pH 7.4) with 0.05% (w/v) sodium azide was used as the receptor medium (6 ml) and the temperature was maintained at 37 °C by circulating heated water. The area of skin exposed to the donor chamber was 0.64 cm^2 and the receptor phase was stirred using a magnetic stir bar. Before starting the experiments, transepidermal water loss (TEWL) (Vapometer, Delfin, Sweden) and skin resistance were measured to ensure that the skin was not damaged. Only skin samples which had a TEWL < 10 $g\,m^{-2}\,h^{-1}$ and a resistance >15 $k\Omega\,cm^{-2}$ were used in the study. Donor chamber was loaded with 0.2 ml of 1 mM dendrimer and drug suspension (20 mg/ml 5FU spiked with 0.1 μCi ^{14}C-5FU) either separately or together in pre-treatment and co-treatment studies respectively. Samples (0.2 ml) were collected from the receptor compartment at regular time intervals up to 48 h and were replaced with fresh buffer. Scintillation cocktail was mixed with the samples and the radioactive counts were measured using a liquid scintillation counter (Beckman Coulter LS 6500).

2.4. TEWL and skin resistance measurement

The TEWL ($g\,m^{-2}\,h^{-1}$) was measured before and after skin treatment with dendrimer by placing the vapometer on the donor chamber. The room temperature was between 20 and 26 °C and the relative humidity was between 46 and 50%. Skin resistance was also measured before and after dendrimer treatment. For measuring the skin resistance, a direct current of 0.3 $mA\,cm^{-2}$ (I) was applied using a constant power supply unit (Phoresor II, Iomed, Inc., UT) through platinum electrodes (Fisher Scientific, NJ). The potential difference (V) across the skin was measured using a multimeter (Fluke, WA) and the skin resistance (R) was calculated from Ohm's law ($V = IR$) (Rastogi and Singh, 2001). To minimize the variations from different skin pieces, the values after treatment were normalized with respect to the values before treatment for the same skin piece. Change in TEWL was represented as percent increase in the normalized value after dendrimer treatment with respect to the normalized value in the control. Similarly, the change in skin resistance was represented as the percent decrease in the normalized value after dendrimer treatment with respect to the normalized value in the control. The control skin was pre-treated with blank buffer.

2.5. Vehicles

Three vehicles including PB, mineral oil (MO) and isopropyl myristate (IPM) were used to study the influence of vehicles on skin permeation of 5FU. A saturated dispersion of 5FU (20 mg/ml) was used for skin permeation studies.

2.6. Dendrimer treatment

To delineate the effect of dendrimer on the skin and the drug, pre-treatment and co-treatment studies were performed respectively. In co-treatment studies dendrimer and 5FU were dispersed in the vehicle and then applied on the skin. The control was 5FU in respective vehicles without dendrimer. In pre-treatment studies, the skin was treated with 0.2 ml of dendrimer in PB for pre-determined time periods (2, 12 and 24 h). After completely removing the dendrimer, a saturated dispersion of 5FU in each of the three vehicles was applied on the skin. Control skin was pre-treated with plain buffer for a similar time period before applying the drug. In all these experiments, 1 mM of PAMAM dendrimer with 64 amine surface groups (G4-NH_2) was used. The influence of dendrimer pre-treatment time (2–24 h) on 5FU permeation was studied only with IPM.

2.7. Solubility studies

The solubility of 5FU was measured with all the three vehicles in presence and absence of dendrimer. An excess amount of 5FU was taken in a glass vial with or without the addition of 1 mM of G4-NH_2 dendrimer. The dispersions were incubated in a shaker water bath at 37 °C for 48 h and then centrifuged at 10,000 rpm for 10 min. This time period has been reported to reach equilibrium solubility for 5FU (Beall et al., 1993). For determining the concentration in PB, an aliquot of the supernatant was diluted with PB and the absorbance was measured at 265 nm in a UV spectrophotometer (Spectra Max, Molecular Devices, CA). For determining the solubility in MO and IPM, the method from Beall et al. (1993), was followed. Briefly, an aliquot of the supernatant was diluted with acetonitrile and the absorbance was measured at 265 nm in UV spectrophotometer. The 5FU concentration was calculated using a standard curve generated in PB (5–100 μg/ml; $r^2 = 0.999$) and acetonitrile (0.5–50 μg/ml; $r^2 = 0.999$) respectively. A similar procedure was used to check the solubility of 1 mM dendrimer in different vehicles. In case of PB, the dendrimer concentration was directly determined, while for IPM and MO, dendrimer was extracted from the supernatant using methanol. Absorbance was measured at 280 nm in UV spectrophotometer and dendrimer concentration was calculated using a calibration curve generated in PB or methanol (0.01–0.05 mM; $r^2 > 0.99$).

2.8. Epidermis/vehicle partition coefficient

The epidermis was hydrated in 1 ml of PB for 6 h prior to use. Then the epidermis was blotted dry using Whatman filter paper and weighed. Epidermis was suspended in 1 ml of 1 mM dendrimer solution in PB and for the control study, the epidermis was suspended in 1 ml of PB. After 2 h, the epidermis was washed with PB to remove any adsorbed dendrimer. Later, the epidermis was placed in a vial containing 5FU (20 mg/ml spiked with 0.1 μCi ^{14}C-5FU) dispersed in PB, MO or IPM and equilibrated in a shaker water bath at 37 °C for 48 h. To simulate the co-treatment studies, in another set of partition experiments, dendrimer and 5FU were dispersed in the vehicle along with the skin. The surface adsorbed 5FU and dendrimer was removed by washing the epidermis with 3 ml of buffer. Then the epidermis was digested using 0.5 ml of tissue solubilizer and placed in a shaker water bath at 37 °C for overnight. Radioactive counts in the vehicle and skin homogenate were measured in the liquid scintillation counter. Partition coefficient was calculated using the following equation.

$$K_{\text{epidermis/vehicle}} = \frac{\text{Drug concentration in epidermis}}{\text{Drug concentration in vehicle}} \quad (1)$$

2.9. Data analysis

For calculating the skin permeation parameters, cumulative amount of 5FU permeated per unit area of skin was plotted against time. Flux (J) was obtained from the slope of the linear portion (pseudo-steady state) of the curve and the lag time (t_{lag}) was calculated by extrapolating the linear portion of the curve to the time axis. To calculate the flux, 1–6 or 6–24 or 24–48 h was used depending on the vehicle. The correlation coefficient of linear portion of the curve was >0.99. Permeability coefficient (K_p) and diffusion coefficient (D) were calculated using the following equations

$$K_p = \frac{J}{C_v} \quad (2)$$

$$D = \frac{h^2}{6 \cdot t_{\text{lag}}} \quad (3)$$

C_v is the concentration of the drug in the respective vehicles with or without dendrimer, D is the diffusion coefficient and h is membrane thickness. Enhancement ratio (ER) was calculated by dividing the permeability coefficient of 5FU in presence of dendrimer with the permeability coefficient of 5FU in absence of dendrimer. Diffusivity ratio (DR) and partition ratio (KR) were calculated similarly by using the values in presence and absence of dendrimer. All the experiments were performed in triplicates, unless specified and the results are expressed as mean ± S.E.M. Student's t-test (Instat, GraphPad software, CA) was used to compare the treatment groups and the results were considered to be significant at $p < 0.05$.

3. Results

3.1. Influence of co-treatment of 5-FU with dendrimer

Fig. 1 shows the permeation of 5FU on co-treatment with dendrimer from different vehicles. Among the three solvents, IPM showed the highest permeation for 5FU both in absence and presence of dendrimer (Fig. 1a and b). There was no significant difference in 5FU permeation between MO and IPM till 24 h in both the control and dendrimer treatment groups. The drug was least permeable from PB and there was no significant effect of dendrimer in this solvent. Flux was calculated from 18 to 36 h for IPM and 12–24 h for MO, while for PB it was calculated from 24 to 48 h. Cumulative amount of 5FU permeated in 48 h (Q_{48}) was in the

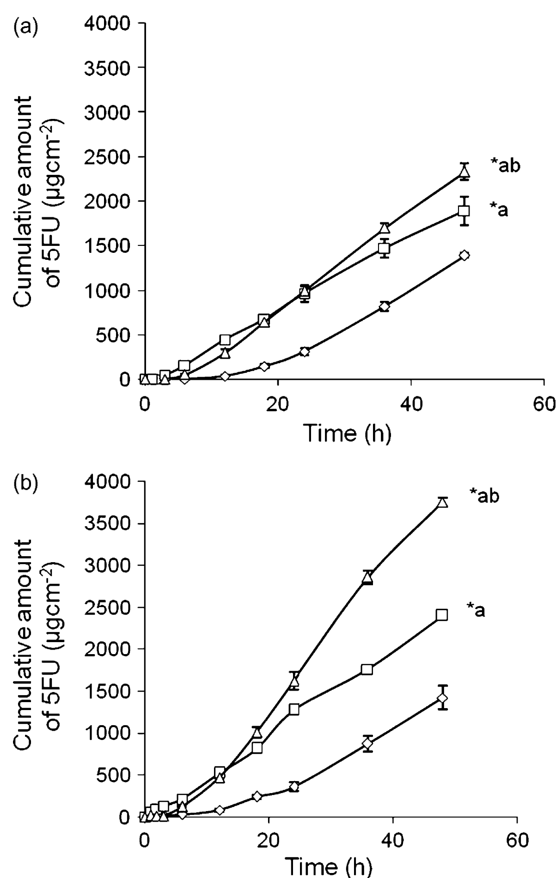

Fig. 1. Skin permeation of 5FU after co-treating the skin with (a) 5FU alone (control) and (b) 5FU in presence of 1 mM dendrimer. The cumulative amount of 5FU permeated from phosphate buffer (PB; ◇), mineral oil (MO; □) and isopropyl myristate (IPM; △). Each data point represents mean of three to four experiments and standard error. Asterisk (*) indicates that the value is significant at $p < 0.05$, 'a' is significant in comparison to PB and 'b' is significant in comparison to MO.

Table 1
Solubility, skin permeation parameters and partition coefficient of 5FU on co-treatment with dendrimer in different vehicles

Vehicle	Treatment	S (mg/ml)	J (μg cm^{-2} h^{-1})	Q_{48} (μg)	t_{lag} (h)	D (cm^2 h^{-1}, ×10^{-4})	K_p (cm h^{-1})	$K_{epidermis/vehicle}$ (×10^{-3})
PB	Control	16.8 ± 1.1	45.0 ± 1.7	886.1 ± 18.3	17.30 ± 1.35	1.1 ± 0.1	0.0027 ± 0.0001	0.8 ± 0.01
	Dendrimer	30.5 ± 3.2^{a}	44.1 ± 4.3	903.9 ± 88.9	15.97 ± 1.02	1.6 ± 0.2	0.0015 ± 0.0001^{a}	0.5 ± 0.01
IPM	Control	0.036 ± 0.004^{d}	58.3 ± 1.5^{d}	1486.1 ± 58.2^{d}	6.95 ± 0.61^{d}	2.6 ± 0.3^{d}	1.62 ± 0.04^{d}	39.7 ± 2.20^{d}
	Dendrimer	0.003 ± 0.001^{ab}	102.5 ± 1.5^{ab}	2390.3 ± 31.9^{ab}	8.18 ± 0.47^{b}	1.8 ± 0.1^{a}	37.55 ± 0.54^{ab}	1.8 ± 0.29^{ab}
MO	Control	0.012 ± 0.002^{de}	43.4 ± 1.5^{e}	1208.0 ± 102.5^{d}	2.17 ± 0.37^{de}	10.3 ± 1.4^{de}	3.49 ± 0.12^{de}	3.0 ± 0.40^{de}
	Dendrimer	0.0008 ± 0.0004^{ab}	59.9 ± 3.4^{abc}	1527.6 ± 18.3^{abc}	5.95 ± 1.00^{abc}	2.7 ± 0.4^{ab}	77.78 ± 7.01^{abc}	1.3 ± 0.17^{ab}

PB: phosphate buffer; IPM: isopropyl myristate; MO: mineral oil; J: flux; t_{lag}: lag time; Q_{48}: cumulative amount of 5FU permeated in 48 h; D: diffusivity; S: solubility in respective vehicle at 37 °C; K_p: permeability coefficient; $K_{epidermis/vehicle}$: partition coefficient between epidermis and vehicle at 37 °C. Results are presented as mean ± S.E.M. (n = 3–4). The superscript represents that the values are significant at $p < 0.05$; 'a' is significant in comparison to respective controls; 'b' is significant in comparison to PB after dendrimer treatment; 'c' is significant in comparison to IPM after dendrimer treatment; 'd' is significant in comparison to PB vehicle control treatment; 'e' is significant in comparison to IPM control treatment.

following decreasing order both in presence and absence of dendrimer IPM > MO > PB (Table 1). However, there was no significant difference in Q_{48} between control and dendrimer treatments in PB.

Solubility and other skin permeation parameters of 5FU after co-treatment with dendrimer in different vehicles are shown in Table 1. The solubility of 5FU was highest in PB followed by IPM and MO. In presence of dendrimer, the drug solubility significantly increased in PB and decreased in the lipophilic vehicles. As expected for a saturated solution, the flux of 5FU in the control group was similar in PB and MO, but was higher in IPM. This indicates that IPM, unlike the other two vehicles has an effect on the skin resulting in a higher flux. When dendrimer and 5FU were simultaneously applied on the skin, the flux increased by 1.77 times in IPM and 1.38 times in MO (Table 1). On the other hand, there was no significant effect of dendrimer in PB. The lag time of 5FU increased in presence of dendrimer when MO or IPM was used, but the increase was not significant in IPM (Table 1). In case of PB there was no effect of dendrimer co-treatment on lag time, but the lag time was highest among the three vehicles. Dendrimer decreased the diffusion coefficient of 5FU from the lipophilic vehicles, while it remained unchanged in PB (Table 1). The K_p value for 5FU in lipophilic vehicles was 3 orders of magnitude higher in comparison to PB. On co-treatment with dendrimer, K_p value further increased from the lipophilic vehicles. Since K_p is flux normalized to drug solubility, a decrease in solubility is expected to increase the value of K_p. This is evidenced by the increase in K_p by 23 and 22 times in IPM and MO respectively in presence of dendrimer (Table 1). In case of PB, K_p decreased by half in presence of dendrimer.

Drug partitioning into epidermis was highest from IPM and lowest from PB. However, in presence of dendrimer, 5FU partitioning decreased from lipophilic vehicles, while there was no significant change in PB. Dendrimer was 10 times more soluble in PB in comparison to lipophilic vehicles, but there was no significant difference in dendrimer solubility between MO and IPM (Table 2). From the results it appears that dendrimer interacts with 5FU and alters its diffusion coefficient, solubility and skin partitioning. To understand the mechanism of skin permeation, the solubility parameter of the vehicle was plotted against the enhancement ratio and solubility ratio (calculated from the 5FU solubility in presence and absence of dendrimer) on the y-axis (Fig. 2). The solubility parameters for the three solvents were taken from the literature (Pfister and Hsieh, 1990; Rosado et al., 2003). It is evident from Fig. 2 that the effect of dendrimer on 5FU solubility is dependent on the vehicle's solubility parameter. The enhancement in K_p was highest from solvent–dendrimer combination in which 5FU was least soluble. Further, dendrimer is also least soluble in the lipophilic solvents, thus providing the driving force for dendrimer partitioning into skin and resultant increase in drug's permeability coefficient.

Table 2
Solubility of dendrimer in different vehicles

Vehicle	Solubility (mM)
PB	1.21 ± 0.09^{a}
IPM	0.12 ± 0.04
MO	0.10 ± 0.00

Each value represents mean of three readings with standard error. 'a' is significant compared to other two vehicles at $p < 0.05$.

3.2. Influence of pre-treatment with dendrimer

The pre-treatment studies were carried out to understand the interaction of dendrimer with the skin independent of its interaction with the drug. Fig. 3 shows the permeation profile of 5FU from the three solvents with and without dendrimer pre-treatment for 24 h. In the control group (Fig. 3a), skin permeation of 5FU from the three solvents was in the following decreasing order IPM > PB > MO. The drug permeation was similar from both PB and MO till 12 h and at later time points the drug permeation was more in PB. After dendrimer treatment, 5FU permeation was in the following decreasing order IPM > MO > PB (Fig. 3b). Among the three solvents IPM showed the highest permeation. In the control groups, Q_{48} was similar in IPM and PB, while it was lower in MO. After dendrimer pre-treatment, Q_{48} was 1.74 and 1.36 times higher in MO

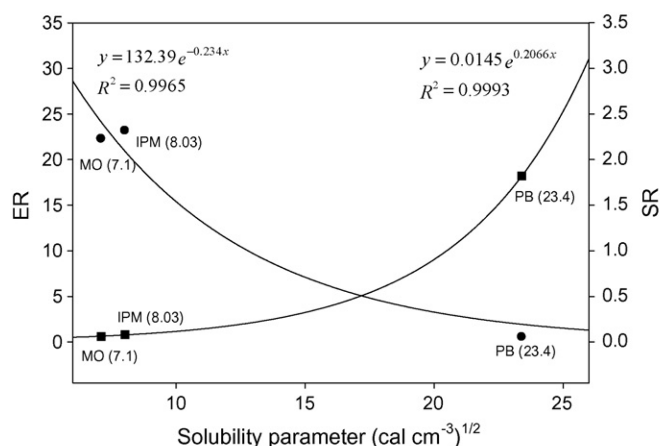

Fig. 2. Relationship between vehicle solubility parameter and enhancement ratio (ER; ●) and solubility ratio (SR; ■). Solubility parameter values for mineral oil (MO), isopropyl myristate (IPM) and phosphate buffer (PB) are 7.09, 8.03 and 23.4 (cal/cm^3)$^{1/2}$ respectively. The values are shown within parentheses in the graph. Each data point represents average of three to four experiments. ER and SR were calculated from ratios of permeability and solubility in presence and absence of dendrimer respectively. The regression equation for ER and SR are represented near their corresponding y-axis.

Table 3
Skin permeation parameters and skin partitioning of 5FU from different vehicles after pre-treatment with dendrimer

Vehicle	Treatment	J (μg cm^{-2} h^{-1})	Q_{48} (μg)	t_{lag} (h)	D (cm^2 h^{-1}, ×10^{-4})	K_p (cm h^{-1})	$K_{epidermis/vehicle}$ (×10^{-3})
PB	Control	49.3 ± 8.4	1442.5 ± 175.0	2.86 ± 1.36	6.3 ± 1.9	0.0029 ± 0.0005	0.6 ± 0.0
	Dendrimer	24.5 ± 5.0^{a}	629.3 ± 124.8^{a}	7.91 ± 1.44^{a}	2.6 ± 0.5^{a}	0.0015 ± 0.0003^{a}	0.4 ± 0.0
IPM	Control	66.9 ± 12.9	1420.6 ± 182.7	1.96 ± 0.28	8.7 ± 3.4	1.86 ± 0.36^{d}	95.2 ± 1.8^{d}
	Dendrimer	167.5 ± 9.7^{ab}	1952.1 ± 61.1^{ab}	1.52 ± 0.01^{b}	12.8 ± 0.8^{b}	4.65 ± 0.27^{ab}	170.7 ± 13.5^{ab}
MO	Control	33.6 ± 7.7	715.5 ± 87.5	1.71 ± 0.01	13.8 ± 0.2	2.16 ± 0.65^{d}	1.4 ± 0.2^{de}
	Dendrimer	103.9 ± 12.1^{abc}	1245.5 ± 193.0^{abc}	0.99 ± 0.14^{abc}	27.5 ± 1.7^{abc}	8.36 ± 0.97^{abc}	3.0 ± 0.5^{abc}

PB: phosphate buffer; IPM: isopropyl myristate; MO: mineral oil; J: flux; t_{lag}: lag time; Q_{48}: cumulative amount of 5FU permeated in 48 h; D: diffusivity; S: solubility in respective vehicle at 37 °C; K_p: permeability coefficient; $K_{epidermis/vehicle}$: partition coefficient between epidermis and vehicle at 37 °C. Results are presented as mean ± S.E.M. (n = 3–4). Pre-treatment was carried out for 24 h with 1 mM dendrimer. The superscript represents that the values are significant at $p < 0.05$; 'a' is significant in comparison to respective controls; 'b' is significant in comparison to PB after dendrimer treatment; 'c' is significant in comparison to IPM after dendrimer treatment; 'd' is significant in comparison to PB vehicle control treatment; 'e' is significant in comparison to IPM control treatment.

and IPM compared to the respective controls (Table 3). In case of PB, Q_{48} was half of the control, but compared to co-treatment studies, Q_{48} was higher for PB in the control group. The Q_{48} in pre-treatment studies was lower in control group and higher in dendrimer treated group respectively than co-treatment studies for MO. However there was no significant difference in Q_{48} between pre-treatment and co-treatment control groups for IPM. Dendrimer pre-treatment resulted in lower Q_{48} than co-treatment studies for IPM (Tables 1 and 3).

There was no significant difference in flux between the three solvents in the control group, although the flux was slightly higher in IPM (Table 3). As can be seen in Fig. 3b, for IPM and MO, the flux increased initially (1–12 h) after dendrimer pre-treatment, which decreased and tailed off later. The initial flux (1–6 h) was highest in IPM followed by MO (Fig. 3b; Table 3). However, the flux value at >24 h was not significantly different from the control flux values in these two vehicles. In contrast, the flux was significantly lower than the control in PB. The flux enhancement in pre-treatment studies was higher than co-treatment studies for the lipophilic vehicles (Tables 1 and 3). After dendrimer pre-treatment, the lag time increased in PB and decreased in MO, but there was no change in IPM. In general, the lag time was less than co-treatment studies. The diffusion coefficient was higher from the two lipophilic vehicles in comparison to PB (Table 3). Dendrimer treatment significantly enhanced the diffusion coefficient of 5FU from MO, but it was not significantly different from the control for IPM (Tables 3 and 4). On the other hand, the diffusion coefficient decreased in PB after dendrimer pre-treatment. Skin partitioning of 5FU was highest from IPM both in absence and presence of dendrimer (Table 3). In MO and IPM, the skin partitioning increased after dendrimer treatment, while it decreased in PB (Table 4). However, the increase in skin partitioning was not significantly different between MO and IPM.

The K_p value for control group in pre-treatment studies was comparable to the corresponding control values in co-treatment studies (Tables 1 and 3). After dendrimer pre-treatment, K_p values increased in MO and IPM, but was reduced by half in PB (Tables 3 and 4). In general, the variation in skin permeation parameters was higher in pre-treatment studies than co-treatment studies (Tables 1 and 3). Probably this is due to the skin hydration for prolonged period (24 h) in pre-treatment studies.

ER was plotted against diffusivity (DR) and partition ratios (KR) in Fig. 4a and b. There was no statistical difference between the correlation coefficients of the two plots even though the correlation coefficient was higher for DR compared to KR. To better define the relative influence of diffusion and partition coefficients on ER, the skin was pre-treated for various time periods with dendrimer followed by 5FU permeation studies in IPM. It was found that the increase in flux was directly proportional to the pre-treatment time (Fig. 5a). Table 5 shows the change in TEWL and skin resistance after pre-treating the skin with dendrimer for different time peri-

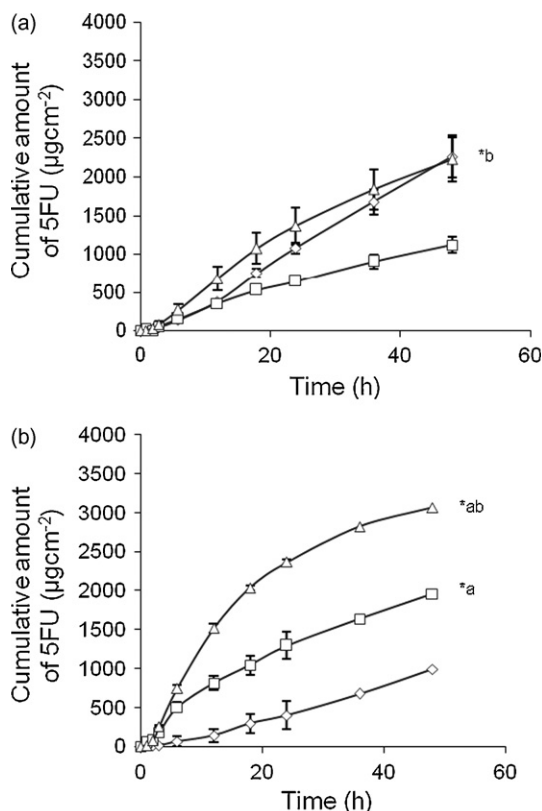

Fig. 3. Skin permeation of 5FU after pre-treating the skin for 24 h with (a) phosphate buffer (control) and (b) 1 mM dendrimer in phosphate buffer. The cumulative amount- of 5FU permeated from phosphate buffer (PB; ◊), mineral oil (MO; □) and isopropyl myristate (IPM; △). Each data point represents mean of three to four experiments and standard error. Asterisk (*) indicates that the value is significant at $p < 0.05$, 'a' is significant in comparison to PB and 'b' is significant in comparison to MO.

Table 4
Ratios of skin permeation parameters for 5FU after pre-treatment with dendrimer

	ER	DR	KR	ER*
PB	0.49 ± 0.13	0.41 ± 0.08	0.67 ± 0.08	0.27
IPM	2.50 ± 0.14^{a}	1.47 ± 0.09^{a}	1.79 ± 0.19^{a}	2.63
MO	3.87 ± 0.36^{ab}	1.99 ± 0.13^{ab}	2.14 ± 0.52^{a}	4.26

PB: phosphate buffer; IPM: isopropyl myristate; MO: mineral oil; ER, DR and KR are the ratios of 5FU permeability coefficient, diffusion coefficient and epidermis/vehicle partition in presence and absence of dendrimer from Table 2. ER* is calculated by multiplying mean DR and mean KR. The ratios are mean ± S.E.M. (n = 3–4). 'a' is significant in comparison to PB and 'b' is significant in comparison to IPM at $p < 0.05$.

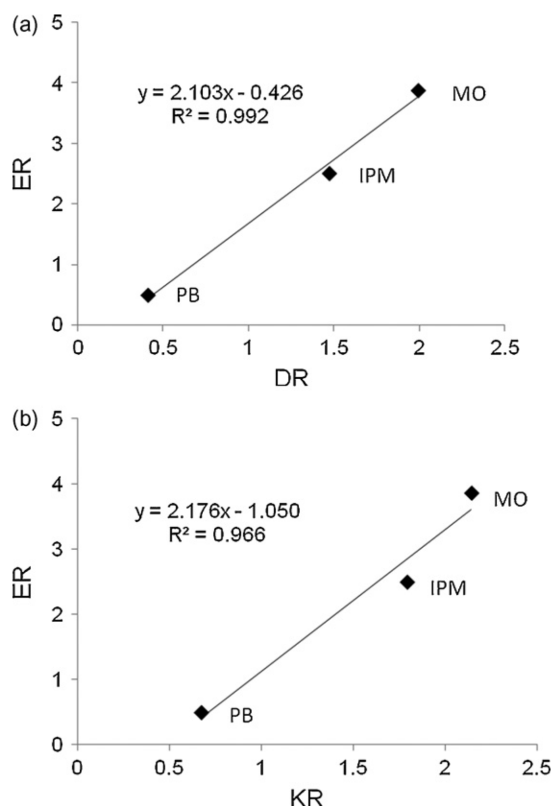

Fig. 4. Relationship of enhancement ratio (ER) with (a) diffusivity ratio (DR) and (b) partition ratio (KR). Each data point represents average of three to four experiments from the three solvent systems. The values are from Table 4.

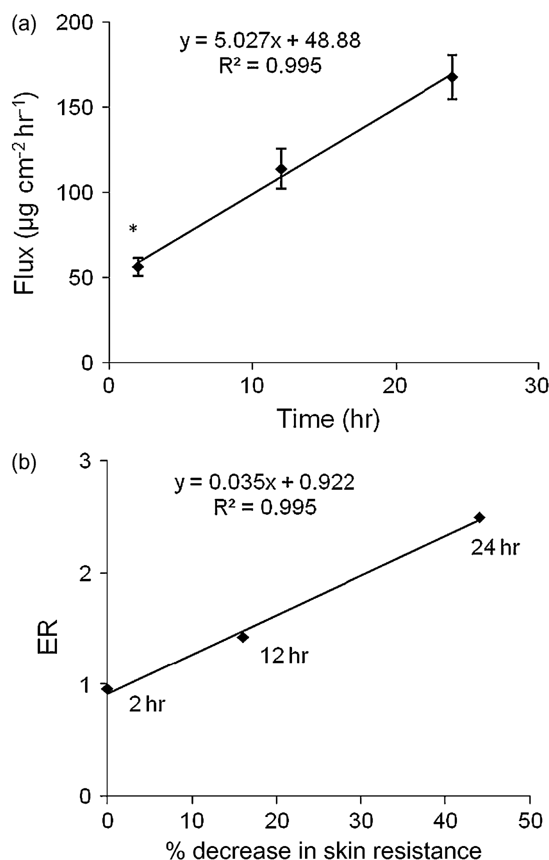

Fig. 5. Influence of dendrimer pre-treatment time on 5FU flux and skin resistance. (a) Flux of 5FU in IPM after pre-treating the skin with 1 mM of dendrimer in phosphate buffer for 2–24 h. Each data point represents mean of three to four experiments and standard error. (b) Relationship between percent decrease in skin resistance and enhancement ratio (ER) after pre-treating the skin with dendrimer for 2–24 h. ER was calculated from the ratio of 5FU permeability coefficient in presence and absence of dendrimer. The percent decrease in skin resistance is from Table 4. Each data point represents mean of three to four experiments. Asterisk (*) represents that flux after 2 h treatment is not significantly different ($p > 0.05$) from control.

ods. There was no significant change in TEWL between 2 and 12 h dendrimer treatments. On the other hand, 24 h dendrimer pre-treatment significantly increased the water loss. Similarly, there was no significant decrease in skin resistance with 2 h of dendrimer pre-treatment and the values were same as the control skin. However, skin resistance significantly decreased after 12 and 24 h of dendrimer pre-treatment (Table 5). The results indicate that dendrimer penetrates and alters the skin permeability in a time dependent manner. This is evident from Fig. 5b, which shows that the enhancement in K_P is directly proportional to the decrease in skin resistance.

4. Discussion

Dendrimers are branched polymers with a high density of functional groups which can undergo multivalent interactions with drug and biological membranes (Mammen et al., 1998). To this end, the main objective of the present study is to understand the effect of dendrimer on skin transport of 5FU, a poorly permeable model hydrophilic drug. Generally, penetration enhancers increase skin permeation by altering one or more of the parameters in the Fick's equation

$$J = \frac{KDC_v}{h} \quad (4)$$

J is flux, K is skin/vehicle partition coefficient, D is diffusion coefficient, C_v is the drug concentration in the vehicle and h is the thickness of the membrane or diffusional path length. To study the influence of these factors on permeation enhancement of 5FU both co-treatment and pre-treatment experiments were carried out.

The effect of a skin penetration enhancer is dependent on the vehicle used for delivering the drug and/or the enhancer. It is generally known that the solubility of the drug in the vehicle has a significant effect on drug permeation through skin (Higuchi, 1960). The thermodynamic activity of a drug is highest at its saturated solubility in the vehicle. Hence, at saturated solubility, the flux is expected to be similar from all vehicles, if the vehicle by itself does not have any effect on the membrane (Higuchi, 1960). In the present study, experiments were conducted at saturated solubility and as expected the flux was similar in PB and MO. However in case of IPM it interacts with the skin lipids and altered the skin barrier resulting in a higher flux (Sherertz et al., 1990). The K_p value of 5FU in IPM has been reported to be 5.4 and 0.22 cm h^{-1} in hairless

Table 5
Change in TEWL and skin resistance after pre-treating the skin with dendrimer for various time periods

Treatment time (h)	%Increase in TEWL*	%Decrease in skin resistance**
2	10.1 ± 3.2	0.0[c]
12	6.3 ± 2.3	16.3 ± 3.2[a]
24	135.9 ± 18.5[ab]	44.1 ± 6.7[ab]

Results are presented as mean ± S.E.M. (n = 3–4). 'a' is significant in comparison to 2 h treatment and 'b' is significant in comparison to 12 h treatment at $p < 0.05$; 'c' the value was similar to control value ($p > 0.05$) and hence there was no decrease in skin resistance.

* %Increase in TEWL = [(Dendrimer treatment − Control)/Control] × 100.

** %Decrease in skin resistance = [(Control − Dendrimer treatment)/Control] × 100.

mouse skin and human skin respectively (Sherertz et al., 1990). The K_p value obtained in the present study (1.86 cm h^{-1}) is consistent with these values taking into account the interspecies differences in skin permeability (Sherertz et al., 1990; Gao and Singh, 1997). Sherertz et al. (1987) found a parabolic relationship between the log K_p of 5FU and solubility parameter (δ) of the vehicle. The vehicle whose δ value was closest to δ value of 5FU (15 (cal/cm^3)$^{1/2}$) showed the highest solubility and lowest K_p value in the parabolic curve. On the other hand, 5FU K_p values were higher from those vehicles in which the drug was less soluble. Furthermore, the solvents whose δ values were closest to the skin's δ value (10 (cal/cm^3)$^{1/2}$) gave higher K_p values for 5FU. Similarly, in the present study the enhancement in K_p was highest from the solvent–dendrimer combinations in which 5FU had the least solubility (Fig. 2). Although there were only three data points, a good correlation was observed between δ and ER. The δ value of PB is 23 (cal/cm^3)$^{1/2}$ which is closest to 5FU among the three vehicles (Sherertz et al., 1987). Hence 5FU is highly soluble in PB and as a result showed lower K_p. The exact solubility parameter of dendrimer is not known, but it is probably in the range of 10–11.4 (cal/cm^3)$^{1/2}$ based on its solubility in different solvents (Uppuluri et al., 1999). Therefore, the addition of dendrimer to lipophilic vehicles is expected to shift the δ value closer to skin and enhance the K_p value of 5FU. This is also consistent with the decreased solubility of 5FU when dendrimer is added to the lipophilic vehicles. Sherertz et al. (1987) reported a decrease in 5FU solubility and an increase in K_p value with increasing concentrations of oleic acid in propylene glycol.

Earlier studies have shown that dendrimer increases the permeation of lipophilic drugs by increasing its water solubility through complexation with the surface functional groups and/or encapsulation within the dendrimer core (Chauhan et al., 2003; Yiyun et al., 2007). In the present study, dendrimer increased the water solubility of 5FU in PB from 17 to 30 mg/ml but this did not result in increased flux. It should be noted that 5FU concentration used for co-treatment studies was 20 mg/ml and therefore the drug was sub-saturated in PB-dendrimer system. Singh et al. (2005) have shown that the solubility and ionization state of 5FU plays an important role in its skin permeation. The drug is a diprotic acid with pK_a 8 and 13. At pH 7.4, 20% of 5FU is ionized, but on addition of dendrimer the pH increases to 9.0 (data not shown). This is not surprising considering the high density of amine groups on the dendrimer surface. The pK_a of surface primary amine groups and tertiary amine groups in dendrimer are 10.29 and 6.85 respectively (Diallo et al., 2004). Therefore, both the dendrimer and 5FU will be ionized in PB resulting in lesser drug permeation. Interestingly, the solubility values in presence of dendrimer is comparable to the values reported by Singh et al at pH > 7.4 (2005), which indicates that dendrimer induced pH shift increased the drug solubility in PB.

On the other hand, 5FU can also be encapsulated in the core or undergo electrostatic interactions or form hydrogen bonds with the surface amine groups in the dendrimer (Chauhan et al., 2003). This may account for the relatively lesser flux enhancement seen with co-treatment studies in comparison to pre-treatment studies. The skin permeation of ionic drugs can be increased by ion pairing with opposite ions (Hadgraft et al., 1968). However, this does not seem to be the case with 5FU, as the octanol/PB partitioning of 5FU decreased in presence of dendrimer (data not shown). Chauhan et al. (2003) suggested a cyclodextrin like mechanism for the increased flux of indomethacin–dendrimer combination. According to the authors, the dendrimer serves as a carrier and transports the drug in the solubilized form to the skin surface from where it partitions into the SC due to the high affinity of the lipophilic drug for skin lipids. Cationic peptides have been found to form salt with negatively charged fluorescein and transport it across the lipid membrane by exchanging with the anions in membrane surface (Rothbard et al., 2005). The authors noted that membrane partitioning and transport is dependent on lipophilicity of the anion. Although there is no direct experimental proof, salt formation is possible between 5FU and dendrimer. However, the skin partitioning was reduced in presence of dendrimer due to the hydrophilic nature of 5FU. Unlike in PB, the dendrimer was present at saturated concentration in the lipophilic vehicles, which results in higher skin partitioning of dendrimer and increased skin permeation of 5FU.

Alternatively, the complexation or encapsulation of 5FU with the dendrimer may serves as a depot and increase its skin permeation. The molecular weight of G4-NH_2 dendrimer is 14.5 kDa and hence the drug-dendrimer complex is expected to show a longer lag time and lower diffusion coefficient in co-treatment studies. With an amine based polymer, Akimoto et al. (1997) found a 3-fold higher enhancement for indomethacin in pre-treatment studies compared to the co-treatment studies and the authors attributed the difference to the drug-enhancer interaction in the later case. In pre-treatment studies, the control skin and dendrimer treated skin was in contact with PB for 24 h and hence the skin is more hydrated than the skin used in co-treatment studies. Hydration is generally known to alter the skin permeation parameters (Wester and Maibach, 1995) and therefore this would also account for the differences in results of pre-treatment and co-treatment studies. The pre-treatment studies showed that dendrimer penetrates and alters the skin barrier. At physiological pH, the skin is negatively charged and hence cationic molecules have greater skin affinity (Burnette and Ongpipattanakul, 1987). Using fluorescent labeled dendrimers we found that amine terminated dendrimers were able to penetrate the SC better than anionic or neutral dendrimers (unpublished results). Studies in model lipid bilayers and cell cultures have shown that amine terminated dendrimers interact with the lipid bilayers and enhance the transport of molecules (Mecke et al., 2004; Shcharbin et al., 2006). Dendrimers have been reported to interact with the negative phosphate head groups of model phospholipids and fluidize the lipid bilayers (Gardikis et al., 2006). Although phospholipids are not present in the skin lipid matrix, dendrimer can interact with the polar head groups of skin ceramides and free fatty acids. To attest to this claim, we found changes in lipid stretching peaks of skin in FTIR spectrum after dendrimer treatment and the results will be reported in a separate publication.

Transepidermal water loss and skin resistance measure the change in skin barrier caused by alterations in intercellular lipid bilayer (Rastogi and Singh, 2001; Gao and Singh, 1997). Significant changes in TEWL values and skin resistance are a clear indication of the interaction of dendrimer with the skin lipids. The skin resistance measurement was more sensitive in detecting the changes in skin barrier after dendrimer treatment in comparison to TEWL. Such differences in the sensitivity of TEWL and skin impedance measurements have been reported earlier (Kalia et al., 1996). Unlike skin resistance, impedance gives a true measure of change in both the resistive and capacitive components of the skin. Nevertheless, skin resistance measurement gives a gross estimate of the change in skin barrier properties. Shcharbin et al. (2006) reported a decrease in electrical resistance of a model lipid bilayer after treatment with amine dendrimer. In the present study, the percent reduction in skin resistance directly correlated to the permeation enhancement caused by dendrimer (Fig. 5b). Therefore, perturbation of the lipid bilayer by dendrimer reduces the diffusional resistance for drug transport.

The large molecular size results in slower skin diffusion of dendrimer and hence its effect is time dependent. A smaller cationic penetration enhancer such as dodecylamine increased 5FU permeability by 75-fold (Aungst et al., 1990) by altering the skin barrier

and increasing the drug solubility in the vehicle. After dendrimer treatment, a rapid rise in flux was followed by a drop in flux. Such effects have been observed with small penetration enhancers as a result of washout of the enhancer from the skin by the vehicle (Goodman and Barry, 1988). This is highly unlikely with dendrimer due to its large molecular size. Alternatively, as suggested by one of the reviewers, the dendrimer may partition back into the vehicle causing a decrease in flux. Using confocal microscopy we found that dendrimers penetrate only to a depth of 100 μm in the skin (unpublished results) which supports this possibility. However, further studies are required to understand this aspect.

From the safety point of view, the dendrimer may be beneficial as it only produces a transient effect, unlike the deeper penetration and skin irritation caused by small penetration enhancers. Aoyagi et al. (1990) compared the skin penetration enhancing properties of a series of alkylammonium monomers and polymeric enhancers synthesized using the same monomers. Both monomers and polymers enhanced the skin permeation of 5FU by altering the skin barrier. However, the monomers penetrated deeper inside the skin to the dermis and caused skin irritation. On the other hand, the polymeric enhancer was retained in SC and did not cause skin irritation (Aoyagi et al., 1991; Akimoto et al., 1997). In contrast to linear polymers, dendrimers are branched monodisperse polymers which allow precise control of size, shape and placement of functional groups. Thus dendrimers combine the typical characteristics of small organic molecules and polymers as a new class of skin penetration enhancers.

Except in PB, the skin partitioning of 5FU increased from other vehicles on pre-treatment with dendrimer. The skin penetration of dendrimer provides a hydrophilic environment for the drug to partition from the lipophilic vehicles and increases the drug's solubility in the skin. On the other hand, PB is more hydrophilic than the hydrophilic environment provided by the dendrimer pre-treated skin and hence the skin partitioning of 5FU was not altered. Earlier Akimoto et al. (1997) reported increase in skin partitioning of a hydrophilic model drug after pre-treatment with an amine based polymeric enhancer. Williams and Barry (1991) have shown that the skin partitioning of 5FU decreased after pre-treating the skin with lipophilic penetration enhancers such as terpenes. On the other hand, terpenes acted as good penetration enhancers for lipophilic drugs by increasing the drug solubility and partitioning in skin (Williams and Barry, 1991). In addition to increasing the drug penetration, the dendrimer may also increase the vehicle penetration into skin and this has been shown with other enhancers (Aungst et al., 1990). As stated earlier, the higher skin partitioning and permeation seen with IPM compared to the other vehicles is because of its ability to interact with skin lipids and alter the barrier (Sherertz et al., 1990). At the same time, the influence of dendrimers on transcellular pathways cannot be ruled out. Moghimi et al. (1996) indicate that transcellular hydrophilic corneocytes also may be an important pathway for the transport of hydrophilic molecules such as 5FU.

The permeability coefficient is a product of partition coefficient and diffusion coefficient divided by the membrane thickness. In the present study, the ER calculated from the product of DR and KR in Table 4 is fairly close to the experimentally determined ER. Other investigators have reported similar findings for 5FU with terpenes (Williams and Barry, 1991; Moghimi et al., 1996). For DR to be directly related to ER, the partition ratio should be constant given by the slope of the line assuming that the skin thickness remains constant. This assumption may not represent the true situation, as the enhancer treatment may alter the effective path length for drug transport (Williams and Barry, 1991). Overall the findings show that dendrimer increases the skin permeation of hydrophilic drugs by altering the skin barrier and increasing drug solubility in the skin.

5. Conclusions

Dendrimer increased the skin permeation of 5FU when delivered using lipophilic vehicles by altering the skin barrier. On the other hand, dendrimer interacts with the drug if applied simultaneously and increases the drug's permeability coefficient by decreasing its solubility in the vehicle. The study demonstrates the use of dendrimer as a polymeric skin penetration enhancer. Influence of dendrimer surface charge, generation and concentration on skin permeation of 5FU will be reported subsequently.

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