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Novel dendritic derivatives of unsaturated fatty acids as promising transdermal permeation enhancers for Tenofovir

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This study was aimed at exploring the potential of unsaturated fatty acids (UFAs) [palmitoleic (PA), linoleic (LA), linolenic (LLA) and arachidonic acid (AA)], and their newly synthesized dendritic esters [PA1E, LA1E, LLA1E and AA1E] having basic tertiary nitrogen as the branching element as transdermal permeation enhancers for the delivery of tenofovir. The structures of the derivatives were confirmed by FTIR, NMR (^{1}H and ^{13}C) and HRMS. The *in vitro* cytotoxicity study revealed their biocompatibility. Amongst the UFAs, only PA and LLA exhibited transdermal enhancer potential [enhancement ratio (ER) of 2.9 and 1.35 respectively]. All synthesized derivatives at 1% w/w were found to be more effective enhancers as compared to their parent UFAs, with LLA1E being identified as the most superior (ER = 5.31). Further, the concentration effect study revealed that at 2% w/w LLA1E had a greater ER (6.11) as compared to its parent (ER=3.85). The permeability data correlated with the observations made in the histomorphological and transepithelial electrical resistance (TEER) evaluations. There was no significant loss in the integrity of the epidermis, transcellular and intercellular route of transport across the epidermis, with drug and enhancer treatment having no permanent damage on the epidermis. The novel dendritic ester derivatives of the UFAs therefore can be considered as effective transdermal permeation enhancers.

Introduction

Transdermal drug delivery (TDD) involves the transport of drugs across the epidermis, into the blood vessels located within the dermis resulting in systemic distribution of a desired drug [1, 2]. This route avoids the first pass effect for drugs that are rapidly metabolized by the liver when taken orally. Drugs that require consistent plasma concentrations are very good candidates, as peaks and troughs in plasma level can be avoided. Since this route avoids direct effects on the gastrointestinal tract (GIT), drugs that cause GIT distress would benefit from TDD. These systems can be used as a substitute delivery method for patients who cannot tolerate oral dosage forms. The relative ease of these systems is due to the possibility of self-administration, with drug therapy being terminated rapidly by removal of medication from site of application. This simple, painless and non-invasive application lends to improved patient compliance and comfort [1, 3-6]. The use of TDD is therefore highlighted as being especially suitable for pediatrics as compared to several oral formulations [7, 8]. TDD has the potential to improve bioavailability of various drugs, decrease dosages required and reduce drug side effects. Controlled drug release can also be achieved using this system, resulting in decreased frequency of administration [9, 10]. TDD can therefore enhance therapeutic effects, compliance and adherence. In light of these benefits various classes of drug and permeants have therefore been explored for their transdermal permeation potential. These include amongst others tenoxicam, aceclofenac, ketoprofen [11], diclofenac sodium [11, 12], peroxicam[13], testosterone [14], niacinamide [15], vitamin B_{12} [16], donepezil [17], lamivudine [18], Stavudine [19], zidovudine [20-22] and pyrimidinedione IQP-410 [23]. There are already many commercially available transdermal drug patches and topical applications in the market for numerous acute and chronic disease conditions. Additionally, many drugs proposed for transdermal delivery are currently within various stages of clinical trials. TDD therefore has the potential to facilitate the transport of a huge selection of drugs that can treat various disease conditions [24, 25].

The major shortcoming of drug delivery across the skin is its natural barrier property. This barrier property of the skin is mainly due to the thin outer layer of the epidermis, stratum corneum (SC). The SC is commonly termed the “brick and motar” structure due to the structurally organized proteins and lipids in this protective layer. The structured lipids found in this layer function to prevent water loss from the body, and thereby impede the movement of most topical applied permeants through the skin, unless they are of a low molecular weight and lipid-soluble [4, 12, 26]. This barrier prevents movement of permeants from the superficial surface of the skin. In order to deliver therapeutically relevant doses of a drug across the skin, strategies need to be developed to overcome the hindrance provided by the SC.

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Literature reports physical and chemical methods employed to modify the barrier properties of the SC, such as the use of chemical penetration enhancers (CPEs) as excipients to promote permeation enhancement is often considered[27-30]. CPEs could act through one or more of the following mechanisms (i) by improving partition of the drug into the SC, (ii) by interaction with the intercellular protein, (iii) alteration of SC highly ordered lipid structure and its fluidity [28, 31, 32].

Being endogenous constituents of skin, fatty acids (FAs) are the most attractive skin permeation enhancers employed for TDD [13, 33]. The physicochemical properties of a compound can determine its efficacy as a permeation enhancer. A variety of contributing factors such as carbon chain length, structural configuration and lipophilicity of a compound have been implicated as factors that determine the effectiveness of a transdermal permeation enhancer [17, 27, 28]. Depending on their subclass, FAs retain differing structural properties which can influence their efficacy as transdermal penetration enhancers. These properties include variations in carbon chain length, number, position and configuration of their double bonds as well as their branching substituents [13]. FAs can decrease the barrier properties of the SC by disturbing the packing of the lipid bilayer, thereby increasing its fluidity[12, 31, 33]. A large selection of saturated and unsaturated fatty acids (UFAs) such as lauric acid, myristic acid, capric acid, oleic acid (OA), palmitoleic acid (PA), linoleic acid (LA), linolenic acid (LLA) and arachidonic acid (AA) have been studied as transdermal permeation enhancers [13, 34]. However, OA, an 18 carbon chain monounsaturated FA has been reported as being the most prevalent FA for having transdermal permeation enhancement. In addition literature shows evidence that saturated FAs are less effective in enhancing transdermal permeation as compared to their unsaturated counterparts [17, 27, 35]. Though there is ongoing intensive search for new chemical entities, discovery and development of new CPEs is at very slow rate [9].

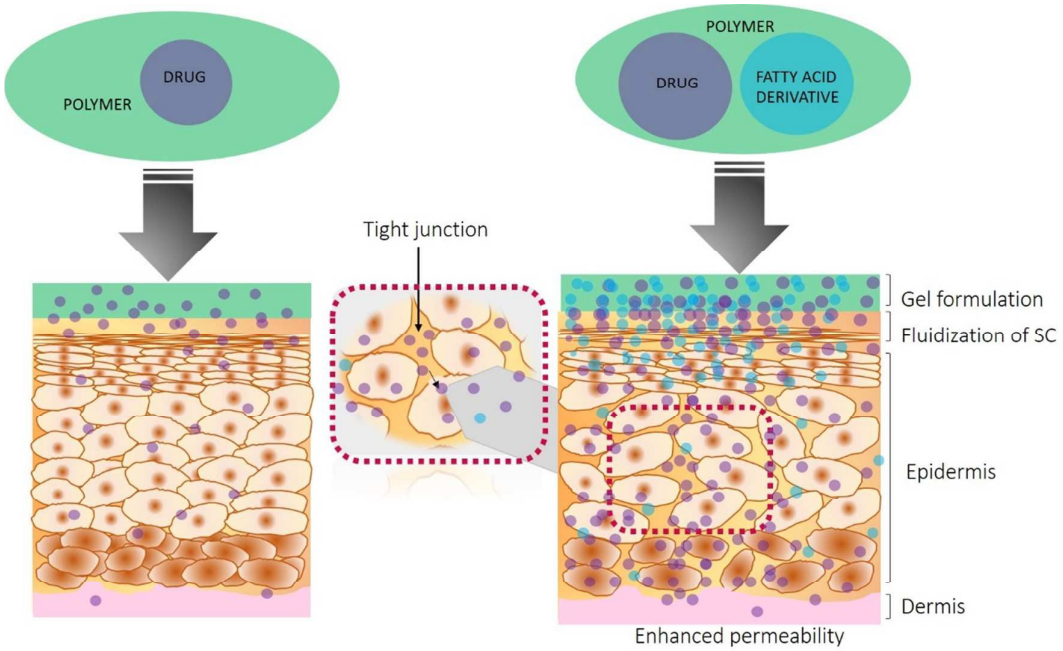
The findings of many reported studies support the notion that derivatives of parent compounds exhibit greater permeation enhancement efficacy[12, 27, 36-39]. Therefore, there is a justified need to synthesize novel derivatives from compounds that display positive CPE properties. The derivatives of FAs such as, diethyl succinate, diethyl adipate, diethyl sebacate, diisopropyl adipate [38] and oleodendrimers from OA [12] have all shown promising transdermal permeation enhancement of the drug studied, which has been attributed to increased lipophilicity of the FA derivatives. FAs and their ester derivatives have been extensively studied as transdermal permeation enhancers for various classes of drugs other than ARVs [12, 38]. The only exception is OA which was studied as an enhancer for the transdermal delivery of zidovudine [22]. Previous findings suggest that functionalization of OA with dendritic esters as head groups significantly improves the transdermal permeation of diclofenac sodium [12] and buccal permeation of didanosine [39], an ARV drug. The possible enhanced drug permeation by dendritic ester derivatives of other UFAs remain to be explored. Therefore, the evaluation of UFAs as well as the synthesis, characterization and to assess the transdermal permeation enhancement efficacy of their novel dendritic ester derivatives will contribute to expanding the pool of available safe and effective excipients for transdermal delivery systems.

Oral antiretroviral (ARV) drug therapy has significantly improved the treatment of human immunodeficiency virus (HIV) infection and acquired immune deficiency syndrome (AIDS) [40, 41]. However, some ARV drugs have low bioavailabilities due to extensive gastrointestinal degradation, first pass metabolism and short half-lives, requiring high doses and frequent drug administration. This leads to severe dose-dependent side-effects, which further exacerbate the decrease in patient compliance [42]. These limitations with oral therapy negatively impact on the effective treatment of HIV and AIDS. The HIV and AIDS crisis coupled with limitations of oral ARV therapy therefore drive the need for identifying innovative drug delivery strategies. In order to address these limitations of oral ARV treatment, formulation scientists have been exploring the use of novel drug delivery systems and alternate routes of drug administration [42]. Alternate routes of drug administration that obviate the gastrointestinal tract as well as hepatic first pass metabolism and deliver drug directly into the systemic circulation will be beneficial to many ARVs that are susceptible to the above mentioned limitations [43, 44]. More specifically, in addition to the buccal [39, 45-47], rectal [48] and vaginal [48] routes, transdermal delivery of ARV drugs is receiving increasing interest and is currently being investigated as an alternate route of administration for overcoming limitations associated with oral ARV therapy [7, 23, 42, 49].

TNF is a nucleotide analog which is widely used in the treatment of HIV and AIDS. Although there are limitations to the administration of TNF, it is still currently considered the most successful drug to HIV infection [50]. Due to its hydrophilicity and poor intestinal permeability, it is currently administered orally as the ester prodrug, tenofovir disoproxil fumarate. The dosage is one 300 mg tablet taken once daily, with a low bioavailability of 25%[51]. Additionally, there are numerous GIT associated side effects reported for oral TNF administration leading to a decrease in patient compliance[52, 53]. Alternate routes of TNF administration that have been reported to overcome its disadvantages include buccal [45] and the rectal and vaginal route [48]. The transdermal permeability potential of TNF has not been reported to date. TDD of TNF could allow administration of the drug directly into systemic circulation in it's basic to native form instead of synthesizing the ester prodrug, thereby decreasing manufacturing costs. It would also obviate the drug from passing through the GIT; thereby eliminating the GIT associated side effects, hence increasing patient compliance. TDD system for TNF could also require lower the loading dose to achieve adequate bioavailability for therapeutic efficacy. Thus there would be benefits both to the patient and product manufacturer.

Fig 1. Illustrates transdermal permeability due to a combination of the specified drug and fatty acid derivative within a polymer medium that supports the formulation. The fatty acid derivative would interact with the integrity of the outer most barrier, the stratum corneum (SC), facilitating enhanced transdermal drug permeability via the two potential routes of transport viz. intercellular or paracellular. The concentration of the fatty acid derivative is a crucial aspect in designing and implementing successful formulations for enhanced transdermal drug delivery. Therefore, the aim of this study was to identify the transdermal permeability potential of TNF and to explore the applicability of various UFAs (PA, LA, LLA and AA) as well as their respective newly synthesized dendritic ester derivatives (PA1E **2**, LA1E **3**, LLA1E **4**, and AA1E **5**) as potential CPEs for the transdermal delivery of TNF.

Fig. 1 Illustrates enhanced transdermal drug permeability through the application of novel fatty acid derivatives and drugs within a polymer



formulation

Results and discussion

Characterization and stability of newly synthesized dendritic esters

FT-IR, ^{1}H NMR, ^{13}C NMR and HRMS techniques were used to characterize the synthesized ester derivatives of UFAs. Transformation of acid function of UFA into ester was easily monitored using FT-IR by observing the peak around 1710-1730 cm^{-1}.

PA1E **2**

^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.89 (t, 3 H, J = 13.6 Hz), 1.25-1.30 (m, 16 H), 1.44 (s, 18 H), 1.59-1.66 (m, 4 H), 1.79 (qt, 2 H, J = 28 Hz), 2.03 (q, 4 H, J = 20 Hz), 2.04-2.35 (m, 4 H), 2.49 (t, 2 H, J = 16 Hz), 2.73 (m, 4 H), 4.1 (t, 2 H, J = 12.9), 5.32-5.36 (m, 2 H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 14.09, 22.64, 24.98, 26.68, 27.16, 27.21, 28.09, 28.98, 29.11, 29.16, 29.18, 29.69, 31.77, 33.80, 34.35, 49.40, 50.14, 62.44, 80.31, 129.76, 129.98, 171.98, 173.86. HRMS (ES-TOF): $[M]^+$ calcd for $C_{33}H_{61}NO_6H^+$ 568.45; found 568.4619.

LA1E **3**

^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.87 (t, 3 H, J = 13.4 Hz), 1.29-1.33 (m, 14 H), 1.42 (s, 18 H), 1.59-1.61 (m, 2 H), 1.72-1.75 (m, 2 H), 2.05 (q, 4 H, J = 19.2 Hz), 2.24-2.33 (m, 6 H), 2.47 (t, 2 H, J = 13.2), 2.67-2.76 (m, 6 H), 4.07 (t, 2 H, J = 12.8), 5.29-5.36 (m, 4 H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 14.04, 22.54, 24.96, 25.6, 26.65, 27.17, 27.87, 28.07, 29.10, 29.13, 29.16, 29.32, 29.58, 31.50, 33.77, 34.32, 49.39, 50.13, 62.41, 80.28, 127.89, 128.01, 130.01, 130.17, 171.91, 173.79. HRMS (ES-TOF): $[M]^+$ calcd for $C_{35}H_{63}NO_6H^+$ 594.47; found 594.4768.

LLA1E **4**

^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.99 (t, 3 H, J = 15.2 Hz), 1.31 (br s, 8 H), 1.44 (s, 18 H), 1.69 (t, 2 H, J = 39.6), 1.71-1.77 (m, 4 H), 1.78-2.09 (m, 2 H), 2.26-2.35 (m, 6 H), 2.45 (t, 2 H, J = 14), 2.69 (m, 4 H), 2.82 (m, 4 H), 4.09 (t, 2 H, J = 12.88), 5.40 (m, 6 H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 14.26, 20.54, 24.97, 25.52, 25.60, 26.68, 27.20, 28.09, 29.12, 29.14, 29.18, 29.58, 33.80, 34.34, 49.40, 50.14, 62.45, 80.30, 127.11, 127.71, 128.25, 128.28, 130.26, 131,95, 171.97, 173.84. HRMS (ES-TOF): $[M]^+$ calcd for $C_{35}H_{61}NO_6H^+$ 592.45; found 592.4572.

AA1E **5**

^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.90 (t, 3 H, J = 12 Hz), 1.25-1.36 (m, 6 H), 1.42 (s, 18 H), 1.68-1.77 (m, 4 H), 2.04-2.35 (m, 6 H), 2.45-2.49 (m, 6 H), 2.73 (t, 2 H, J = 40 Hz), 2.79-2.82 (m, 4 H), 2.83-2.85 (m, 4 H), 4.10 (t, 2 H, J = 16 Hz), 5.34-5.40 m (8 H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 14.10, 22.56, 24.83, 25.61, 26.59, 26.91, 27.21, 28.09, 28.28, 29.69, 31.51, 33.72, 34.66, 49.39, 50.14, 62.53, 80.30, 127.54, 127.86, 128.17, 128.20, 128.57, 128.79, 128.99, 130.47, 171.96, 173.57. HRMS (ES-TOF): $[M]^+$ calcd for $C_{37}H_{63}NO_6H^+$ 618.47; found 618.4776.

All the synthesized dendritic derivatives are prone to oxidation and hydrolysis on long term storage due to the unsaturation in aliphatic chain and the presence of ester linkages. Therefore, samples were stored in an inert atmosphere in amber coloured bottles at 4 ^{o}C to avoid their degradation. The stability of samples stored at these conditions for > 2 years was confirmed by comparing their physical appearance and ^{13}C NMR data with the freshly prepared samples. There was no change in physical appearance and the spectra were

identical. The most important observations were presence of unsaturated and ester carbons in the range of 127-130 and 171-174 ppm respectively (Table S1 and Fig. S1-S8 of Supporting Information). The presence of these characteristic peaks and absence of any other extra peaks in the samples stored in an inert atmosphere for > 2 years was a strong indication of the stability of the synthesized materials under the given storage conditions.

Acid-base titration

The synthesized dendritic lipids were expected to be basic in nature due to the presence of tertiary nitrogen in their structure. An Acid-base titration study was performed to confirm their basicity. In the case of the blank sample (0.1 M NaCl), addition of only 10 µl of 1 M HCl resulted in a steep decrease in pH from 13 to 3.4 indicating no buffering capacity. On the contrary, there was no sudden fall of the pH upon addition of 1 M HCl to the solutions of dendritic lipids. The horizontal trend of the plot for the dendritic lipids confirmed the considerable buffering capacity of the dendritic lipids over almost the
entire pH range (Fig 2). The results obtained proves the basic nature of the synthesized dendritic lipids and are in good agreement with previous findings where pH responsive poly(L-histidine) exhibited the same trend in the graph of HCl versus pH [54].

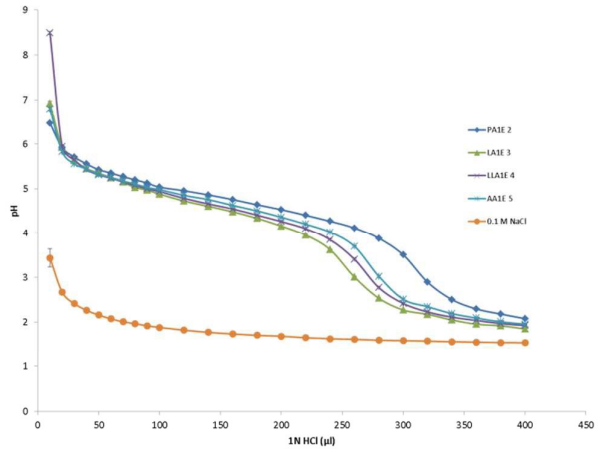


Fig. 2 Acid-base titration profile of dendritic derivatives and 0.1 M NaCl (n = 3; RSD < 10%).

Cytotoxicity evaluation

Following the successful synthesis and characterization of all the FA derivatives (PA1E **2**, LA1E **3**, LLA1E **4**, and AA1E **5**), an *in vitro* cell culture system was used to determine the biological safety of these derivatives. Cytotoxicity evaluations were performed on HeLa cells using the MTT assay. The assay is based on the biochemical reduction of MTT dye by viable cells by testing their enzymatic dehydrogenase activity [55]. The MTT assay results showed that none of the synthesized FA derivatives were toxic against the HeLa cell line. The percentage cell viability was between 85 to 95 % (Fig 3) for all derivatives for the concentration ranges studied. There were also no dose dependent trends observed on the % cell viability of HeLa cells within the concentration range studied (Fig 3). A one-way ANOVA, with a non-parametric Kruskal-Wallis test revealed that there were no significant differences between the results obtained for all the synthesized derivatives when compared to the control (p = 0.7625). These findings therefore suggest that the use of the synthesized FA derivatives in biological studies would be safe.

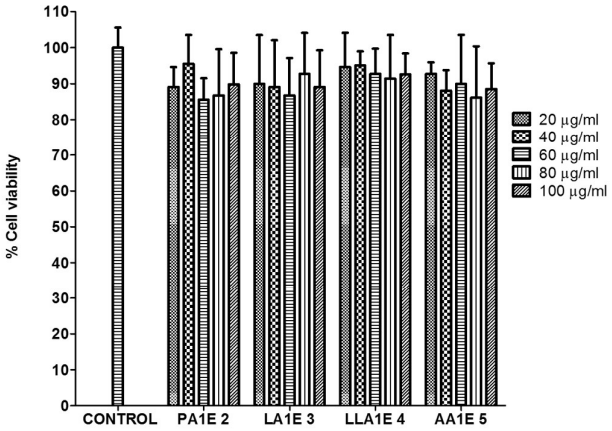


Fig. 3 Cytotoxicity assay displaying percentage cell viability after exposure to various concentrations of the FA derivatives on HeLa cells. Results are presented as mean ± SD. (n = 6).

***In Vitro* transdermal permeation**

I) Permeability of TNF in absence of enhancers

In this study we report for the first time the transdermal permeability potential of TNF. The *in vitro* experiments revealed that at the end of six h, the cumulative amount of TNF that permeated through the skin was 226.85 ± 33.89 µg/cm^2 (Table 1, Fig 4). TNF was able to permeate the skin without any permeation enhancer with a steady state flux value of 31.48 ± 6.21 µg/cm^2 h (Table 1). This permeability parameter is similar to a study by Singh *et al.*, where the transdermal permeability potential of the ARV zidovudine was confirmed. This study reported a flux value of 38.2 µg/cm^2 h for the zidovudine treatment without enhancer [22]. Permeants can transverse the skin either via the intercellular or the paracellular route, or both routes simultaneously, to cross the SC and reach the blood vessels located in the dermis. The route across the SC by the permeant is determined by its physicochemical properties[31]. Since TNF is hydrophilic its preferred route of transport could be through the intracellular route, via passive diffusion [56]. The permeability parameters displayed by TNF suggest that it can be exploited for delivery via the skin as an alternate drug delivery route to overcome the limitations presented when administered orally.

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Table 1. Effect of the various derivatives as compared to their parent lipid on the transdermal permeability properties of TNF. * Indicates significant difference i.e $p < 0.05$ (all values compared to control)

	Treatment	*Amount permeated* ($\mu g/cm^2$)	*Jss (flux)* ($\mu g/cm^2.h$)	*Permeability* ($P \times 10^{-2}$)	*ER*	*P value*
Control	TNF	226.85 ± 33.89	31.48 ± 6.21	0.157 ± 0.03	1	
Enhancer 1	LA	196.18 ± 22.46	29.398 ± 2.53	0.146 ± 0.01	0.93	0.1658
Enhancer 2	LA1E **3**	514.79 ± 26.89	82.308 ± 4.33	0.411 ± 0.02	2.61	0.2039
Enhancer 3	LLA	560.72 ± 20.01	*91.316 ± 3.244	0.456 ± 0.16	2.9	0.0243
Enhancer 4	LLA1E **4**	1014.57 ± 24.90	*167.3 ± 3.49	0.836 ±0.17	5.31	0.0066
Enhancer 5	AA	189.67 ± 19.30	29.82 ± 3.78	0.149 ± 0.18	0.94	0.3262
Enhancer 6	AA1E **5**	510.18 ± 15.19	83.466 ± 1.58	0.417 ± 0.008	2.65	0.0832
Enhancer 7	PA	269.99 ± 5.16	42.588 ± 0.69	0.212 ± 0.004	1.35	0.6860
Enhancer 8	PA1E **2**	707.84 ± 37.36	*110.3 ± 10.73	0.551 ± 0.05	3.5	0.0039

II) Effects of UFAs on TNF permeability

Due to the barrier properties of the skin being an obstacle to the movement of drugs across the SC of the skin, permeation enhancers are employed to aid the transport of drugs to ensure therapeutically relevant doses reach the systemic circulation [17, 27]. Numerous CPE's have been described in the literature, with FAs being advocated as the most promising for the enhanced delivery of hydrophilic compounds through skin [27, 31, 57]. This study therefore explored the use of PA, LA, LLA and AA, and their respective newly synthesized derivatives PA1E **2**, LA1E **3**, LLA1E **4**, and AA1E **5** as potential permeation enhancers for the transdermal delivery of TNF.

The results indicate that amongst the four FAs studied at a concentration of 1% w/w; only LLA and PA were able to enhance the permeability of TNF across the skin (Table 1, Fig 4). LLA increased the steady state flux and cumulative amount of TNF permeated from 31.48 ± 6.21 to 91.316 ± 3.244 $\mu g/cm^2$ h and 226.85 ± 33.89 to 560.72 ± 20.01 $\mu g/cm^2$, respectively with an ER of 2.9 (Table 1, Fig 3). The increases in the permeability parameters due to the addition of LLA were statically significant ($p = 0.0243$) (Table 1). The ER displayed by LLA is comparable to a report by Wen *et al*., where an ER of 3.10 was achieved using span 80 as a transdermal enhancer for the delivery of daphnetin across rat skin [58]. PA was also able to increase the permeability of TNF with an increase in the steady state flux to 42.588 ± 0.69 $\mu g/cm^2$ h and cumulative amount permeated to 269.99 ± 5.16 $\mu g/cm^2$, with an ER of 1.35 (Table 1, Fig 4). However, these increases in the permeability parameters were not statistically significant ($p = 0.6860$) (Table 1). Although the increases are not statistically significant, the ER is still within the reported range for transdermal permeation enhancers, such as methanol (ER=1.17) and Oleic acid (ER=1.52) for the delivery of daphnetin [58]. In a separate study, tween 80 as an enhancer was also able to increase the ER of zidovudine to 1.4 as compared to the unenhanced control [22].

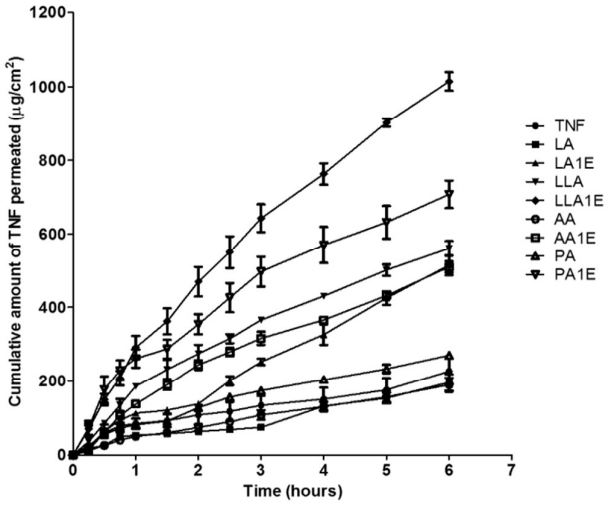
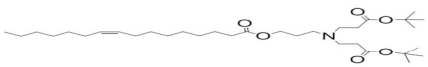


Fig 4. The effect newly synthesized derivatives as compared to their parent FA on the cumulative amount of TNF permeated across the skin. Results are presented as mean ± SD. (n = 6).

The literature reports that permeation enhancement using UFAs is related to carbon chain length, the degree of unsaturation i.e. the number of double bonds present and the "kink" isomer at the double bond [17, 27, 30, 59, 60]. Of the four FAs studied, PA has the lowest molecular weight of 254.41 $g.mol^{-1}$ (Table 2).

Table 2. Structure, molecular formula, number of unsaturations and molecular weight of the UFA/derivative studies

UFA/Derivative	*Structure*	*Molecular formula*	*Molecular weight (g.mol^{-1})*	*Number of unsaturations*
PA		$C_{16}H_{30}O_2$	254.41	01
LA		$C_{18}H_{32}O_2$	280.45	02
LLA		$C_{18}H_{30}O_2$	278.44	03
AA		$C_{20}H_{32}O_2$	304.47	04
PA1E **2**		$C_{33}H_{61}NO_6$	567.85	01
LA1E **3**		$C_{35}H_{63}NO_6$	593.89	02
LLA1E **4**		$C_{35}H_{61}NO_6$	591.87	03
AA1E **5**		$C_{37}H_{63}NO_6$	617.91	04

It is probable that the low molecular weight of PA was responsible for the slight degree of enhancement observed, as lower molecular weight compounds permeate the skin more readily [32, 61]. Although LLA has a slightly higher molecular weight (278.44 g.mol^{-1}) than PA (Table 2), its molecular weight is still lower than that of LA and AA which therefore places LLA at a slighter advantage. A combination of carbon chain length and number of double bonds present in LLA sets it apart from the other FAs studied (Table 2). Reports from extensive experiments show that UFAs with a carbon length of C_{18} are optimal for permeation enhancement [27]. Studies also describe the bent *cis*-configuration of UFAs disturbs intercellular lipid packing and creates mobile free volumes that could increase the fluidity, allowing molecules to enter the free volumes of the kinks at the double bonds and migrate across the membrane thereby lending to the permeation enhancement effect of these compounds. The number of kinks tends to increase with the number of double bonds in the UFA. Therefore the permeation enhancement effect of UFAs are expected to increase with the increase in the number of double bonds [17, 27, 59, 60]. Although LA and LLA have the same carbon chain length of C_{18}, LLA has a slightly lower molecular weight, and also possesses one more double bond than LA (Table 2). On the other hand AA has a greater number of double bonds, a longer carbon chain length of C_{20}, and a higher molecular weight of 304.46 g.mol^{-1} (Table 2) when compared to LLA. PA has a shorter carbon chain length of C_{16}, and only one double bond (Table 2) which therefore renders PA, LA and AA less effective as transdermal permeation enhancers when compared to LLA. Amongst the FAs studied LLA possesses the ideal properties that constitute an optimal UFA transdermal permeation enhancer as described above, and therefore displayed superior permeation enhancement of TNF.

III) Effects of novel dendritic derivatives on TNF permeability

The study further determined the effect of structural modifications to the UFAs via the transformation of carboxylic acid function into a branched tert-butyl ester function on the permeation enhancement efficacy. The findings of the study were compared to both the respective parent material as well as to the control. The addition of the branched tert-butyl ester functional group to the periphery of the parent FAs increases their liphophilicity [12, 39]. All the derivatives synthesized in this study had a higher lipophilicity as compared to their respective parent FA as indicated by the calculated log *P* values (Table 3) [62].

Table 3. Log *P* values of UFAs and their respective newly synthesized derivatives.

UFA/ Derivative	Log *P*
PA	5.89
PA1E	7.95
LA	6.42
LA1E	8.48
LLA	6.06
LLA1E	8.11
AA	6.59
AA1E	8.64

This is a desirable property as the greater the lipophilic nature of an enhancer, the greater the permeability across the skin, as there is a greater disruption of the lipids in the SC thereby decreasing the barrier properties [12, 38, 63]. Indeed, permeation results showed that all of the synthesized derivatives at a concentration of 1% w/w were able to increase the permeability of TNF across the rat skin (Table 1, Fig 4) and it was to a greater extent than the respective parent FA at similar concentrations. Interestingly, these results show that the addition of the *tert*-butyl ester functional group to the periphery of the parent FAs increases the permeability enhancement potential of even the FAs which had originally displayed no permeation enhancement (LA and AA) (Table 1, Fig 4). When compared to the control, LA1E **3** increased the steady state flux and the cumulative amount permeated from 31.48 ± 6.21 to 82.308 ± 4.33 µg/cm^2 h and 226.85 ± 33.89 to 514.79 ± 26.89 µg/cm^2 respectively, with an ER of 2.61 (Table 1, Fig 3). The data

highlights the fact that LA1E **3** displayed an ER of 1.68 higher than that obtained by its parent LA. The increases displayed by the derivative were however not statistically significant when compared to the control (p = 0.2039) (Table 1). AA1E **5** was able to increase the steady state flux and cumulative amount permeated to 83.466 ± 1.58 μg/cm^2 h and 510.18 ± 15.19 μg/cm^2 respectively and presented an ER of 2.65 (Table 1, Fig 4). AA1E **5** increased the ER of TNF across the skin by 1.71 times than its parent (AA). The enhanced permeability due to AA1E **5** was also not significantly different when compared to the control (p = 0.0832) (Table 1). Although the increases in the permeability parameters by the parent PA were not significantly different from the control, the increases in the permeability parameters by the derivative PA1E **2** were significantly different from the control (p = 0.0039) (Table 1). PA1E **2** increased both the steady state flux and cumulative amount to 110.3 ± 10.73 μg/cm^2 h and 707.84 ± 37.36 μg/cm^2 respectively, and displayed an ER of 3.5 (Table 1, Fig 4). The derivative has an ER of 2.15 higher than that of the parent (PA). LLA displayed the best enhancement potential when compared to the other FAs studied. In the same way, this was also true for its derivative LLA1E **4**, which showed the greatest increase in the permeability parameters of TNF when compared to the rest of the synthesized derivatives. LLA1E **4** exhibited an ER of 5.31 (Table 1). LLA1E **4** increased the steady state flux to 167.3 ± 3.49 μg/cm^2 h and the cumulative amount to 1014.57 ± 24.90 μg/cm^2 (Table 1, Fig 4). The difference between the ER of derivative and parent was 2.41 in the case of LLA.

The ER range acquired by these derivatives was equivalent and higher than the ER (2.02 to 3.39) reported by an independent study, that employed dendrimers with either amide or ester linkages as transdermal permeation enhancers for the delivery of diclofenac sodium (DS). Interestingly, that report showed that the dendrimers with ester linkages displayed superior enhancement of DS, which was attributed to their greater lipophilicity due to the ester linkage between UFA and the dendron, as well as the presence of *tert*-butyl ester at the periphery [12]. The physicochemical properties of an enhancer plays a major role in its ability to increase the permeability of a drug/permeant across the membrane. It is evident from this data that the structural modification by addition of the *tert*-butyl ester function augments the enhancement activity of the parent FAs. This may be due to the increase in the lipophilicity of the FAs with the addition of this functional group (Table 3). Studies have shown that an increase in enhancer lipophilicity results in an increase in permeation [12, 38, 39, 63], also possible ionic conjugation between basic dendritic derivatives and the acidic drug could aid in the movement of the TNF across the epidermis of the skin. This rational could explain the increase in permeation observed for LA1E **3** and AA1E **5**, as their parent materials displayed no enhancement activity. For LLA1E **4** and PA1E **2**, the increase in lipophilicity has had an additive effect to the already superior enhancement activity displayed by their respective parent material. From the current study it was observed that LLA1E **4** had all the ideal characteristics viz., optimal FA chain length (C_{18}), number of unsaturations (3) and lipophilicity. Therefore, further concentration effects on transdermal permeation enhancement efficacy were carried out on LLA and LLA1E **4**.

IV) Concentration effect of LLA and LLA1E 4 on the permeability of TNF

The effects of varying concentrations of LLA and LLA1E **4** on the transdermal permeability parameters of TNF were then investigated (Table 4, Fig 5) to facilitate the establishment of the optimal concentrations of either LLA or LLA1E **4** to be potentially incorporated as an enhancer into a future TDD system for TNF. The results showed that all the concentrations (0.5, 1, 2, 4, 6 % w/w) both LLA and LLA1E **4** were able to enhance the permeability of TNF (Table 4, Fig 5). There was a statistically significant difference between all the concentrations studied for both the parent and the derivative when compared to the control. Although both LLA and LLA1E **4** displayed enhanced permeation, the derivative LLA1E **4** exhibited a greater degree of enhancement at all similar concentrations as compared to its parent material LLA (Table 4, Fig 5). The profile of permeation enhancement across the concentration range studied was similar for both LLA and LLA1E **4** with an initial increase in permeation enhancement at the lower concentrations (0.5 and 1% w/w), reaching optimal permeation enhancement at a concentration of 2% w/w with a subsequent decrease in permeation as the enhancer concentration was further increased to 4 and 6% w/w. For the optimal enhancement, LLA at 2% w/w increased the steady state flux to 121.47 ± 10.20 μg/cm^2 h and the cumulative amount to 773.37 ± 45.13 μg/cm^2 displaying an ER of 3.85 when compared to the control. The derivative LLA1E **4** at 2% w/w increased the steady state flux of the control to 192.37 ± 17.58 μg/cm^2 h and the cumulative amount to 1214.88 ± 87.33 μg/cm^2 with an ER of 6.11 (Table 4, Fig 5).

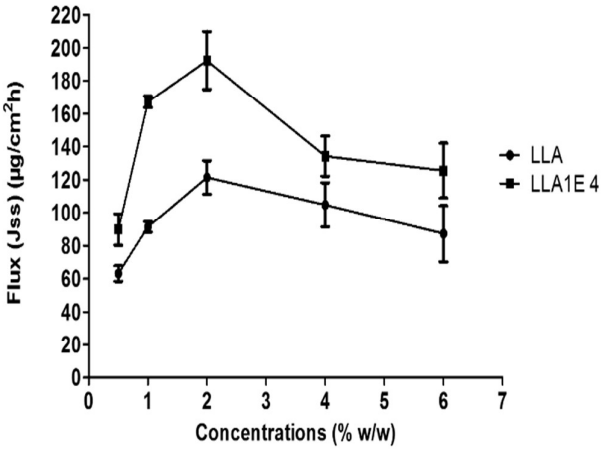


Fig. 5 Concentration effects of LLA and LLA1E 4 on the flux of TNF (n=6)

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Table 4. Effect of the various concentrations of LLA and LLA1E 4 on the transdermal permeability properties of TNF. *Indicates significant difference i.e *p* < 0.05 (all values compared to control)

	Cumulative amount permeated (µg/cm²)		*Flux, Jss (µg/cm².h)*		*Enhancement ratio (ER)*	
Control	226.85 ± 33.89		31.48 ± 6.21		1	
Concentrations [%w/w]	LLA	LLA1E **4**	LLA	LLA1E **4**	LLA	LLA1E **4**
[0.5]	415.83 ± 25.36	592.37 ± 64.70	*63.104 ± 4.69	*89.823 ± 9.59	2	2.85
[1]	560.72 ± 20.01	1014.57 ± 24.94	*91.316 ± 3.244	*167.3 ± 3.49	2.9	5.31
[2]	773.37 ± 45.13	1214.88 ± 87.33	*121.47 ± 10.20	*192.37 ± 17.58	3.85	6.11
[4]	691.17 ± 68.08	805.42 ± 70.94	*104.87 ± 13.04	*134.32 ± 12.13	3.33	4.26
[6]	584.82 ± 43.72	775.31 ± 94.83	*87.209 ± 17.06	*125.56 ± 16.62	2.77	3.98

The IC_{50} range for tenofovir is reported to be between 0.55 – 2.2 µM[64], which is 158 – 632 µg. The amount of tenofovir permeated per cm^2 in this study using the newly synthesized dendritic ester derivatives as CPEs is between 510.18 – 1214.88 µg which is adequate to achieve the desired therapeutic effect.

The flux values and ER obtained with 2% w/w LLA were similar to that obtained in a separate study where 5 % w/v cineole displayed a flux value of 122.4 µg/cm^2 and ER of 3.13 when it was employed as a transdermal enhancer for the delivery of zidovudine [22]. The ER achieved by 2% w/w LLA1E **4** is comparable to that achieved by 5 % (w/w) of either *N*-methyl-2-pyrrolidone (NMP) or transcutol P, which in an independent study displayed ER of 6.99 and 6.28 respectively for the transdermal delivery of daphnetin [58]. Even at lower concentrations, both LLA and LLA1E **4** displayed similar or higher ER to reported compounds that show promise as transdermal permeation enhancers. With an increase in the concentration of the lipophilic enhancer in the drug formulation, there would be a corresponding increase in the lipophilicity of the formulation. The increase in lipophilicity should thereby allow a greater disruption of the lipids in the SC with a corresponding decrease in the barrier properties provided by these lipids. This assertion is corroborated by the trend observed in this study where there was a direct correlation with the increase in permeability of the TNF with an increase in the concentration of enhancer in the formulation from 0.5 to 2% w/w. Further increases to 4 and 6% w/w of the enhancers didn't elicit a similar trend in the permeability; rather there was a progressive decrease in the permeability of the drug as the concentrations of the enhancers were increased. This could be due to the increased viscosity at the epidermal surface as concentrations of the enhancers were increased, which could thereby reduce the movement of the drug [39]. Previous studies report that FA at higher concentrations in gel formulations could also slow down the partitioning of the drug from the formulation thereby decreasing the amount of drug permeating [39, 65]. The findings in this study are consistent with findings from other studies that reported that increasing enhancer concentrations displayed an initial increase in the permeability, however further increases in enhancer concentrations led to a decrease in permeability [39, 45, 65].

TEER studies

TEER measurements are useful in establishing the integrity of cellular barriers. The values obtained quantitatively measure the integrity of tight junction dynamics that maintain the barrier properties. TEER measurements across biological barriers taken before they are evaluated for the transport of chemicals or drugs are strong indicators of its original integrity [66, 67]. A reduction in the resistance values would be indicative of the opening of transport pathways that could decrease the barrier properties of the membrane. The difference in TEER measurements pre and post experiment can quantifiably establish the change in the barrier properties brought on by the drug treatment [66, 67].

This study aimed to establish if the TNF gel formulation containing the optimal enhancers, either LLA or LLA1E **4**, at the optimal concentrations of 2% w/w did not affect the integrity of the skin during the permeation experiment. The results indicated that as compared to the control, both LLA and LLA1E **4** gel formulations at a concentration of 2% w/w were able to decrease the TEER values after 6 h permeation study (Fig 5). LLA treatment reduced its control resistance from 326 ± 28 to 311 ± 28 Ω /cm^2, which represents a 4.6% reduction in the resistance of the barrier. LLA1E **4** treatment was able to reduce the resistance of its control from 348 ± 15 to 319 ± 22 Ω /cm^2, which signifies an 8.3% reduction in the resistance of the barrier (Fig 6). These results correlate with the data from the permeability studies that display that LLA1E **4** had a greater permeability enhancement effect than LLA at a

concentration of 2% w/w (Table 4). This may have been due to LLA1E **4** being able to decrease the barrier properties to a larger extent when compared to LLA. Interestingly, the factor by which LLA1E **4** has greater ER over LLA is similar to the factor by which LLA1E **4** has greater reduction in TEER as compared to LLA. The findings from both these separate studies are therefore in good agreement with each other. One of the characteristics of an ideal transdermal permeation enhancer is that upon its removal, the skin should regain its barrier properties i.e. its effects on the barrier should be temporary[27-29, 31].

The 2 h post experiment evaluation of TEER was assessed to determine if there was any recovery of the barrier function upon removal of the drug formulations. The results displayed that of the original decrease recorded for LLA (4.6%) there was a 1.33 % increase in resistance after 2 h post experiment (Fig 6). This represents a 28 % recovery in the barrier property of the skin after removal of the gel formulation. In the LLA1E **4** study, of the original decrease (8.3%) there was a 2.1% increase in the resistance after 2 h post experiment (Fig 6). This signifies a 25% recovery in the barrier property. These findings are a reflection of the recovery of the barrier properties, and suggest a return to the initial measured integrity over time. The TEER values obtained in this study indicates that dermal integrity was not irreversibly affected by the drug formulations [46, 47].

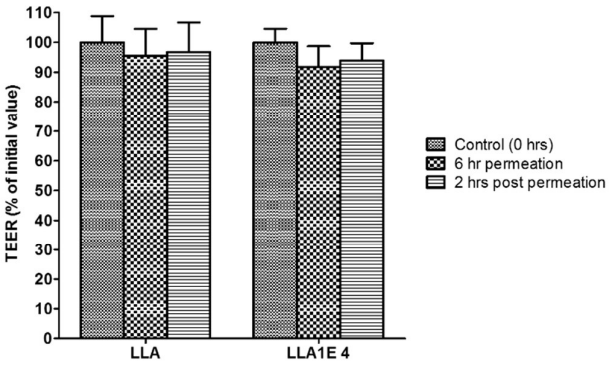


Fig 6. Percentage change in TEER values after TNF permeation with either LLA or LLA1E 4 at a concentration of 2% w/w (n = 6).

Light and Transmission Electron Microscopy

Histomorphological evaluations of the rat skin treated with TNF gel formulations were performed to establish whether tissue morphology or cellular integrity of the skin was compromised. These investigations were undertaken on the enhancer free TNF gel, and on TNF gel containing enhancer, either LLA or LLA1E **4** respectively at a concentration of 2% w/w. Two major routes have been proposed for movement of molecules across the skin i.e. intercellular and transcellular [30, 35, 56]. From these studies it may be possible to make inferences on the route of transport of this drug, or the mode of action of the enhancers[68-71]. Permeants need to transverse the epidermis of the skin to get to blood vessels found in the dermis to enter the systemic circulation. The epidermis is composed of four layers: 1) the basal layer which is the germinal layer of the epidermis supplying new keratinocytes to replace those lost by normal wear and tear; 2) the prickle cell layer which contains cells that is in the process of growth and early keratin synthesis, 3) the granular layer which contain cells with intracellular granules which contribute to the process of keratinization and 4) the SC, which has been identified as the main barrier, consisting of flattened, fused remnants composed mainly of fibrous protein and lipids, with the intracellular keratin having an ordered pattern [5, 72]. The area of skin chosen for this study was from the abdominal region of the rat which is analogous with other reports that performed transdermal permeation studies [12, 58, 73, 74]. Skin from this region is termed "thin skin" and the individual cellular layers are more difficult to discern as compared to "thick skin". In comparison with thick skin, the SC is thin and the combined thickness of the other layers is reduced to a lesser extent [72].

This description above closely resembles the control image (Fig 7a) demonstrating that the skin used in the study represented healthy tissue with normal morphology.

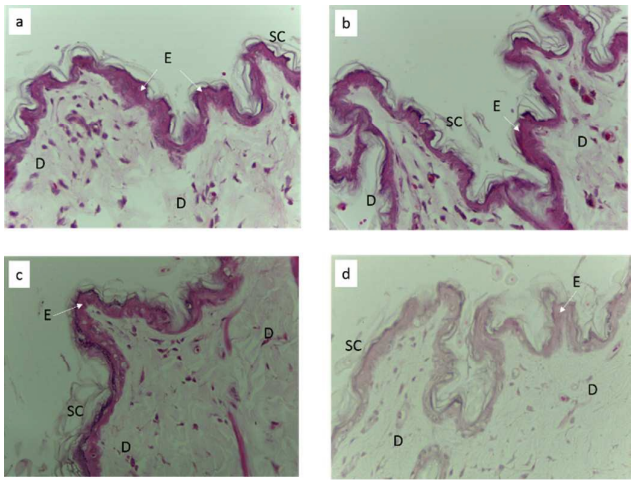


Fig 7. Photomicrographs of the control and the treated skin selections for light microscopy (LM) stained with H&E ; (× 40) a control/untreated, b treated with TNF gel, c treated with 2% w/w LLA TNF gel, d treated with 2% w/w LLA1E **4** TNF gel. (D: dermis; E: epidermis; SC: stratum corneum).

All of the four layers described above were visible in the H&E preparations and were distinguishable from one another. The enhancer free TNF gel treatment (Fig 7b) is similar to the control image, with no signs of morphological changes to the tissue which indicates no adverse effect of TNF gel treatment. The micrographs of the enhancer treatments (Fig 7c and 7d) were comparable to that of the control, with cell layers showing normal morphology with no observable cellular distortions. The basal layer is intact; with the granular layer appearing darkly stained depicting no decrease in the activity of either the regenerative cells or cells in the granular layer as compared to their counterparts in the control image. There was however some observable distortions of the SC in both the LLA (Fig 7c) and the LLA1E **4** (Fig 7d) treated images. These distortions were of a lesser extent in the LLA treatment as compared to LLA1E **4**. These changes could be due to the disruption of the highly ordered lipids in the SC by the permeation enhancers. The disruptions or fluidization of the SC by these enhancers thereby reduce the barrier property of the SC and enhance the permeation of TNF across the skin. These findings also correlate with the results of the permeability and TEER studies that displayed an increase in

the permeation parameters of TNF (Table 4) and decrease in the resistance of the skin (Fig 6) in the presence of these enhancers.

LM evaluations do not permit ultrastructural analysis of the treated tissue. TEM analyses were thus performed to allow for closer evaluations which would give greater insight to the cellular response/reaction of the cells that make up the tissue. The assessments of these images can also contribute to the establishment of potential routes of transport for the drug and the enhancer across the epidermis of the skin. The comparison of the histomorphological components that make up the barriers to the transport of molecules across the epidermis between control and the treated samples can aid in establishing the potential routes of transport. Similar to the LM investigations, the TEM control sample also displayed normal skin morphology. The particular areas of interest in this study were the SC which provides the barrier function of the epidermis, as well as the tight junctions/desmosomes which connect adjacent cells to each other preventing free movement of molecules between cells [75].

In the control images the SC appeared to be uniform and intact and depicted a functional barrier system as described in the literature (Fig 8a). The control images display typical tight junctions/desmosomes with no spaces between them, therefore implying the minimal movement of molecules between these cells via intercellular transport (Fig 8b). There were no significant observable changes in the morphology of the enhancer free drug formulation when compared to the assessments made on the control images. It should be noted that, as described above, since the skin samples used in the study were of thin skin that have reduced thickness of the cellular layers, the minute changes needed in the structural morphology to allow the movement of small amounts of the drug through the epidermis may not be identified as significantly different from that of the control. However, in the LLA and LLA1E **4** treated samples there were structural changes observed in the SC layer of the epidermis. The SC appears to be more loosely packed with larger spaces and disorganization between the sheets of keratin (Fig 8c and 8e respectively). The effects appear to be more prominent in the LLA1E **4** treatment as compared to the LLA treatment, which also correlate with the assessments from the LM study for these samples. These morphological changes could have been brought about by the lipophilic enhancers (LLA and LLA1E **4**) by disrupting the lipids within the SC, thereby causing fluidization of the lipids within this layer. There were also noticeable changes in the size of the intercellular spaces between cells in the LLA1E **4** treated sample, which were caused by the stretching/expansion of the tight junctions/desmosomes (Fig 8f). These effects were minimal to none in the LLA treated group (Fig 8d). The increase in intercellular spaces enables the movement of molecules through the paracellular route, which is therefore suggestive that TNF utilizes this route across the epidermis. The contributing factors leading to the decrease in the barrier properties of the epidermis identified in this study explains the reductions in resistance values observed for the respective treatments in the TEER study. Interestingly, there was also some degree of vacuolization observed within the LLA1E **4** sample (Fig 8f). The appearance of vacuoles in drug treated epidermal tissue has been previously reported as being an indication of possible transcellular transport of the drug across the membrane [45, 76]. The basal cell layer and basement membrane appeared to be unaffected and intact in the treated samples.

The findings of the TEM study corroborate the findings of the permeability, the TEER as well as the LM studies. Histomorphologically the superior ER of the LLA1E **4** sample in this study could be attributed to a combination of factors that allowed greater permeability of TNF across the epidermis. The observed disruptions of the SC would have decreased the main barrier of the epidermis thereby facilitating easier transport of TNF across the epidermis. The formation of larger intercellular spaces caused by the stretching of the tight junctions/desmosomes would have aided the movement of the drug through the cellular layers, and the formation of vacuoles could be a potential indication that the drug was also being transported via the intracellular route as well. The enhancing effect of LLA could be largely credited to its disruption of the SC and the consequent decrease in its barrier properties thus allowing the increased permeability of TNF.

Morphological changes observed to the superficial layers caused by the drug treatment should not be considered permanent as these layers are being continuously replaced by the cell layers beneath it. Data from the TEER study also suggest that the skin would recover its barrier properties upon removal of the enhancer (Fig 6). Both LM and TEM evaluations revealed no loss in cellular integrity of the treated samples as compared to the control. These studies therefore confirm that the exposure of TNF gel formulations with or without the incorporation of either LLA or LLA1E **4** as an enhancer at a concentration of 2% w/w do not have any adverse effect on the skin.

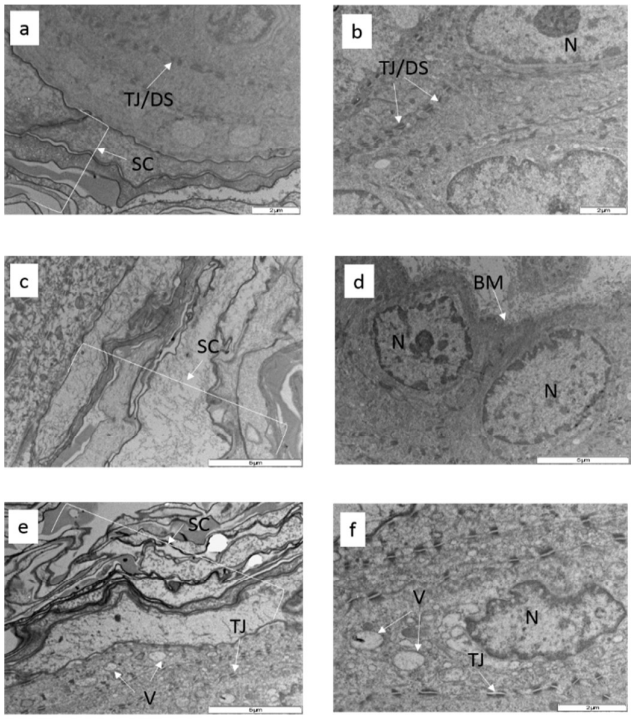


Fig. 8 Electron micrographs of the control and the treated skin selections for transmission electron microscopy (TEM) : a/b control/untreated (× 8000), c treated with 2% w/w LLA TNF gel (× 8000), d treated with 2% w/w LLA TNF gel (× 10000), e treated with 2% w/w LLA1E **4** TNF gel (× 8000), f treated with 2% w/w LLA1E **4** TNF gel (× 12000). (SC: stratum corneum; TJ/DS: tight junctions/desmosomes; N: nucleus; BM: basement membrane; V: vacuoles).

Experimental section

Materials

TNF was purchased from Sinobright Pharmaceutical Co.Ltd (Nanshan, China). Male Wistar rats (200-250 g) were obtained from the Biomedical Resource Unit (BRU), University of KwaZulu Natal (UKZN). PA, LA, α-LLA, AA, N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDAC.HCl), p-dimethylaminopyridine (DMAP) and 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma (St. Louis, MO). 3- Amino-1-propanol and tert-butyl acrylate were purchased from Alfa-Aesar (Karlsruhe, Germany). Acetyl chloride (AcCl) and dichloromethane (DCM) were from Merck Chemicals (Hohenbrunn, Germany). Merck precoated Silica-gel 60F254 plates were used for thin layer chromatography and hydroxypropyl methyl cellulose (HPMC) was purchased from Sigma-Aldrich (Steinheim, Germany). All other reagents and solvents used were of analytical grade and were procured from Merck Chemicals. Milli-Q purified water was obtained from the purification system (Millipore Corp., Billerica, MA) in our laboratories.

Methods

Synthesis and characterization of dendritic esters of UFAs

Synthesis of dendritic esters of UFAs as shown in scheme 1.

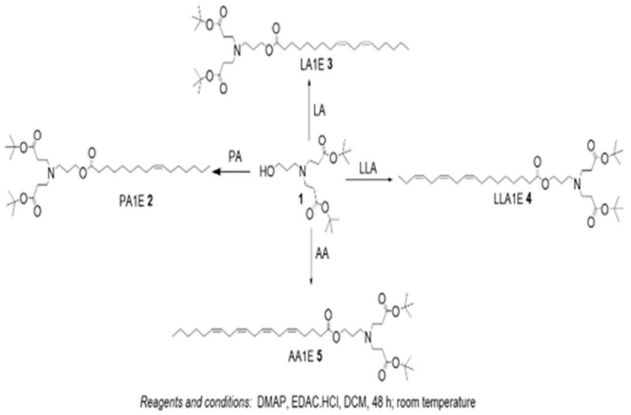
To a stirred mixture of dendron **1** (1.2 equiv.), synthesized using a previously reported method [12] and DMAP (0.75 equiv.) in DCM was added EDAC.HCl (1.5 equiv.) followed by an UFA (1 equiv.). The resulting reaction mixture was magnetically stirred at room temperature for a 48 h period. The organic layer was washed with brine solution and concentrated in vacuo. The crude product obtained was purified by column chromatography (silica gel # 60-100 mesh, hexane/EtOAc; 9:1).

Structural characterization and stability of dendritic esters

FT-IR spectra of all the compounds were recorded on a Bruker Alpha-*p* spectrometer with diamond ATR (Germany). ^{1}H NMR and ^{13}C NMR measurements were performed on a Bruker NMR spectrometer (United Kingdom) at 400 and 100 MHz respectively. HRMS was performed on a Waters Micromass LCT Premier TOF-MS (United Kingdom). The stability of the synthesized derivatives was confirmed by ^{13}C NMR analysis of samples stored at 4 ^{o}C in air tight amber coloured bottles.

Calculation of log *P* values

The lipophilicity of the parent UFAs as well as their respective dendritic ester derivatives was determined by calculating their log *P* values using an online tool, chemicalize [62].



Scheme 1. Synthesis of dendritic esters of UFAs.

Acid-base titration

As the synthesized dendritic ester derivatives have a tertiary nitrogen in their structure, their ability to protonate over a 13 to 2 pH range was determined by acid-base titration [54]. A 20 ml solution of each dendritic ester in ethanol (10 mg/ml) was adjusted to pH 13 by using aqueous 1 M NaOH. This solution was then titrated with aqueous 1 M HCl solution in various volume increments and the pH was recorded at each addition point. The graph of volume of HCl added versus pH was generated for all the dendritic lipids. All the experiments were performed in triplicate.

In vitro cytotoxicity

Cell culture

Human cervix cancer cell line (HeLa) was cultured with complete medium supplemented with 10 % bovine calf serum, 100 units/ml of penicillin, and 100 mg/ml of streptomycin. The cells were maintained at 37˚C in a humidified atmosphere of 5 % CO_2 in air.

Solutions

The derivatives (PA1E **2**, LA1E **3**, LLA1E **4** and AA1E **5**) were dissolved in DMSO and distilled water as a stock solution, and further diluted in the culture medium to give final concentrations of 20, 40, 60, 80 and 100 µg/ml [77].

MTT assay

The culture medium was removed and replaced with fresh medium (100 µL per well) together with the solutions described above to achieve final concentrations in the wells seeded equivalently (2.4 x 10^3) with HeLa cells into a 96-well plate and incubated for 24 h. The control wells were prepared by addition of culture medium only whereas wells containing culture medium without cells were used as blanks. After the 48 h incubation, the culture medium and compounds were removed and replaced with fresh medium (100 µL) and MTT solution (100 µL of 5 mg/mL in PBS) in each well. The media and MTT solution were removed and 100 µL of DMSO was added to each well to solubilize the MTT formazan after 4 h of incubation. The optical density of each well was measured on a microplate spectrophotometer (Mindray MR-96A) at a wavelength of 540 nm (A540: absorbance at a wavelength of 540 nm) [77]. All the

experiments were performed with six replicates. The percentage cell viability was calculated as follows:

$$\%\ Cell\ Viability\ = \frac{A540\ nm\ treated\ cells}{A540\ nm\ untreated\ cells}\ x100 \qquad eq.(1)$$

Ethical Clearance

Ethical clearance was obtained from the University of KwaZulu-Natal Ethics committee in 2014 (054/14/Animal), and renewed in 2015 (015/15/Animal).

Transdermal permeation studies

Formulation of gels for permeation studies. To determine the transdermal permeability of TNF across skin, a gel containing 2% w/w TNF and 4% w/w HPMC were prepared and served as the control. To determine the enhancement effect of the UFAs or their respective derivatives, 1% w/w UFA or their respective dendritic ester derivatives, 4% w/w HPMC and 2% w/w TNF gels were prepared. For the concentration effect experiments, varying concentrations of either LLA or LLA1E **4** were added to the control formulation at concentrations of 0.5, 2, 4, 6% w/w [39].

Preparation of rat skin. The rats were housed at the BRU (UKZN) in polycarbonate cages in a room with controlled temperature and humidity[45, 46], and a 12-h light/dark cycle. They were fed a rodent pellet food and water ad libitum. The rats were sacrificed by CO_2 euthanasia followed by removal of the hair by shaving. Full thickness abdominal skin was harvested and then excised using surgical scissors to remove any subcutaneous fat. The tissues were stored at -20˚C and used within three months [12]. PBS (pH 7.4) was used to thaw the skin at room temperature before the start of permeation studies.

In vitro permeation studies. *In vitro* permeation studies were conducted at 37 ± 1˚C using modified vertical Franz type diffusion cells [5, 66] (PermeGear, Inc., Bethlehem, USA) with a diffusional area of 0.786 cm^2. A circular section of the skin was mounted onto the diffusional area between the donor and receptor cells, and was equilibrated with PBS (pH 7.4) at 37 °C for 30 minutes. The donor compartment contained either TNF loaded gel or TNF loaded gel in the presence of the various FAs or their respective derivatives (PA, PA1E **2**, LA, LA1E **3**, LLA, LLA1E **4**, AA, AA1E **5**) at a concentration of 1% w/w in respective experiments for the enhancer screening study. The receptor compartments were filled with PBS and stirred with a teflon-coated magnetic bar. Samples were removed from the receptor compartments at predetermined time intervals and replaced with the same volume of drug–free (fresh) PBS. Each experiment represents a minimum of six replicates. The drug was quantified by a validated UV spectrophotometry method at a λ_{max} of 262 nm using UV Spectrophotometer 1650 (Shimadzu, Japan) [45]. Once the optimal enhancer (LLA1E **4**) was identified, for the subsequent concentration effect studies TNF gels were prepared using 0.5, 2, 4, 6% w/w of either LLA1E **4** or the parent FA LLA [39].

Permeability data analysis

The cumulative amount of drug (TNF) permeated per unit surface area was plotted against time. The steady state flux (Jss) was determined from the linear part of the permeability curve by linear regression analysis (Microsoft Excel 2010, USA). The permeability coefficient (P) was calculated as follows [39, 45, 46]:

$$\boldsymbol{P} = \frac{\left(\frac{dQ}{dT}\right)}{A}\ x\ Cd = \frac{Jss}{Cd} \qquad eq.(2)$$

dQ/dt is the cumulative amount permeated per unit time, A is the diffusion area and Cd is the drug concentration in the donor compartment. The enhancement ratio (ER) was calculated using the following equation [45, 46]:

$$\boldsymbol{ER} = \frac{P\ of\ the\ drug\ in\ the\ presence\ of\ enhancer}{P\ of\ the\ drug\ in\ the\ absence\ of\ enhancer} \qquad eq.(3)$$

Transepithelial electrical resistance (TEER) studies

TEER measurements using a Millicell ERS meter (Millipore, USA) connected to a pair of chopstick electrodes (STX01) were used to determine the integrity of the skin. TEER measurements were taken across the skin prior to, and at the end of the permeation experiment. TEER values at time zero were used as 100%. The rebound effect of the skin post drug treatment was measured by removing the drug loaded gel from the donor compartment after a period of 6 h and replacing it with fresh PBS for a period of 2 h with subsequent TEER measurements [46, 47].

Light and Transmission Electron Microscopy

Histological evaluations were performed on freshly harvested excised skin. Untreated skin was transferred directly after excision from normal saline into 10 % buffered formalin without any equilibration in PBS and served as the control. Treated samples comprised of skin that were exposed to enhancer free drug loaded gel, and drug loaded gels containing either LLA or LLA1E **4** at a concentration of 2 % w/w. Permeation experiments were carried out with these gels in the donor compartment; and with the freshly excised skin placed between donor and receptor compartments as described above, without drug quantification [45-47]. For light microscopy (LM) evaluations, the skin was removed from the Franz diffusion cells at the end of the experiment, cut into cross sections and fixed in 10 % buffered formalin. Both the control and treated skin were fixed in formalin for 7 days at room temperature. Skin was dehydrated using an ethanol gradient ranging from 50 % up to 96 % and embedded in paraffin wax. The skin sections were collected on slides, dried and stained with hematoxylin and eosin (H&E). Sections were examined using a light microscope (Nikon 80i, Japan) and bright field images were digitally captured using NIS Elements D software and a camera (Nikon U2, Japan). The samples for transmission electron microscopy (TEM) were obtained after the above mentioned permeation experiments. The samples were then cut into pieces not exceeding 0.5 mm^3, and fixed for 24 h (4 °C) using Karnovsky's fixative [78] buffered to pH 7.2. For TEM, each sample was processed and embedded in epoxy resin using standard protocols. Ultrathin sections (90 nm) were cut and contrasted with uranyl acetate and lead citrate and viewed with a TEM (JEOL 1010, Japan). All experiments were performed using a minimum of three replicates [39, 45-47].

Statistical Analysis

The results, expressed as mean ± standard deviation (SD), were analyzed using one-way analysis of variance (ANOVA) followed by the non-parametric Kruskal-Wallis test and t tests followed by the non-parametric Mann-Whitney test were performed using GraphPad Prism® (Graph Pad Software Inc., Version 5., USA). A *p* value of less than 0.05 was considered statistically significant.

Conclusions

This study reported the effect of novel dendritic ester derivatives of UFAs on the transdermal permeability parameters of TNF. TNF gel formulations with 1% w/w of PA, LA, LLA or AA showed that only LLA and PA had transdermal enhancing potential. The novel dendritic derivatives of the studied UFAs were successfully synthesized. *In vitro* cytotoxicity studies revealed the safety of these derivatives for biological applications. All the newly synthesized dendritic derivatives PA1E **2**, LA1E **3**, LLA1E **4** and AA1E **5** at a concentration of 1% w/w displayed permeation enhancement of TNF across the skin. LLA and LLA1E **4** were identified as most effective permeation enhancers with ERs of 2.9 and 5.31 respectively. The highest ER by LLA1E **4** was attributed to carbon chain length, optimum number of double bonds and the increase in the lipophilicity by the addition of the *tert*-butyl ester function. The concentration dependent study revealed that both LLA and LLA1E **4** at 2% w/w displayed the highest ER of 3.85 and 6.11 respectively.

The histomorphological studies suggest enhancement of TNF permeability by 2% w/w LLA and LLA1E **4** was due the disruption and fluidization of lipids in the SC. The highest permeation achieved by LLA1E **4** was strongly supported by the LM and TEM evaluations which display a greater disruption and fluidization of the SC and stretching/expansion of the tight junctions/desmosomes thereby facilitating better intercellular transport of TNF across the epidermis. Vacuole formation was also suggestive of TNF transport using the intracellular pathway. LM and TEM evaluations showed no adverse effects on the tissue after exposure to the 2% w/w concentration of either LLA or LLA1E **4**.

This study has therefore identified the transdermal delivery properties of TNF and has confirmed the superiority of newly synthesized dendritic ester derivatives over their parent UFAs as transdermal permeation enhancers. LLA1E **4** was identified as the superior transdermal permeation enhancer and is therefore a promising CPE candidate for the development of transdermal drug delivery systems of TNF.

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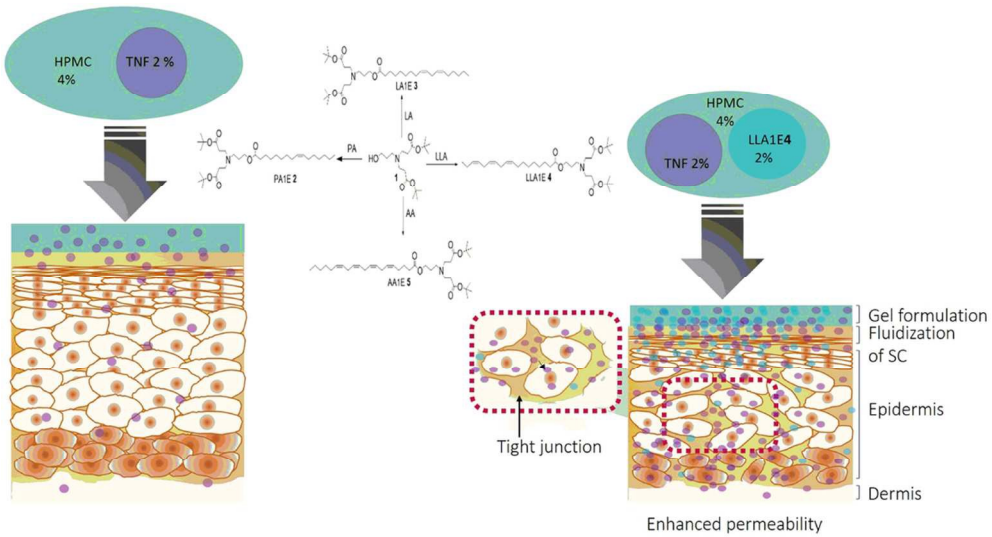

Novel dendritic ester derivatives of unsaturated fatty acids as potential transdermal permeation enhancers.
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