Novel lipidic materials to enhance the transdermal delivery of Tenofovir

by

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Doctor of Philosophy (Pharmaceutics) in the
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“Intellectual growth should commence at birth and cease only at death’’

-Albert Einstein-

*"**This thesis is dedicated to my beloved parents, who have supported, motivated, and prayed throughout my studies. Thank you for always believing in me and encouraging me to pursue my dreams”***

DECLARATION 1 - PLAGIARISM

I, Mr. Sanjeev K. Rambharose, declare that

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DECLARATION 2 – PUBLICATIONS/MANUSCRIPTS

Details of contribution to publications/manuscripts that form part and/or include research presented in this thesis:

1. **S. Rambharose, R.S. Kalhapure, K.G. Akamanchi, T. Govender, Novel dendritic derivatives of unsaturated fatty acids as promising transdermal permeation enhancers for tenofovir. J. Mater. Chem. B 2015, 3 (32), 6662-6675.**

   Mr. S. Rambharose contributed to the design of the project, modification and optimisation of methods as well as synthesis and characterisation of UFA ester dendritic derivatives. Mr. S. Rambharose conducted all experimental work including *in vitro* cytotoxicity, rat skin tissue harvesting, *in vitro* transdermal permeation experiments, TEER experiments and light and transmission electron microscopy evaluations. Mr. S. Rambharose also wrote the paper. Dr. R.S. Kalhapure assisted with the overall design of the project and the methods of preparation and characterisation as well as editing of the paper. Prof K.G. Akamanchi supervised the synthesis of the UFA ester dendritic derivatives in this work. Prof. T. Govender served as supervisor and was responsible for project conceptualisation, editing of paper and abstract and general supervision of the study.

2. **S. Rambharose, R.S. Kalhapure, M. Jadhav, T. Govender, Exploring unsaturated fatty acid cholesteryl esters as transdermal permeation enhancers. Drug Del. Trans. Res. 2017, 7 (2), 333-345**

   Mr. S Rambharose contributed to the design of the project, modification and optimisation of methods as well as synthesis and characterisation of UFAs and SFAs esters of cholesterol. Mr. S. Rambharose conducted all experimental work including rat skin tissue harvesting, in vitro transdermal permeation experiments, TEER experiments and light microscopy evaluations. Mr. S. Rambharose also wrote the paper. Dr. R.S. Kalhapure and Dr. M. Jadhav assisted with the overall design of the project and the methods of preparation and characterisation as well as editing of the paper. Prof. T. Govender served as supervisor and was responsible for project conceptualisation, editing of paper and abstract and general supervision of the study.

3. **S. Rambharose, R.S. Kalhapure, M. Jadhav, T. Govender, Novel mono, di and tri- fatty acid esters bearing secondary amino acid ester head group as transdermal permeation enhancers. Eur. J. Pharm. Sci. Manuscript number: EJPS-D-16-01525.**

   Mr. S. Rambharose contributed to the design of the project, modification and optimisation of methods as well as synthesis and characterisation of all the novel mono, di and tri-ester derivatives of FAs. Mr. S. Rambharose conducted all experimental work including *in vitro* cytotoxicity, rat skin tissue harvesting, *in vitro* transdermal permeation experiments, TEER experiments and light microscopy evaluations. Mr. S. Rambharose also wrote the paper. Dr. R.S. Kalhapure and Dr. M. Jadhav assisted with the overall design of the project and the methods of preparation and characterisation as well as editing of the paper. Prof. T. Govender served as supervisor and was responsible for project conceptualisation, editing of paper and abstract and general supervision of the study.

4. **S. Rambharose, R.S. Kalhapure, T. Govender, Nanoemulgel using a bicephalous heteolipid as a novel approach to enhance transdermal permeation of tenofovir. Colloids Surf. B. 2017, DOI: http://dx.doi.org/10.1016/j.colsurfb.2017.03.040**

   Mr. S. Rambharose contributed to the design of the project, modification and optimisation of methods as well as synthesis of the linolenic acid ester dendritic derivative (LLA1E). Mr. S. Rambharose also conducted the experiments for the construction of the pseudo-ternary phase diagrams, prepared, and characterized all NE and NEG formulations in terms of mean globule diameter, polydispersity index, zeta potential, rheology, morphology and stability. Mr. S. Rambharose conducted the incorporation efficiency studies and all transdermal permeation experimental work including, rat skin tissue harvesting, in vitro permeation experiments, TEER experiments and light microscopy evaluations. Mr. S. Rambharose also wrote the paper. Dr. R.S. Kalhapure assisted with the overall design of the project and the methods of preparation and characterisation as well as editing of the paper. Prof. T. Govender served as supervisor and was responsible for project conceptualisation, editing of paper and abstract and general supervision of the study.

RESEARCH OUTPUT FROM THE THESIS

A. Patent

1. UK Patent (Application No. 1614120.2) pertaining to "pH-responsive lipids" (Co-Inventors: Mahantesh Jadhav, Rahul S. Kalhapure, Sanjeev Rambharose, Chunderika Mocktar, Thirumala Govender). Filing Date: 18 August 2016.

B. Publications

The following papers were published/ submitted in international ISI journals from work done during this study.

a. Primary authored published papers:

1. S. Rambharose, R.S. Kalhapure, K.G. Akamanchi, T. Govender, Novel dendritic derivatives of unsaturated fatty acids as promising transdermal permeation enhancers for tenofovir. J. Mater. Chem. B 2015, 3 (32), 6662-6675. (Impact Factor = 4.872).

2. S. Rambharose, R.S. Kalhapure, M. Jadhav, T. Govender, Exploring unsaturated fatty acid cholesteryl esters as transdermal permeation enhancers. Drug Del. Trans. Res. 2017, 7 (2), 333-345 (Impact Factor = 1.887).

3. S. Rambharose, R.S. Kalhapure, T. Govender, Nanoemulgel using a bicephalous heteolipid as a novel approach to enhance transdermal permeation of tenofovir. Colloids Surf. B. 2017, DOI: http://dx.doi.org/10.1016/j.colsurfb.2017.03.040 (Impact Factor = 3.902).

   *The published papers can be found in Chapter 2, 3 and 5.

b. Primary authored submitted paper:

1. S. Rambharose, R.S. Kalhapure, M. Jadhav, T. Govender, Novel mono, di and tri- fatty acid esters bearing secondary amino acid ester head group as transdermal permeation enhancers. Eur. J. Pharm. Sci. Manuscript number: EJPS-D-16-01525. (Impact Factor = 3.773).

   *The manuscript of the submitted paper can be found in Chapter 4.

2. **Conference presentations**

The following conference presentations were produced from data generated during this study:

a. International:

1. **S. Rambharose,** R.S. Kalhapure, K.G. Akamanchi, T. Govender. Novel Bicephalous Lipids for Transdermal Permeation Enhancement of Tenofovir. 4th African Pharma Conference, 20-22 June 2016, Cape Town, South Africa.

b. Local:

1. **S. Rambharose**, R.S. Kalhapure, K.G. Akamanchi, T. Govender. Novel dendritic derivatives of unsaturated fatty acids as promising transdermal permeation enhancers for tenofovir. College of Health Science Research Symposium, 2015. K-RITH tower building, UKZN, Durban, South Africa.
2. **S. Rambharose**, R.S. Kalhapure, T. Govender. Dual use of a novel dendritic heterolipid: a transdermal permeation enhancer and an oil phase in nanoemulsion of tenofovir. College of Health Science Research Symposium, 2016. K-RITH tower building, UKZN, Durban, South Africa.
3. **S. Rambharose**, R.S. Kalhapure, T. Govender. Dual use of a novel dendritic heterolipid: a transdermal permeation enhancer and an oil phase in nanoemulsion of tenofovir. UKZN Nanotechnology Platform Workshop, 2016. UKZN, Westville Campus, Durban, South Africa.

*The posters can be found in Appendix A & B.

ABSTRACT

The global burden of HIV and AIDS coupled with the limitations of current oral tenofovir (TNF) administration drives the need to develop strategies such as the use of alternate routes of administration to improve drug therapy. Transdermal drug delivery (TDD) offers numerous advantages and is an attractive alternative for the systemic delivery of TNF. Although the inherent protective barrier property of skin is one of the major challenges in the design of TDD systems, the use of chemical permeation enhancers (CPEs) such as fatty acids (FA) and their esters or the use of nano drug delivery systems have the potential to overcome this limitation. To date there are no reports on TDD permeation enhancement strategies or a nanoemulgel (NEG) as a TDD formulation for TNF. Novel lipidic approaches that reversibly decrease the barrier properties of the skin as well as the use lipid based nano drug delivery systems such as NEGs to enhance the TDD of TNF remain to be investigated. The broad aim of this study was therefore to explore the potential of novel lipid-based strategies for enhancing transdermal permeation of TNF. The specific research aims of this study were to: (1) Synthesize and characterize novel biocompatible dendritic ester derivatives of unsaturated FAs (UFAs) and explore their potential as promising permeation enhancers for the transdermal delivery of TNF. (2) Evaluate the novel application of UFA esters of cholesterol as promising transdermal permeation enhancers using TNF as a model drug. (3) Synthesize and characterize novel biocompatible mono, di and tri-ester derivatives of FAs and explore their potential as promising transdermal permeation enhancers using TNF as a model drug. (4) Explore the potential of novel linolenic acid based heterolipid, LLA1E (a novel transdermal permeation enhancer), as an oily phase in the development of a nanoemulgel for the transdermal drug delivery of TNF.

UFAs [palmitoleic (PA), linoleic (LA), linolenic (LLA) and arachidonic acid (AA)] were used to synthesize novel dendritic ester derivatives [PA1E, LA1E, LLA1E and AA1E]. The structural features of the biosafe derivatives were confirmed by FTIR, NMR (^{1}H and ^{13}C) and HRMS. All synthesized novel dendritic ester derivatives at 1% w/w were found to be more effective enhancers with LLA1E being identified as the most superior with an ER of 6.11 at 2% w/w. Histomorphological analysis displayed no significant loss in the integrity of the skin and also indicated that TNF utilized both the transcellular and intercellular route of transport, with the drug and enhancer treatment having no permanent effects on the epidermis. Therefore these novel dendritic ester derivatives of UFAs can be considered as effective transdermal permeation enhancers for TNF.

The TDD potential of TNF using UFA esters of Cholesterol (Chol) viz., oleate, linoleate and linolenate, as CPEs showed that all Chol UFA esters at 1% w/w were found to be more effective enhancers when compared to their respective parent FAs and saturated FAs counterparts. Cholesteryl linolenate (Chol-LLA) showed the most superior performance with the greatest ER of 5.93 being achieved at a concentration of 2% w/w. The histomorphological and transepithelial electrical resistance (TEER) evaluations displayed no damage to the integrity of the epidermis and skin exposure to the permeation enhancer had only temporary effects on its barrier property. Therefore Chol UFA esters can be considered as new CPEs for exploitation in transdermal formulations for various classes of drugs.

The synthesized mono, di and tri-ester derivatives were non-toxic and displayed better transdermal permeation enhancement capabilities as compared to their respective parent FAs. The *in vitro* permeation results showed that the mono oleate derivative (MOAPE) displayed the greatest ER for TNF (5.87) at 1% w/w. Histological investigations of the rat skin treated with MOAPE revealed fluidization of the stratum corneum. Histological and TEER studies revealed no significant change to the viable epidermis of the skin after 1% MOAPE exposure. The TEER findings also suggested that the permeation enhancement effects of MOAPE were not permanent and showed a return towards original skin integrity after removal of the enhancer formulation. These findings therefore indicate that the novel mono ester derivative of OA (MOAPE) adds to the pool of CPEs available to formulation scientists and can be safely incorporated into TDD systems for several classes of drugs.

LLA1E served as an effective oily phase in the formulation of nanoemulsions (NEs). TNF loaded nanoemulsions (TNEs) were prepared and incorporated into TNF nanoemulgels (TNEGs) which were subsequently evaluated for their *ex vivo* transdermal permeation efficacy. TNEs had a mean globule diameter (MGD) of 129.06 ± 3.35 nm, polydispersity index (PDI) of 0.192 ± 0.038 and zeta potential (ZP) of 20.9 ± 2.02 mV with an incorporation efficiency of 91.94 ± 0.84%. There was no significant change is these properties after incorporation of TNEs into a hydrogel as MGD, PDI and ZP of TNEGs were found to be 136.13 ± 5.21 nm, 0.182 ± 0.020 and -20.9 ± 2.08 mV respectively. *Ex vivo* permeation studies showed that the TNEG significantly enhanced the TNF permeation by 39.65-fold, with a cumulative amount of 1866.54 ± 108.62 $\mu g.cm^{-2}$. Histological and TEER assessments showed no permanent effects on the skin by TNEG. Therefore, this novel TNEG nanosystem has the potential of further translation into clinical trials for optimal treatment alternatives for HIV/AIDs patients.

The findings of this study therefore identified and developed novel lipid based approaches that were successful in reversibly decreasing the barrier of the skin and was able to promote the transdermal delivery of TNF. Novel lipid based strategies such as dendritic ester derivatives of UFAs; UFA esters of cholesterol; mono, di and tri-ester derivatives of FAs and TNF loaded NEGs therefore have the potential of enhancing TDD of TNF. This study has therefore made significant contributions towards improving TDD and TNF therapy for optimal treatment alternatives for HIV/AIDs patients.

Keywords: fatty acids; synthesis; esterification; chemical permeation enhancers; tenofovir; transdermal; cholesterol; nanoemulsion; nanoemulgel

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- My loving parents, for your complete support, motivation, and prayers throughout my studies. You have moulded me into the person that I am and I will continue to achieve my goals to make you proud. Thank you for always believing in me and pushing me for greatness.

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LIST OF ABBREVIATIONS

A549	Human lung carcinoma cell line	LM	Light microscopy
AA	Arachidonic acid	LuA	Lauric acid
AIDS	Acquired immune deficiency syndrome	MCF-7	Human breast adenocarcinoma cell line
ANOVA	one-way analysis of variance	MGD	Mean globule diameter
ARV	Antiretroviral	MLAPE	Mono linoleic acid ester derivative
Chol	Cholesterol	MLLAPE	Mono linolenic acid ester derivative
Chol-LA	Cholesteryl linoleate	MOAPE	Mono oleic acid ester derivative
Chol-LLA	Cholesteryl linolenate	MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Chol-LuA	Cholesteryl laurate	NE	Nanoemulsion
Chol-OA	Cholesteryl oleate	NEG	Nanoemulgel
Chol-PA	Cholesteryl palmitate	NMR	Nuclear magnetic resonance
Chol-SA	Cholesteryl stearate	o/w	Oil in water
CPEs	Chemical permeation enhancers	OA	Oleic acid
D	Dermis	PA	Palmitoleic acid
DLAPE	Di- linoleic acid ester derivative	PBS	Phosphate buffered saline
DLLAPE	Di- linolenic acid ester derivative	PDI	Polydispersity index
DLS	Dynamic light scattering	RT	Room temperature
DMAP	p-dimethylaminopyridine	SA	Stearic acid
DMSO	Dimethyl sulfoxide	SB	Stratum basale
DOAPE	Di- oleic acid ester derivative	SC	Stratum corneum
EDAC.HCI	N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride	SD	Standard deviation
ER	Enhancement ratio	SFA	Saturated fatty acids
FA	Fatty acid	SG	Stratum granulosum
FT-IR	Fourier-transform infrared spectroscopy	SS	Stratum spinosum

GIT	Gastrointestinal tract	TDD	Transdermal drug delivery
H&E	Hematoxylin and eosin	TEER	Transepithelial electrical resistance
HeLa	Human cervix cancer cell line	TEM	Transmission electron microscopy
HepG2	Human liver hepatocellular carcinoma	TLAPE	Tri- linoleic acid ester derivative
HIV	Human immunodeficiency virus	TLLAPE	Tri- linolenic acid ester derivative
HLB	Hydrophilic-lipophilic balance	TNEG	Tenofovir nanoemulgel
HPMC	Hydroxypropyl methyl cellulose	TNF	Tenofovir
HRMS	High resolution mass spectroscopy	TOAPE	Tri- oleic acid ester derivative
IE	Incorporation efficiency	UFA	Unsaturated fatty acids
LA	Linoleic acid	WHO	World health organization
LLA	Linolenic acid	ZP	Zeta potential

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CHAPTER 1

INTRODUCTION

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1.1 Introduction

This chapter includes the background to the study, describing the prevalence of HIV and AIDS and the limitations of current oral tenofovir (TNF) administration. It further describes the use and advantages of TDD as an alternate route of drug administration and highlights the limitations of this route. It provides strategies to overcome the limitations and details of promising approaches to optimize TDD. Details of the aims and objectives, novelty and significance of the study are also included.

1.2 Background to this study

Globally, human immunodeficiency virus (HIV) infection and acquired immune deficiency syndrome (AIDS) is still one of the most serious public health diseases. Although antiretroviral (ARV) therapy has revolutionised the treatment of HIV and AIDS [1, 2], some ARVs require frequent administration at high doses due to their low bioavailability as these drugs are prone to extensive gastrointestinal (GIT) degradation, first pass metabolism and have a short half-life [3]. This treatment regimen leads to severe dose-dependent adverse effects which results in a decreased patient compliance. These limitations therefore hinder the effective treatment of HIV and AIDS. The prevalence of HIV and AIDS together with the drawbacks of current oral ARV treatment necessitates the identification of innovative and alternate ARV drug delivery strategies [3].

TNF is classified as a nucleotide analog type ARV drug that has the ability to specifically inhibit reverse transcriptase thereby terminating/blocking the process of viral replication. The oral administration of TNF has numerous limitations and is therefore currently administered as an ester prodrug (tenofovir disoproxil fumarate) to overcome its hydrophilicity and poor intestinal permeability [4, 5]. TNF is administered in high doses due to its poor bioavailability which results in multiple dose-dependent GIT associated adverse effects such as irritation of the intestinal lining, diarrhea, nausea and inflammation. [6, 7]. These limitations lead to a significant

decrease in patient compliance. Although there are several disadvantages associated with its current oral administration, TNF is still considered as the most successful ARV to treat and prevent HIV and AIDS and appears on the world health organization's (WHO) list of essential medicines [8]. In an attempt to improve the therapy of TNF, alternate routes of administration and novel drug delivery systems have been explored as strategies to overcome the above mentioned limitations. The vaginal, rectal and buccal route have attracted interest as alternate routes of TNF administration as they are capable of preventing exposure to the GIT by delivering drugs directly into the systemic circulation [9, 10]. Transdermal drug delivery (TDD) offers an attractive alternative for the delivery of TNF directly into systemic circulation as it has shown promise for several classes of drugs by avoiding several limitations associated current oral drug delivery [11, 12].

TDD has numerous advantages as the application and removal of dosage forms are convenient and stable plasma drug levels can be easily achieved by administering long acting TDD formulations. TDD avoids the hepatic first-pass effect and passage through the GIT, which results in a higher drug bioavailability and decreased GIT associated adverse effects [11, 12]. Furthermore, TDD is reported for the alternate administration of donepezil, diclofenac sodium, ketoprofen and peroxicam which are drugs that cause GIT distress in patients [13-16]. The administration of TNF via the transdermal route could therefore ensure adequate drug bioavailability and a decrease in the dose dependent GIT adverse effects, leading to an increase in patient compliance.

Despite the numerous advantages of TDD, a critical limitation to this route is the natural protective barrier layer of the skin, the stratum corneum (SC) [17]. The SC is the uppermost layer of the epidermis and is composed of corneocytes that are surrounded by extracellular lipids such as ceramides, cholesterol and free fatty acids. This layer presents a formidable barrier and only allows substances that are either lipid soluble and of a low molecular weight to pass through [18-20]. This barrier does not allow therapeutically relevant quantities of many drugs to transverse the skin unaided, therefore strategies need to be developed to overcome the hindrance provided by the SC. To increase the number of drugs that can be delivered in therapeutically relevant quantities, it is necessary to temporarily decrease the barrier properties of the skin. There have therefore been significant efforts using chemical, biochemical or physical approaches

to develop new strategies to reduce skin's barrier properties and improve TDD [18]. Chemical permeation enhancers (CPEs) have often been considered as excipients used to promote the transdermal penetration of drugs [21, 22]. CPEs have the ability to enhance TDD by increasing the partition of the drug into the SC, or interacting with the corneocytes, or by reversibly disrupting the extracellular lipid matrix thereby altering its nanostructure and increasing its fluidity (Figure 1) [21-23]. While the corneocytes forms the framework for the extracellular lipid matrix in the SC, the focal point of TDD approaches has been on the manipulations of the extracellular lipid region. While lipid soluble drugs are able to accumulate in the lipid matrix thereby facilitating their slow release, the presence of aqueous pores in this region also provides additional novel drug delivery opportunities in this area[12, 18].

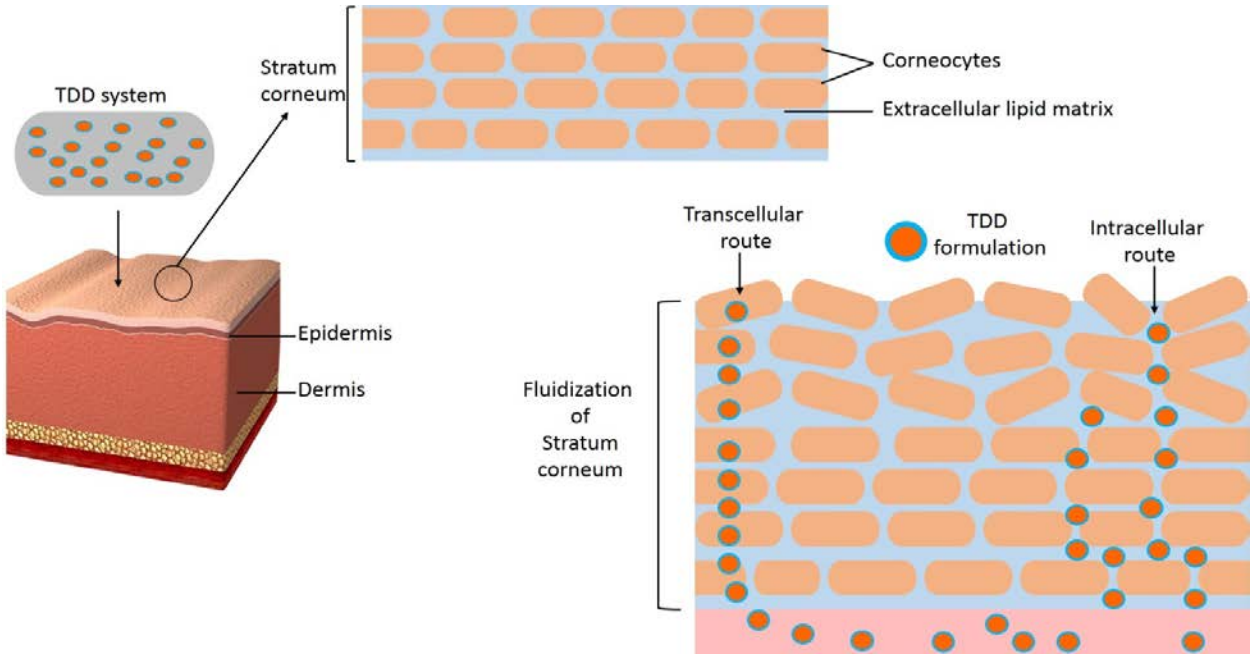

Figure 1. Mechanism of permeation enhancement: fluidization and transport pathways across the SC layer.

Fatty acids (FAs) are natural components of skin and have progressed as one of the most promising classes of transdermal CPEs. By altering the organization of the extracellular lipid matrix and increasing the fluidity of the SC, FAs are able to promote TDD by expanding the intercellular domains which effectively decreases the barrier properties [21, 24, 25]. Physicochemical characteristics such as carbon chain length, lipophilicity and structural

orientation of FAs have been implicated as features that determine their performance as a transdermal permeation enhancer [16, 21, 22]. Although saturated fatty acids (SFAs) and unsaturated fatty acids (UFAs) such as lauric acid (LuA), stearic acid (SA), oleic acid (OA), palmitoleic acid (PA), linoleic acid (LA), linolenic acid (LLA) and arachidonic acid (AA) have all been studied as transdermal permeation enhancers [15, 24, 26, 27]; literature reports that UFAs are more effective as compared to their SFAs counterparts[15, 28, 29]. Further, UFAs with an 18 carbon chain length have been reported as being the most successful transdermal permeation enhancers [21]. The notion that derivatives display greater enhancement than their parent materials is well supported by the literature [14, 21, 30-33]. FA derivatives such as diethyl sebacate, diisopropyl adipate and oleodendrimers of OA have all displayed significant permeation enhancement across the skin. One of the contributing factors to these derivatives displaying a greater performance has been attributed to their greater lipophilicity as compared to their parent material [14, 32]. Studies report that the formation of ester derivatives of FAs yields new superior chemical entities which display significantly greater permeation enhancement potential than their respective parent material. FA esters such as ethyl oleate, isopropyl decanoate and sucrose laurate have therefore shown promise as effective transdermal permeation enhancers for both hydrophilic and hydrophobic drugs [24]. Although FAs and their ester derivatives have been explored as enhancers for TDD of various classes of drugs, only one ARV drug (zidovudine) has been investigated using a FA as a transdermal permeation enhancer [34]. Recent reports further confirm the applicability of FA esters as novel dendritic esters of OA have displayed superior transdermal and buccal permeation enhancement of diclofenac sodium and didanosine respectively [14, 33]. Although there is a continuous search for new chemical entities, the identification of new CPEs is slow. Therefore, the modification of existing CPEs to produce novel chemical entities with superior performance is highly desirable.

Biocompatible nanomaterials have emerged as one of the recent strategies developed to enhance TDD. Nanomaterials offer unique properties that are not present in their bulk counterparts. Features such as size, biocompatibility, shape, surface chemistry, optical and electrical properties play key roles in improving many medical applications [35-38]. The formulation of nanomaterials as an approach to improve drug delivery and therapeutics are gaining huge interest in pharmaceutical sciences. Literature reports that nano sized transporters/ vehicles below 150

nm are able to effectively deliver drugs transdermally despite the natural barrier properties of the SC [38]. Lipid based nano formulations such as nano or microemulsions and nanoemulgels have shown promise to enhance the delivery of drugs across the skin [35-37, 39].

Nanoemulsions (NEs) have been explored for TDD applications as they display unique size features ranging from 20- 500 nm and have favorable morphological and chemical properties [40]. NEs are formulated through the combination of oil and water interface that is simultaneously stabilized using one or more surfactant or co-surfactants. The application of NEs in drug delivery offers many benefits such as decreased preparation costs, the absence of organic solvents or other toxic by-products, improved thermodynamic stability and increased storage stability [41, 42]. NEs also display large surface areas to volume ratios, a feature common to all nano-sized formulations. This inherent property can be utilized for the efficient delivery of drugs through the dermal route. The larger surface area of the nano-sized emulsion allows for increased and rapid penetration through the SC layer, promoting the entry of the drug into systemic circulation [41, 43].

The formulation of oil in water (o/w) NEs have an oil core surrounded by water and a combination of surfactants/ co-surfactants that stabilize the oil-water interface [41, 42]. The oil phase of an o/w NE is critically important due to its capacity to solubilize lipophilic drug molecules, thereby increasing its absorption through lipid membranes [43]. Interestingly it has been demonstrated that nanoemulsions have higher drug permeation when compared to conventional transdermal formulations such as gels and emulsions [41, 42]. Despite the advantages of NEs, the clinical applications of NEs are limited mainly due to the inconvenience of its low viscosity properties [44]. Literature illustrates that the use of hydrogels to thicken and increase the viscosity of NEs have displayed better rheological features and higher stability which leads to more effective TDD systems that display improved permeation properties [44]. The term nanoemulgels (NEGs) is derived from the combination of NEs and hydrogel matrices.

NEGs exhibit unique features from both NEs and gels that synergistically function to increase drug permeation enhancement across the skin tissue and improve transdermal applications [45, 46]. These advantages have promoted the use of NEGs for TDD of various classes of drugs.

Despite several classes of drugs explored using NEGs, there is no report for the formulation of TNF NEs or NEGs for transdermal drug delivery applications. Although the literature is expanding with myriad research on novel biomaterials that increase, improve and enhance drug permeation through the transdermal route, there still remains limited information on their translation into a dosage form or delivery systems that are essential for the development of successful therapeutics.

1.3 Problem statement

HIV and AIDS is one of the most serious, prevalent public health diseases globally. TNF is currently the most successful ARV drug to HIV infection. The oral administration of TNF has numerous limitations which necessitates its current administration as an ester prodrug, tenofovir disoproxil fumarate. Due to its high dose administration there are several GIT associated adverse effects which lead to a decrease in patient compliance. There is therefore a justifiable need to explore alternate strategies to improve the systemic delivery of TNF. In order to address the limitations of TNF, the use of novel drug delivery systems and alternate routes of drug administration have been investigated, however the transdermal permeability potential of TNF is yet to be explored. TDD of TNF could allow administration of the drug directly into systemic circulation in it's basic to native form which would also prevent the drug from passing through the GIT. This would eliminate the GIT associated side effects and result in an increased patient compliance. TDD system for TNF could also require lowering of the loading dose to achieve adequate bioavailability for therapeutic efficacy. Thus there would be benefits for both the patient and product manufacturer. Although there are numerous benefits to TDD, a major limitation to this route is the upper most layer of the skin, the SC, which is a natural barrier that prevents the entry of drugs through the skin. The use of transdermal permeation enhancers that are able to reversibility decrease the barrier property of the SC have shown promise to overcome this limitation and offer an effective means of increasing the number of drugs that can be delivered via this route. CPEs are most frequently used as transdermal permeation enhancers; however the identification of new CPEs is slow. Chemically modified entities have displayed greater permeation enhancement capabilities; therefore the modification of existing CPEs to produce superior novel derivatives will increase the pool of available excipients for TDD systems.

1.4 Aims and objectives of this study

The broad aim of this study was to explore the potential of lipid-based strategies for enhancing transdermal permeation of TNF. The specific research aims and objectives of two series of novel FA ester derivatives and the novel application of UFA esters of cholesterol as CPEs for TDD, as well as the novel formulation of a TNF nanoemulgel for transdermal application are highlighted in the chapters they are presented in.

1.4.1 Chapter: 2

The specific aim of this study was to synthesize and characterize novel biocompatible dendritic ester derivatives of UFAs and explore their potential as promising permeation enhancers for the transdermal delivery of TNF.

In order to achieve this aim, the objectives of the study were to:

1.4.1.1 Synthesize novel ester dendritic derivatives of palmitoleic (PA), linoleic (LA), linolenic (LLA) and arachidonic acid (AA) and characterize the derivatives using FTIR, NMR (^{1}H and ^{13}C) and HRMS techniques.

1.4.1.2 Determine the lipophilicity, pH responsiveness and the *in vitro* biosafety of the novel ester derivatives.

1.4.1.3 Explore the *in vitro* potential of the novel FA ester derivatives as transdermal permeation enhancers for TNF and assess the effects of the enhancer treatment on the skin using transepithelial electrical resistance as well as light and transmission electron microscopy.

1.4.2 Chapter: 3

The specific aim of this study was to evaluate the novel application of UFA esters of cholesterol as promising transdermal permeation enhancers using TNF as a model drug.

In order to achieve this aim, the objectives of the study were to:

1.4.2.1 Synthesize cholesterol ester derivatives of oleic (OA), linoleic (LA), linolenic (LLA), Lauric (LuA), Palmitic (PA) and stearic acid (SA) and characterize the derivatives using FTIR, NMR (^{1}H and ^{13}C) techniques as well as determining their lipophilicity.

1.4.2.2 Explore the *in vitro* potential of the cholesterol ester derivatives as transdermal permeation enhancers for TNF and assess the effects of the enhancer treatment on the skin using transepithelial electrical resistance and light microscopy.

1.4.3 Chapter: 4

The specific aim of this study was to synthesize and characterize novel biocompatible mono, di and tri-ester derivatives of FAs and explore their potential as promising transdermal permeation enhancers using TNF as a model drug.

In order to achieve this aim, the objectives of the study were to:

1.4.3.1 Synthesize novel mono, di and tri-ester derivatives of FAs bearing β-alanine *t*-butyl ester head group using oleic (OA), linoleic (LA), linolenic (LLA) and stearic acid (SA) and characterize the derivatives using FTIR, NMR (^{1}H and ^{13}C) and HRMS techniques.

1.4.3.2 Determine the lipophilicity and the *in vitro* biosafety of the novel ester derivatives.

1.4.3.3 Explore the *in vitro* potential of the novel ester derivatives as transdermal permeation enhancers for TNF and assess the effects of the enhancer treatment on the skin using transepithelial electrical resistance and light microscopy.

1.4.4 Chapter 5:

The specific aim of this study was to explore the potential of novel linolenic acid based heterolipid, LLA1E (a novel transdermal permeation enhancer), as an oily phase in the development of a nanoemulgel for the transdermal drug delivery of TNF.

In order to achieve this aim, the objectives of the study were to:

1.4.4.1 To determine the optimal ratio of surfactant and co-surfactant (S_{mix}) to oil (LLA1E) by constructing pseudo-ternary phase diagrams.

1.4.4.2 Formulate and evaluate the TNF NE and TNF NEG in terms of mean globule diameter, polydispersity index, zeta potential, viscosity, morphology and stability.

1.4.4.3 Explore the *ex vivo* potential of the novel TNF nanoemulgel to enhance the transdermal delivery of TNF and assess the effects of the nano formulation on the skin using transepithelial electrical resistance and light microscopy.

1.5 Novelty of the study

The novelty of the research is presented in the four different experimental chapters as follows:

1.5.3 Chapter:2

The research performed in this study is novel for the following reasons:

- The synthesized dendritic ester derivatives explored in this paper as chemical permeation enhancers are novel, non-cytotoxic to mammalian cells and have not been previously reported for any drug delivery applications.
- The novelty of the study also lies in the fact that this is the first report on transdermal permeation efficacy of TNF, an ARV drug with potential clinical significance.
- Additionally, all the synthesized dendritic ester derivatives consist of tertiary nitrogen in their structure and their potential to show pH dependency has been proven experimentally. Therefore, these derivatives can also be considered as a novel class of biocompatible pH responsive excipients for pH dependant drug delivery systems.

1.5.4 Chapter:3

The research performed in this study is novel for the following reasons:

- This is the first report that utilizes UFAs esters of cholesterol as transdermal permeation enhancers for any drug class.

1.5.5 Chapter: 4

The research performed in this study is novel for the following reasons:

- The synthesized mono, di and tri-ester derivatives of FAs bearing β-alanine *t*-butyl ester head group are novel, non-cytotoxic to mammalian cells and have not been previously reported for any drug delivery applications.
- Additionally, a UK Patent (Application No. 1614120.2) was registered for the synthesis and transdermal permeation enhancement application of these novel derivatives.

1.5.6 Chapter: 5

The research performed in this study is novel for the following reasons:

- This study identified the potential of a novel dendritic heterolipid, LLA1E previously synthesized and reported by our group, as an oily phase excipient in the preparation of a performance efficient novel transdermal TNF NEG. This study reports for the first time the applicability of LLA1E in a pharmaceutical preparation.
- In addition to the novelty of the formulated nanosystem, this is the first report on the formulation of TNF NEs and NEGs for transdermal delivery.

1.6 Significance of the study

All four TNF transdermal permeation enhancement approaches proposed in this study are novel and could contribute to overcoming the natural barrier properties of the skin thereby allowing a larger variety of drugs to be delivered via this route. The novel approaches of this study could also demonstrate that therapeutically relevant doses of TNF can be delivered transdermally and therefore this route could function as an attractive alternative to overcome the limitations of current oral TNF administration. The significance of this study is highlighted below:

New pharmaceutical products:

The proposed ester dendritic derivatives of PA, LA, LLA and AA and the mono, di and tri-ester of OA, LA, LLA and SA which bear β-alanine *t*-butyl ester head group as well as the TNF loaded NEG are new pharmaceutical products not yet reported that can promote and improve innovate drug delivery approaches in industrial pharmaceutical R&D.

Improved patient therapy and disease treatment:

All the proposed permeation enhancement approaches could decrease the barrier properties of the skin, which should enhance the transdermal delivery of TNF. This could improve patient therapy by minimizing doses, lowering side effects and increasing patient compliance. These approaches may therefore improve patient amenability to this treatment regimen.

Creation of new knowledge to the scientific community:

The proposed research would lead to the dissemination of new scientific knowledge within the domains of pharmaceutical sciences and biomaterials chemistry.

- In the novel dendritic ester derivatives study, new knowledge from the development of superior biocompatible novel CPEs from parent FAs will be generated. Additionally, the applicability of TNF for transdermal delivery will be established, and its possible route of transport across the skin could be elucidated from light and transmission electron microscopy.
- In the UFAs esters of cholesterol study, new knowledge can be generated from the identification and combination of existing materials which possess advantageous inherent features which would contribute favourably to their novel applicability in TDD approaches.
- In the mono, di and tri-ester FA derivatives study, new knowledge can be generated from the design and synthesis of novel biosafe derivatives that target the lipid domains of the SC to decrease the barrier properties of the SC.
- In the TNF NEG study, new knowledge can be generated from the translation of previously identified novel CPEs into nano TDD dosage forms which is essential to realise their downstream pharmaceutical applications.

Stimulation of new research:

The proposed strategies designed for the enhancement of TNF permeation across the skin has the potential to improve drug delivery via the transdermal route. Additionally, this work has the capacity to stimulate innovative approaches in various research areas, such as:

- Due to the likely pH titratable nature of the synthesized novel FA dendritic ester derivatives they are potential excipients in the development of pH responsive drug delivery systems for antimicrobial or cancer therapeutic applications.

- If the UFAs esters of cholesterol show transdermal permeation enhancement potential they can therefore also be considered as multifunctional lipids (as CPEs and membrane stabilizers) in the future formulation development of transdermal liposomes for the treatment of multiple disease conditions.
- Additionally, the novel FA dendritic ester derivatives and the mono, di and tri-esters of FAs could also be further exploited for their potential applications in lipid based colloidal drug delivery systems such as nanoemulsions, self-microemulsifying drug delivery systems and nanostructured lipid carriers for several classes of drugs.
- The formulated NEG would demonstrate the translation of previously identified excipients into efficient nano drug delivery systems. This would encourage further research utilising reported materials in the design and implementation of effective dosage forms for various biomedical applications.

1.7 Overview of thesis

The research presented in this thesis is in the manuscript format, which is in line with the University of Kwa-Zulu Natal, College of Health Sciences guidelines, which specifies the inclusion of a brief introductory chapter, manuscripts in preparation/submitted/published and a final chapter on the conclusions. A PhD study requires at least 3 first authored manuscripts/papers, 2 of which must be experimental.

CHAPTER 1. INTRODUCTION: This chapter includes the background to the study, describing the prevalence of HIV and AIDS and the limitations of current oral TNF administration. It further describes the use and advantages of TDD as an alternate route of drug administration and highlights the limitations of this of this route. It provides strategies to overcome the limitations and details of promising approaches to optimize TDD. Details of the aims and objectives, novelty and significance of the study are also included.

CHAPTER 2. EXPERIMENTAL PAPER 1: This chapter addresses aim 1.4.1 and is a first authored experimental article published in the Journal of Materials Chemistry B (Impact

Factor = 4.872) which is an ISI international journal. This article reports the synthesis, characterization, and *in vitro* permeation enhancement effect of a novel series of UFA ester dendritic derivatives for the transdermal delivery of TNF.

CHAPTER 3. EXPERIMENTAL PAPER 2: This chapter addresses aim 1.4.2 and is a first authored experimental article manuscript published in an ISI international journal i.e. Drug Delivery and Translational Research (Impact Factor = 1.887). This article reports the synthesis, characterization and novel TDD application of a series of UFA esters of cholesterol using TNF as the model drug.

CHAPTER 4. EXPERIMENTAL PAPER 3: This chapter addresses aim 1.4.3 and is a first authored experimental article manuscript submitted the European Journal of Pharmaceutical Sciences (Impact Factor = 3.773) which is an ISI international journal, manuscript number: EJPS-D-16-01525. This manuscript reports the synthesis, characterization, biosafety and *in vitro* permeation enhancement effect of a novel series of mono, di and tri-ester derivatives of FAs bearing β-alanine *t*-butyl ester head group for the transdermal delivery of TNF.

CHAPTER 5. EXPERIMENTAL PAPER 4: This chapter addresses aim 1.4.4 and is a first authored experimental article published in Colloids and Surfaces B: Biointerfaces (Impact Factor = 3.902) which is an ISI international journal. This article reports the use of a novel linolenic acid based heterolipid, LLA1E (a novel transdermal permeation enhancer), as an oily phase in the development of a nanoemulgel for the transdermal drug delivery of TNF.

CHAPTER 6. CONCLUSION: This chapter details the general conclusions from the research outcomes of the study. It describes the significance and impact of the research and further recommends future research in the field of TDD and improved ARV therapeutics.

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CHAPTER 2

Experimental paper 1

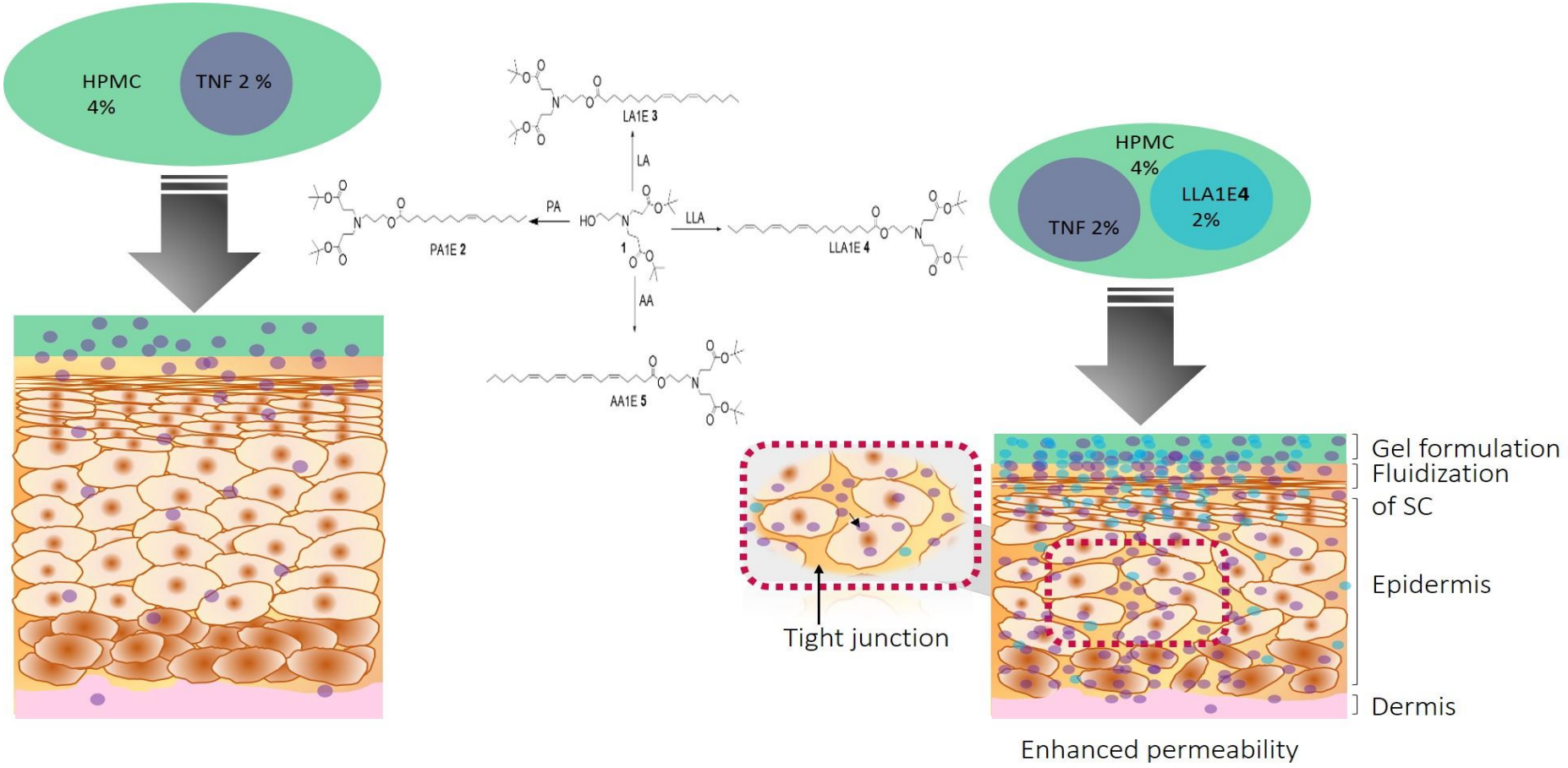
CHAPTER 2
Experimental paper 1

2.1 Introduction

This chapter addresses aim 1.4.1 and is a first authored experimental article published in the Journal of Materials Chemistry B (Impact Factor = 4.872) which is an ISI international journal. This article reports the synthesis, characterization, and in vitro permeation enhancement effect of a novel series of UFA ester dendritic derivatives for the transdermal delivery of TNF.

Mr. S. Rambharose contributed to the design of the project, modification and optimisation of methods as well as synthesis and characterisation of UFA ester dendritic derivatives. Mr. S. Rambharose conducted all experimental work including *in vitro* cytotoxicity, rat skin tissue harvesting, *in vitro* transdermal permeation experiments, TEER experiments and light and transmission electron microscopy evaluations. Mr. S. Rambharose also wrote the paper. Dr. R.S. Kalhapure assisted with the overall design of the project and the methods of preparation and characterisation as well as editing of the paper. Prof K.G. Akamanchi supervised the synthesis of the UFA ester dendritic derivatives in this work. Prof. T. Govender served as supervisor and was responsible for project conceptualisation, editing of paper and abstract and general supervision of the study.

2.2 Graphical abstract



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

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Abstract

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.

Keywords: Tenofovir, transdermal, fatty acids, 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.

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 designingand 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.

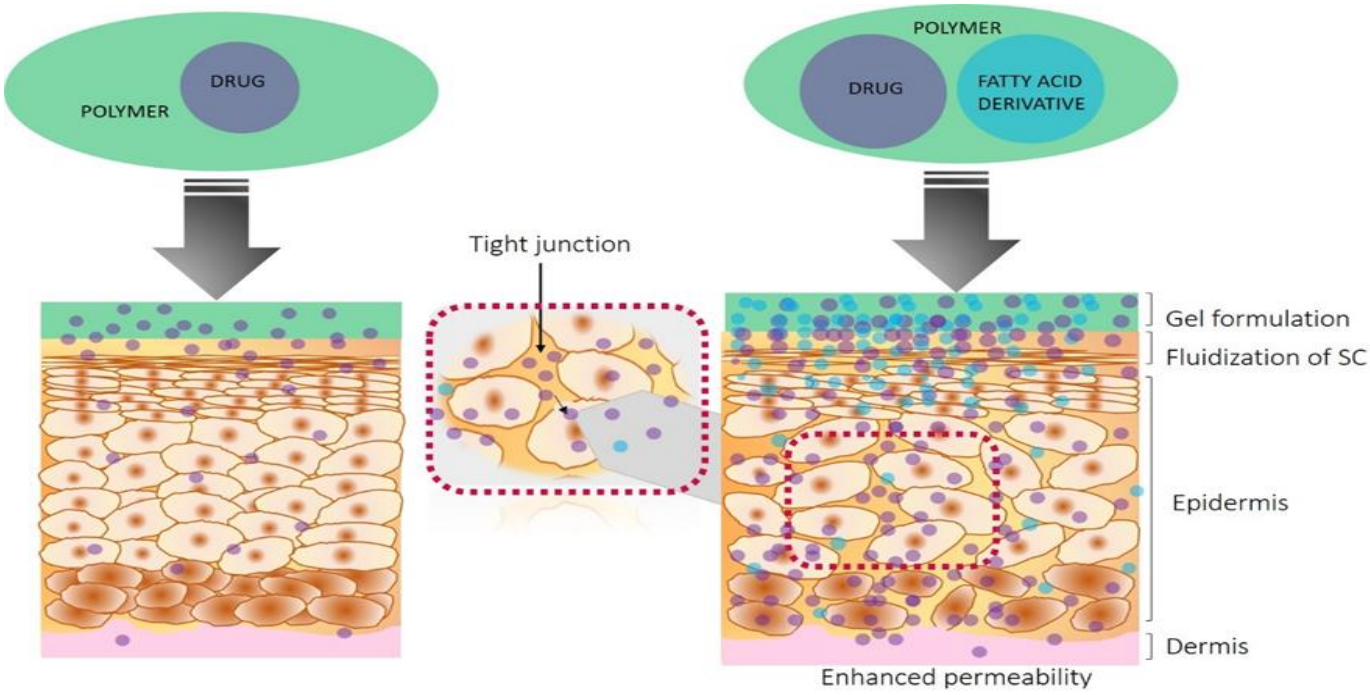


Fig. 1 Illustrates enhanced transdermal drug permeability through the application of novel fatty acid derivatives and drugs within a polymer formulation

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].

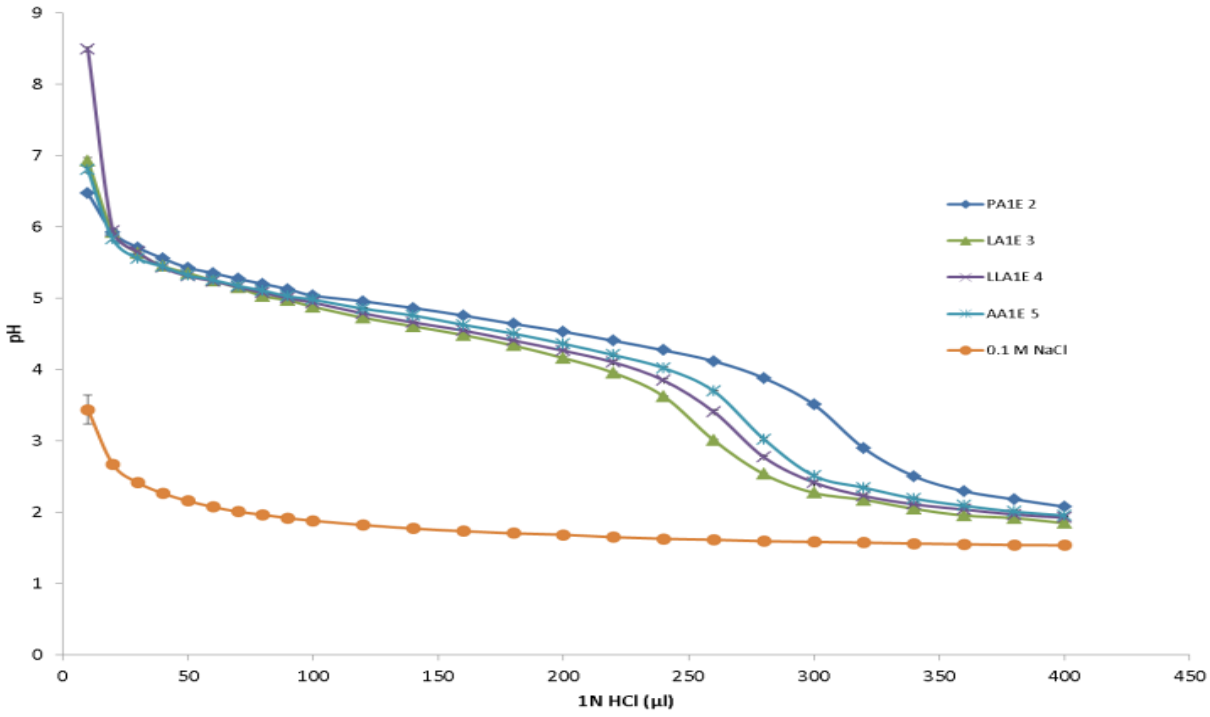


Fig. 2 Acid-base titration profile of dendritic derivatives and 0.1 M NaCl (n = 3; RSD < 10%).

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

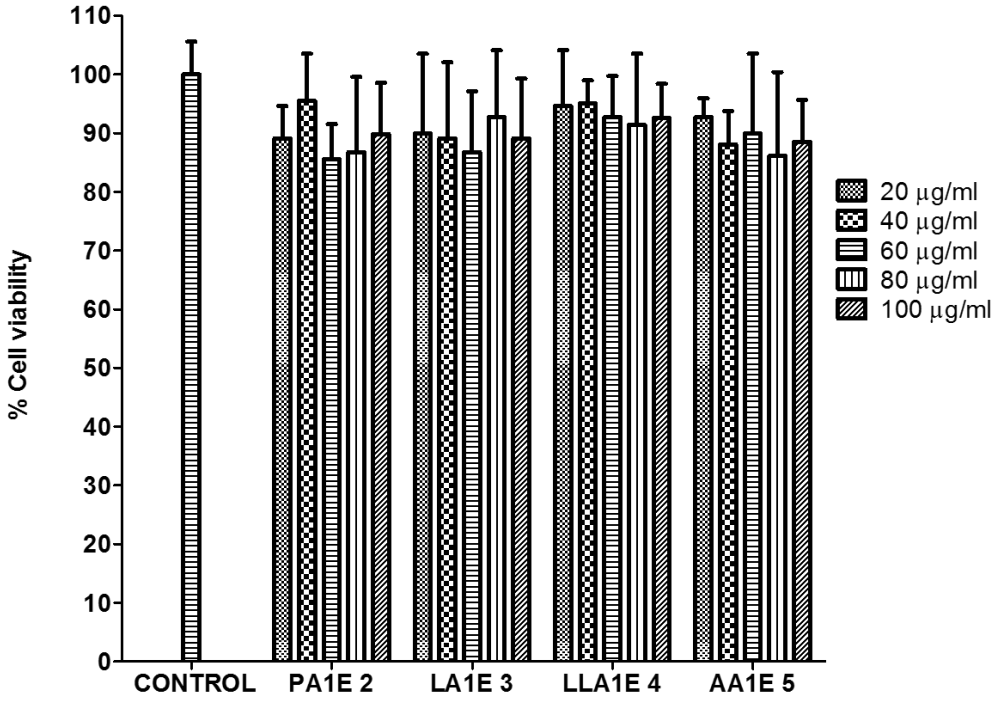


Fig. 3 Cytotoxicity assay displaying percentage cell viability after exposure to various concentrations of the FA derivatives on HeLa cells. Results are presented as mean ± SD. (n = 6).

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.

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 (µg/cm²)*	*Jss (flux) (µg/cm².h)*	*Permeability (P x 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 µg/cm^2 h and 226.85 ± 33.89 to 560.72 ± 20.01 µg/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 µg/cm^2 h and cumulative amount permeated to 269.99 ± 5.16 µg/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].

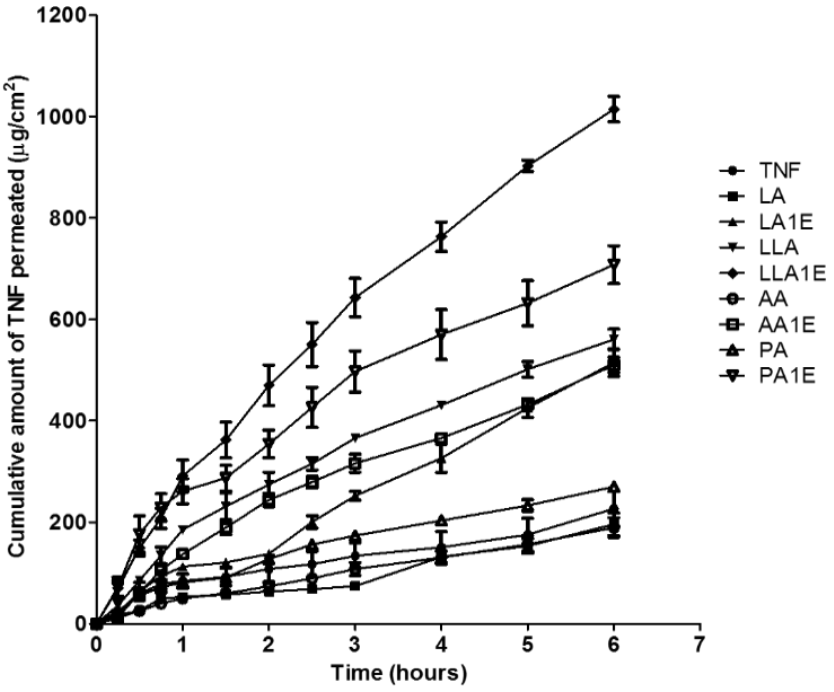
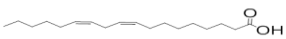
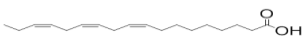
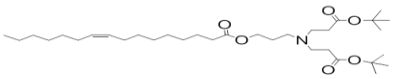
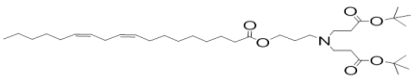
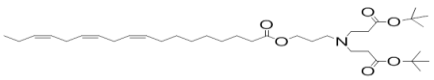
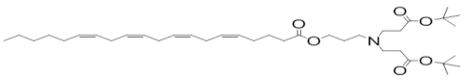


Fig 4. The effect newly synthesized derivatives as compared to their parent FA on the cumulative amount of TNF permeated across the skin. Results are presented as mean ± SD. (n = 6).

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).

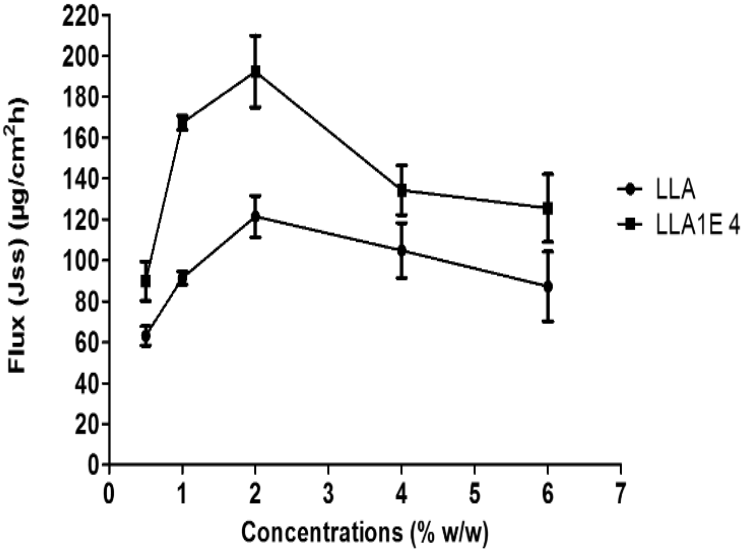


Fig. 5 Concentration effects of LLA and LLA1E 4 on the flux of TNF (n=6)

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].

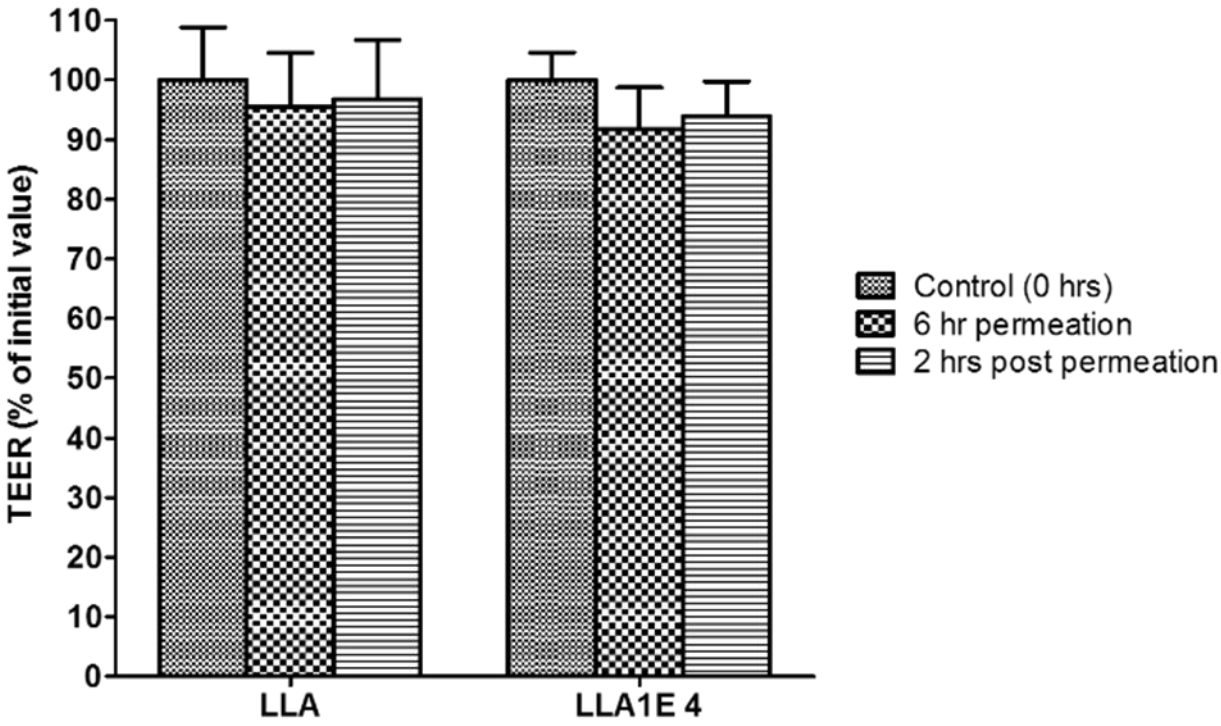


Fig 6. Percentage change in TEER values after TNF permeation with either LLA or LLA1E 4 at a concentration of 2% w/w (n = 6).

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.

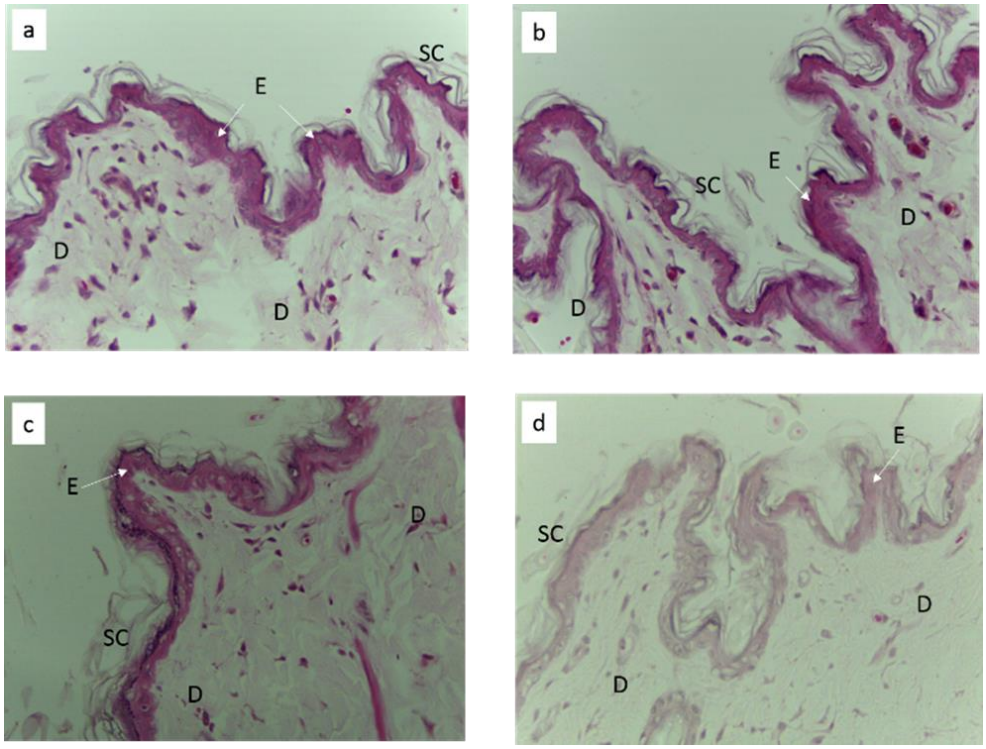


Fig 7. Photomicrographs of the control and the treated skin selections for light microscopy (LM) stained with H&E ; (× 40) a control/untreated, b treated with TNF gel, c treated with 2% w/w LLA TNF gel, d treated with 2% w/w LLA1E **4** TNF gel. (D: dermis; E: epidermis; SC: stratum corneum).

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.

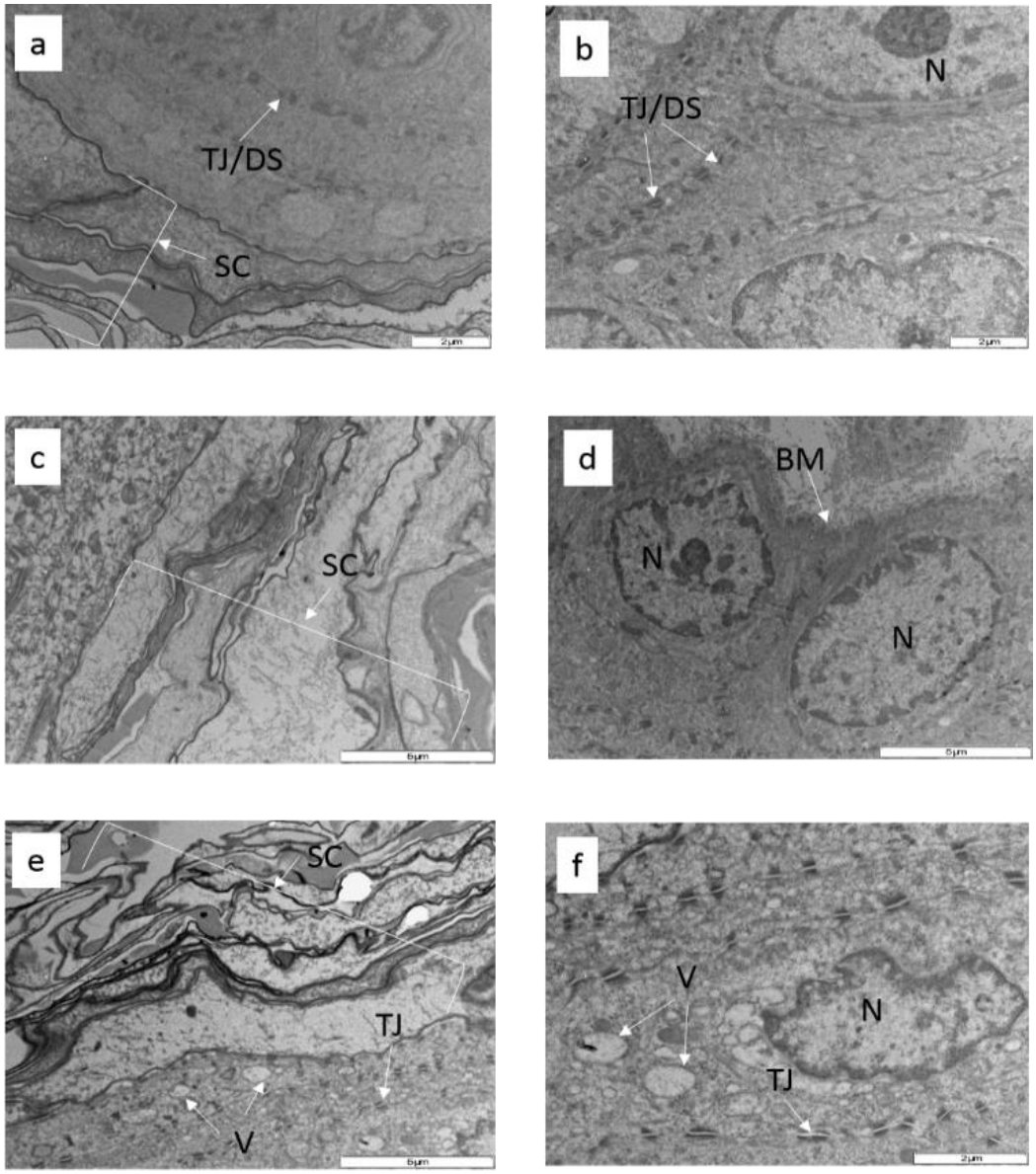


Fig. 8 Electron micrographs of the control and the treated skin selections for transmission electron microscopy (TEM) : a/b control/untreated (× 8000), c treated with 2% w/w LLA TNF gel (× 8000), d treated with 2% w/w LLA TNF gel (× 10000), e treated with 2% w/w LLA1E **4** TNF gel (× 8000), f treated 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.

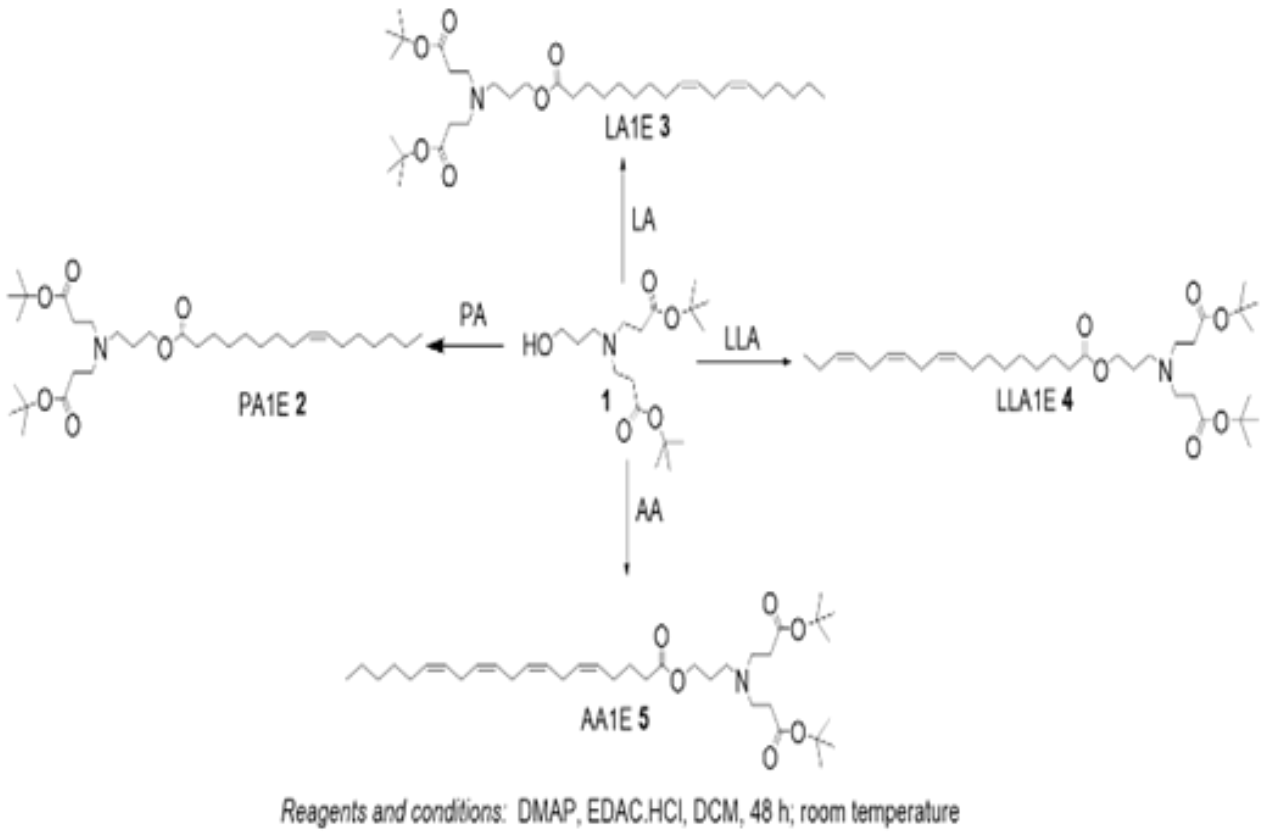
To a stirred mixture of dendron **1** (1.2 equiv.), synthesized using a previously reported method [12] and DMAP (0.75 equiv.) in DCM was added EDAC.HCl (1.5 equiv.) followed by an UFA (1 equiv.). The resulting reaction mixture was magnetically stirred at room temperature for a 48 h period. The organic 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.

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 (2.4×10^3) 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]:

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

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.

Acknowledgements

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SUPPORTING INFORMATION

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

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Contents:

Table S1. ^{13}C NMR spectra of dendritic ester derivatives before and after storage.

Dendritic ester derivative	^{13}C NMR	
	Fresh	2 year storage
PA1E **2**	14.09	14.09
	22.64	22.65
	24.98	24.98
	26.68	26.61
	27.16	27.17
	27.21	27.22
	28.09	28.09
	28.98	28.98
	29.11	29.11
	29.16	29.15
	29.18	29.18
	29.69	29.69
	31.77	31.77
	33.80	33.71
	34.35	34.34
	49.40	49.36
	50.14	50.15
	62.44	62.41
	80.31	80.37
	129.76	129.76
	129.98	129.98
	171.98	171.91
	173.86	173.86
LA1E **3**	14.04	14.06
	22.54	22.56
	24.96	24.97
	25.60	25.62
	26.65	26.67
	27.17	27.19
	28.07	28.09
	29.10	29.12
	29.13	29.14
	29.16	29.18
	29.32	29.33
	29.58	29.60
	31.50	31.51
	33.77	33.79
	34.32	34.33
	49.39	49.40
	50.13	50.14
	62.41	62.42
	80.28	80.30

Continued……

	127.89	127.90
	128.01	128.02
	130.01	130.04
	130.17	130.19
	171.91	171.94
	173.79	173.81
LLA1E **4**	14.26	14.26
	20.54	20.54
	24.97	24.97
	25.52	25.52
	25.60	25.61
	26.68	26.63
	27.20	27.20
	28.09	28.09
	29.12	29.12
	29.14	29.14
	29.58	29.18
	33.80	33.74
	34.34	34.33
	49.40	49.38
	50.14	50.15
	62.45	62.41
	80.30	80.33
	127.11	127.11
	127.71	127.71
	128.25	128.24
	128.28	128.27
	130.26	130.26
	131.95	131.94
	171.97	171.91
	173.84	173.81
AA1E **5**	14.10	14.06
	22.56	22.56
	24.83	24.82
	25.61	25.61
	26.59	26.52
	26.91	26.58
	27.21	27.20
	28.09	28.08
	29.69	29.31
	31.51	31.50
	33.72	33.63
	34.66	33.71
	49.39	49.31
	50.14	50.12
	62.53	62.50
	80.30	80.37
	127.54	127.53
	127.86	127.86

Continued……

128.17	128.16
128.20	128.20
128.79	128.56
128.99	128.80
130.47	130.47
171.96	171.88
173.57	173.58

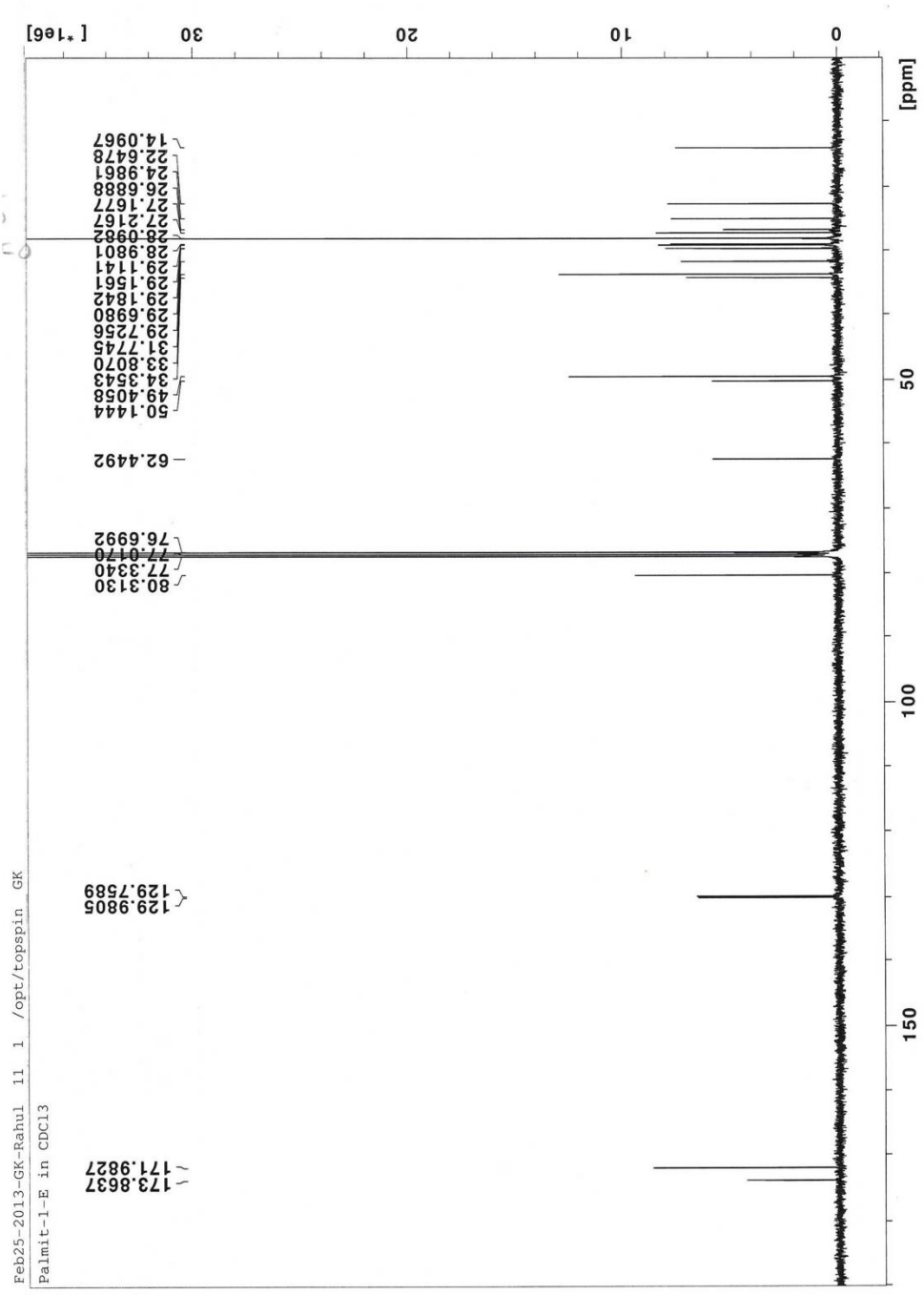


Fig. S1. ^{13}C NMR of PA1E **2** (day 0).

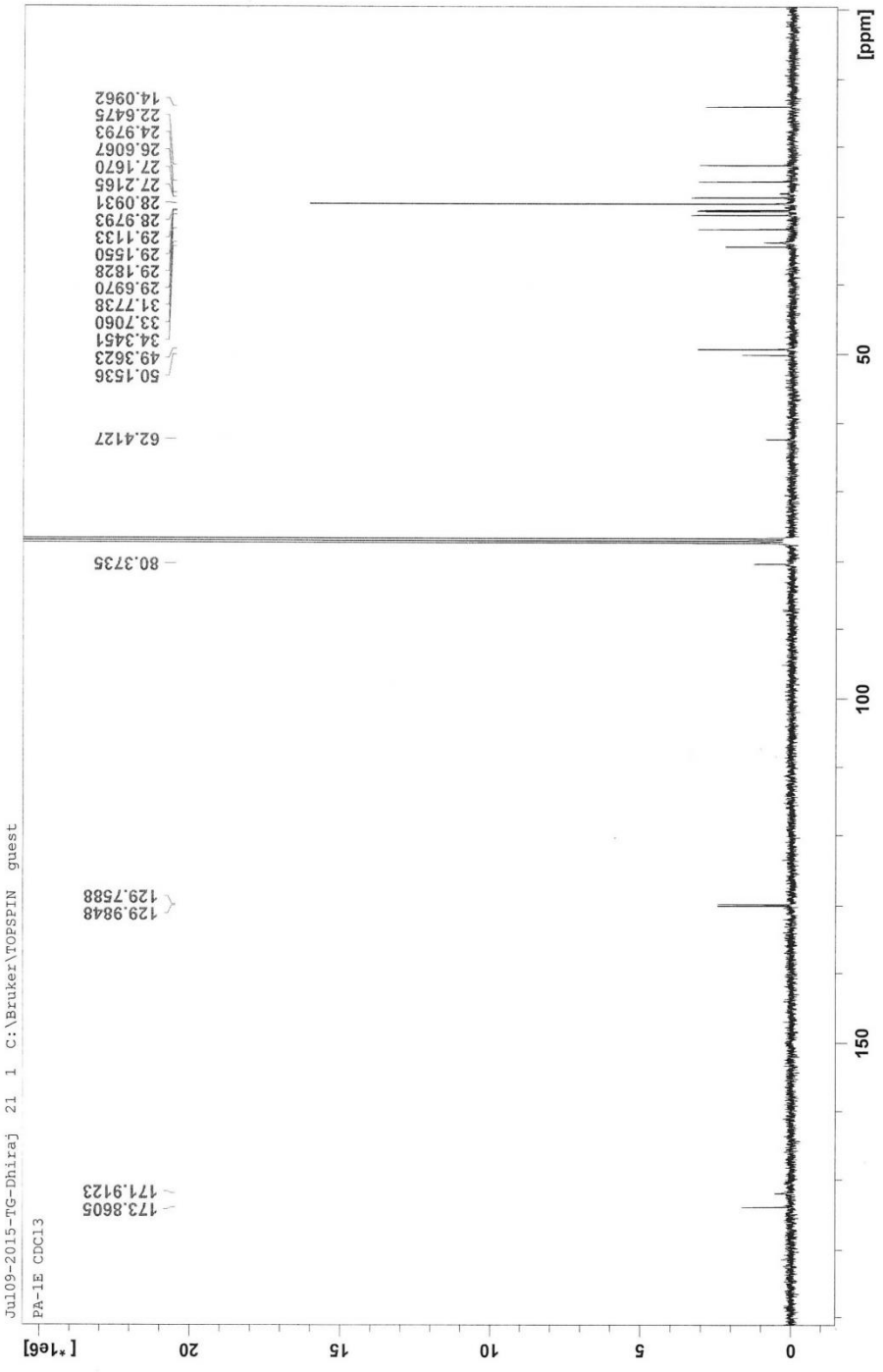


Fig. S2. ^{13}C NMR of PA1E **2** (2 years).

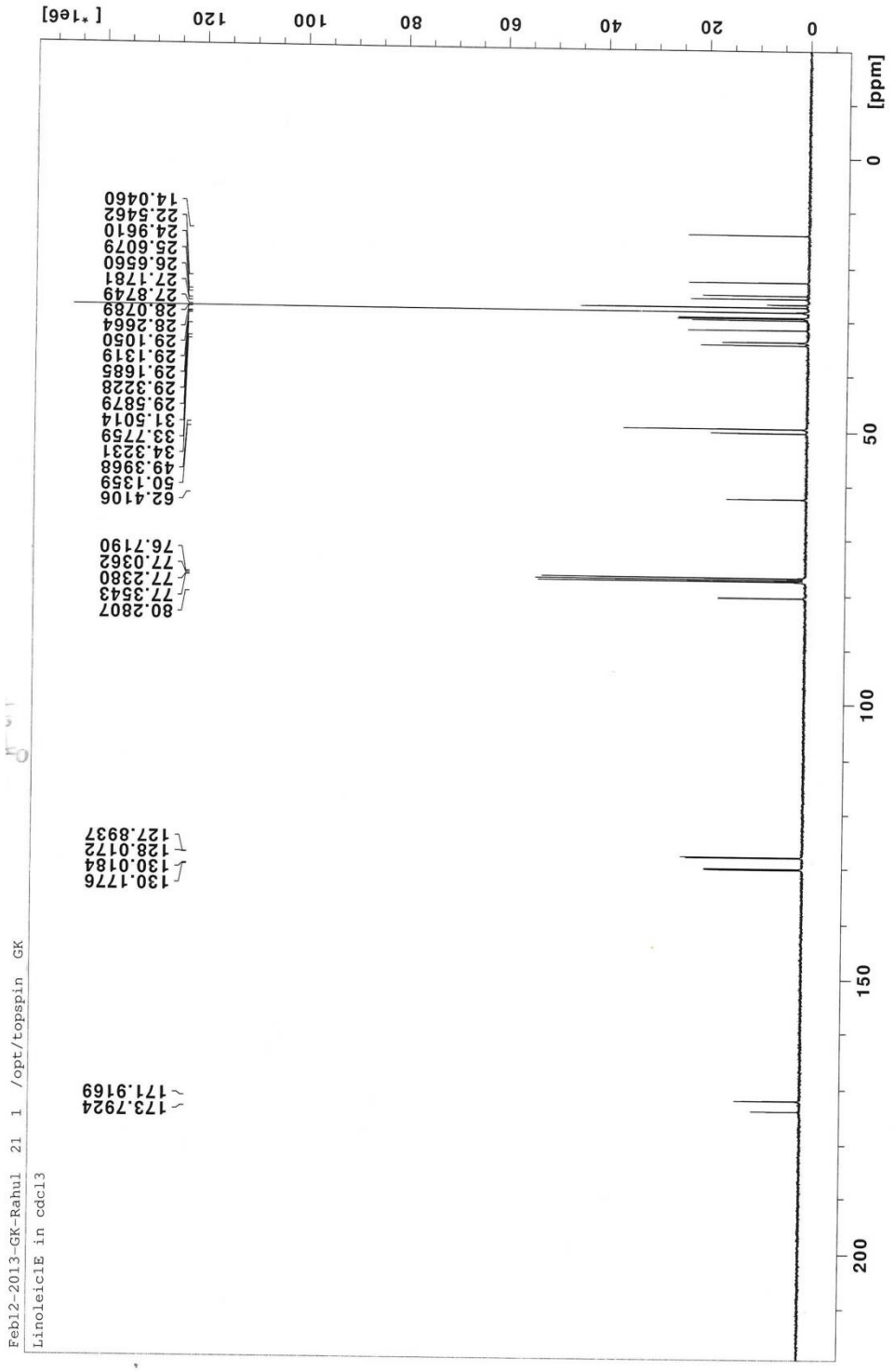


Fig. S3. ^{13}C NMR of LA1E **3** (day 0).

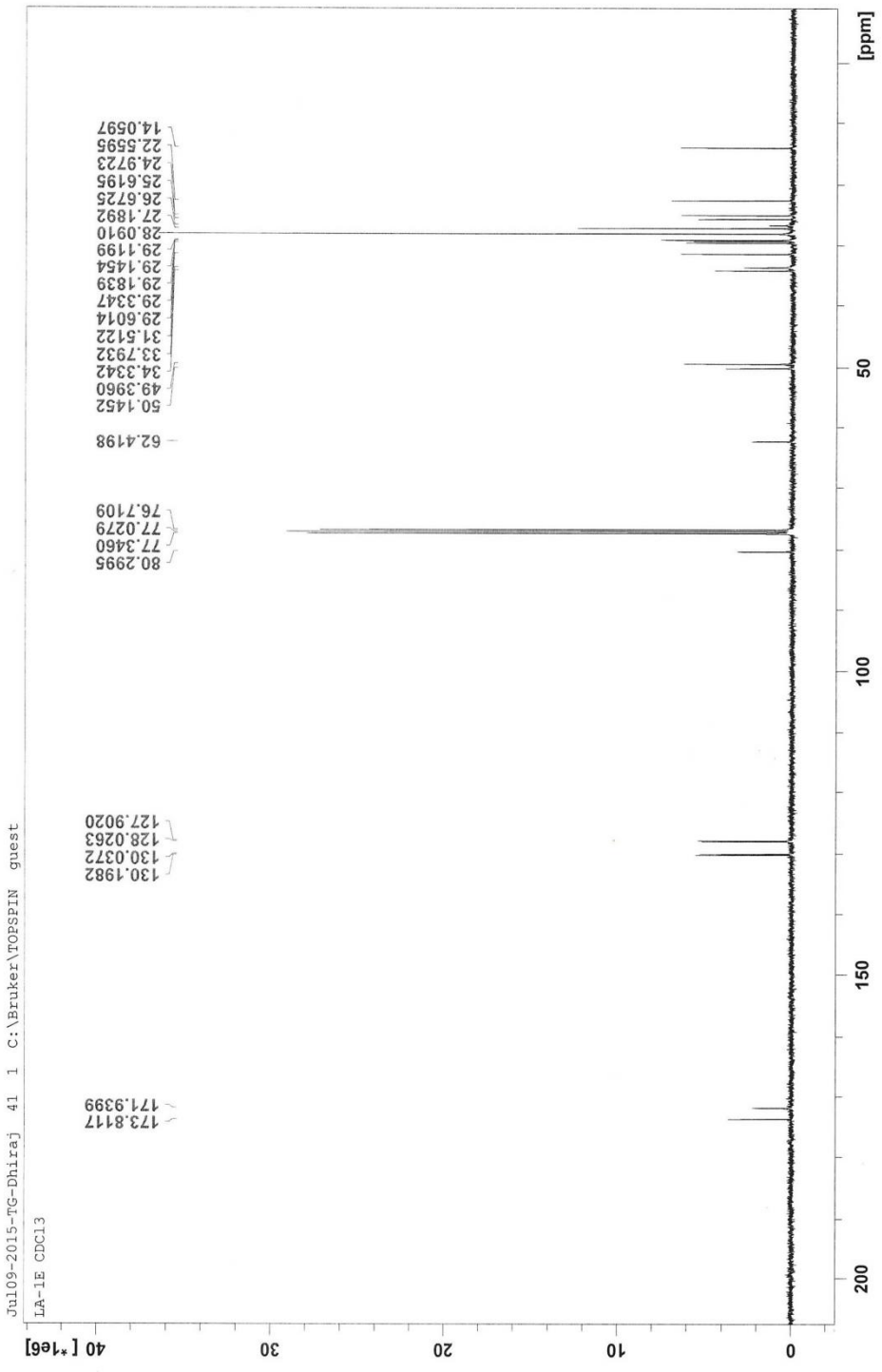


Fig. S4. ^{13}C NMR of LA1E **3** (2 years).

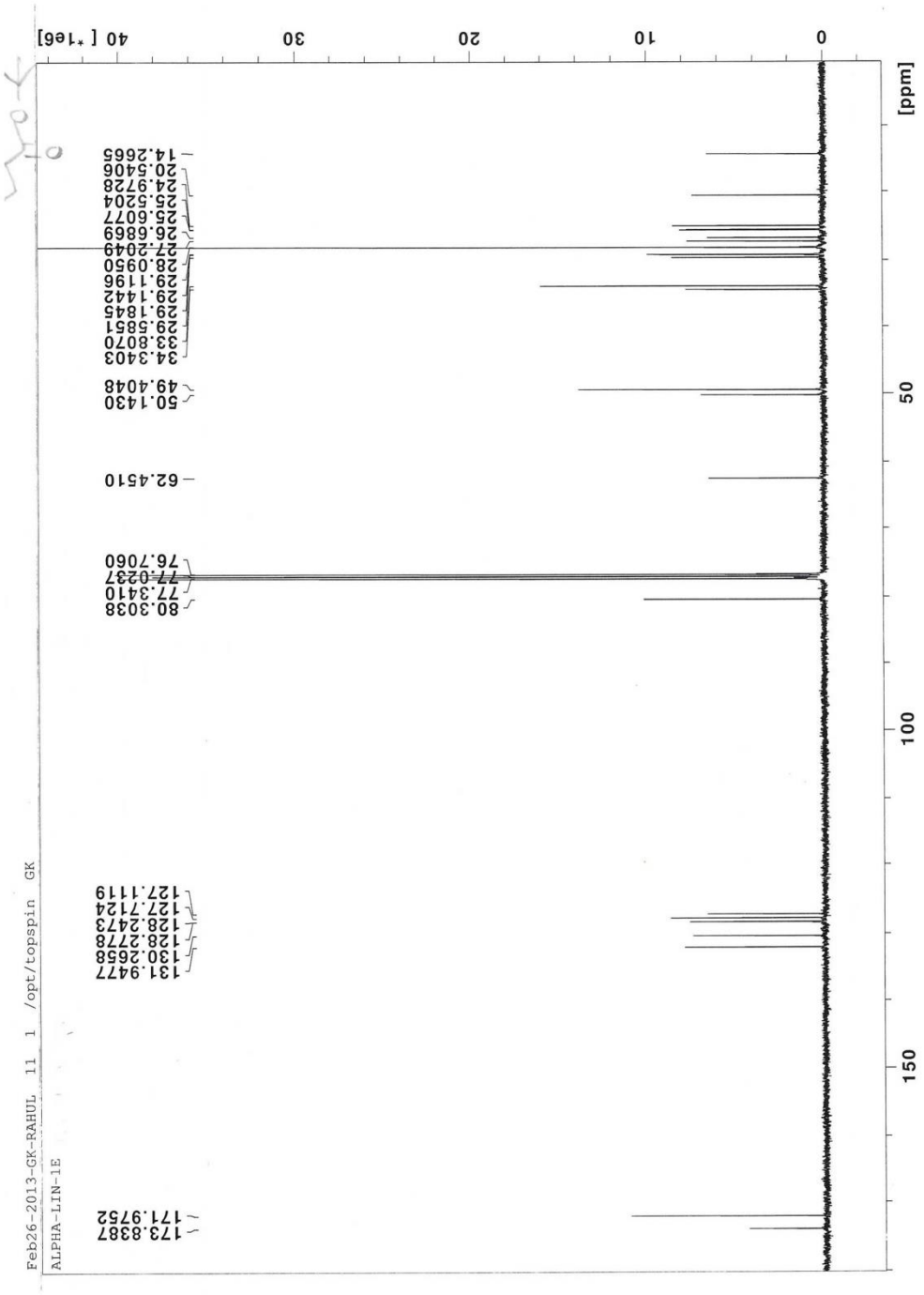


Fig. S5. ^{13}C NMR of LLA1E **4** (day 0).

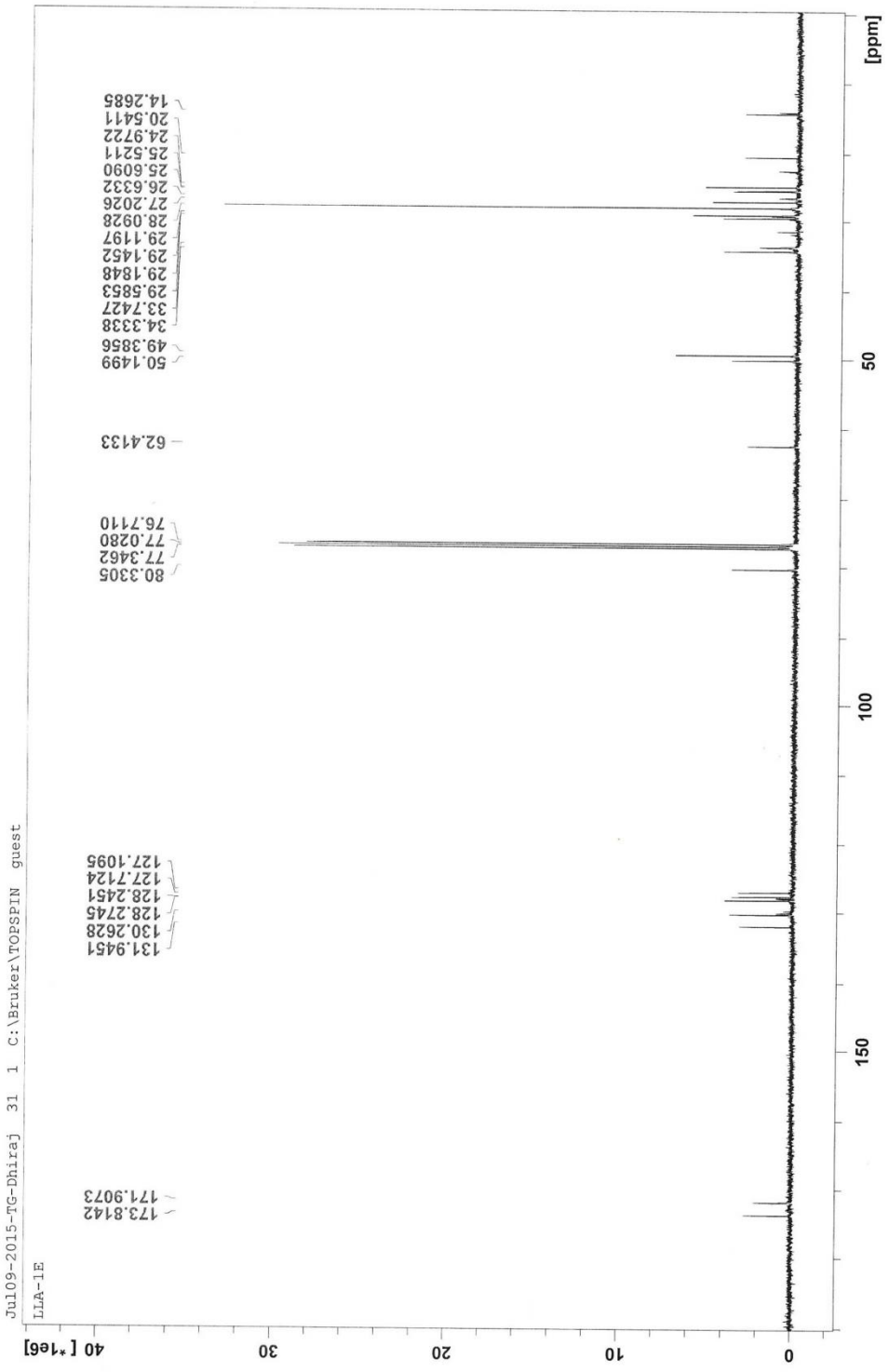


Fig. S6. ^{13}C NMR of LLA1E **4** (2 years).

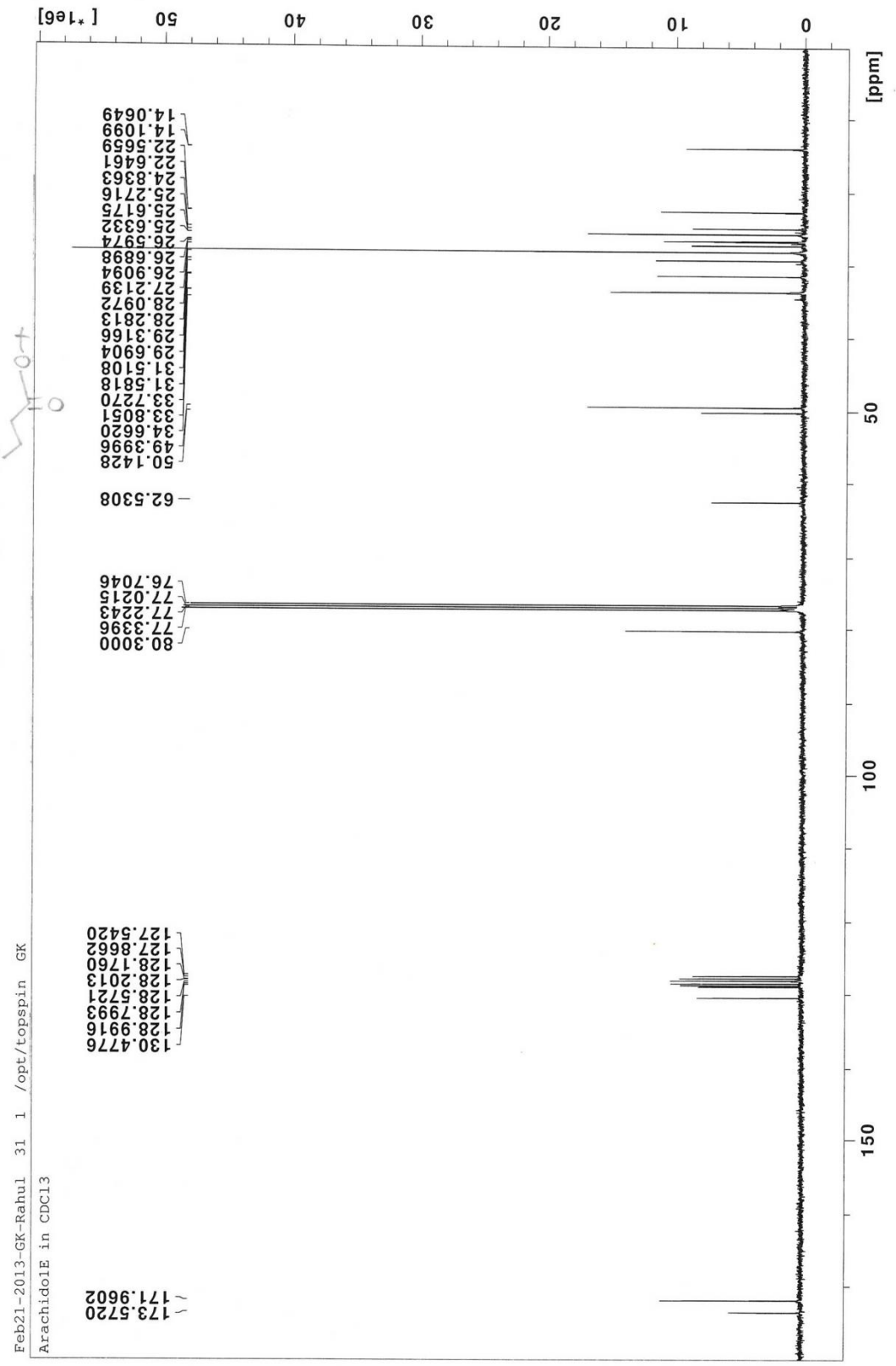


Fig. S7. ^{13}C NMR of AA1E **5** (day 0).

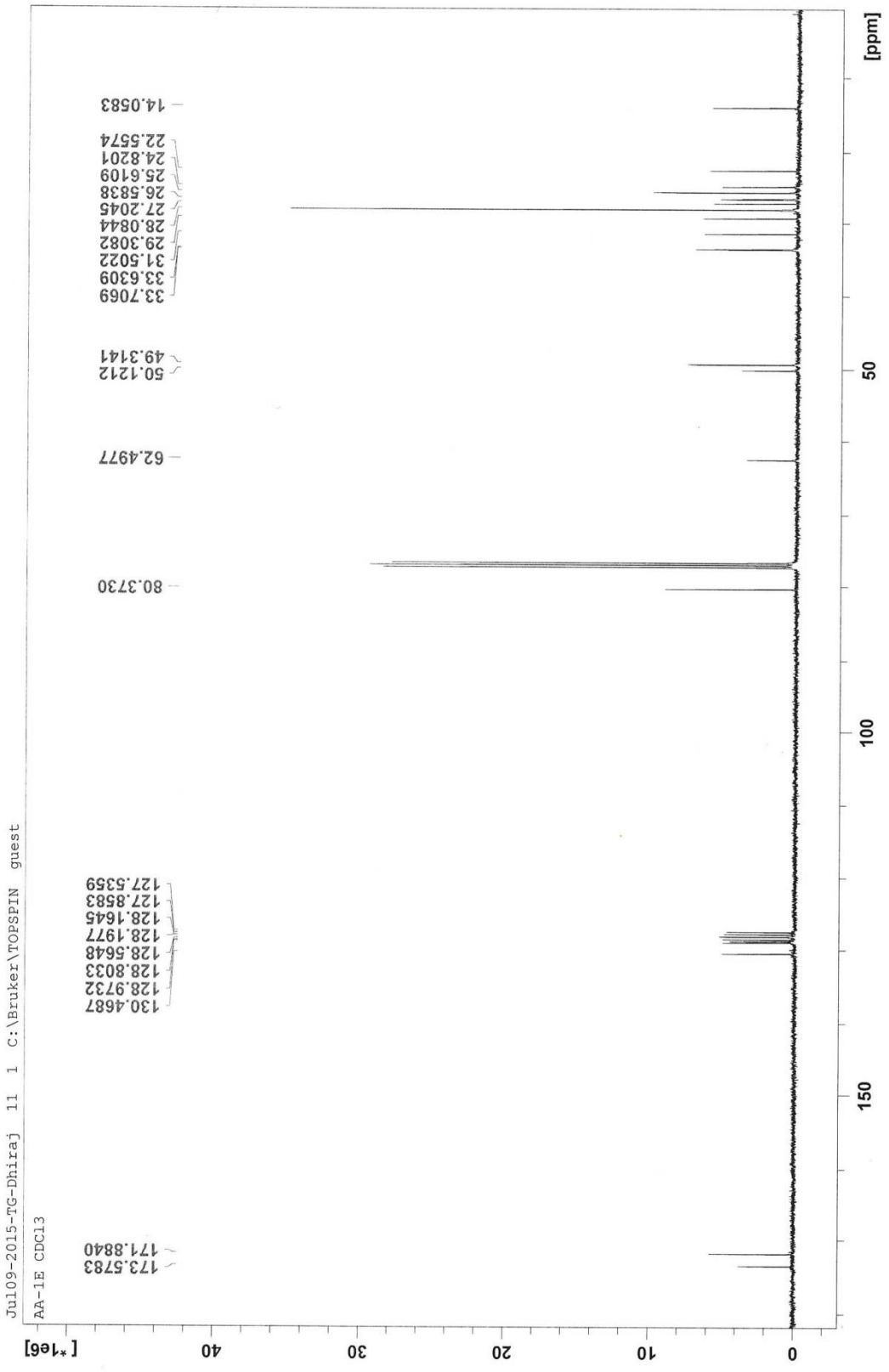


Fig. S8. ^{13}C NMR of AA1E **5** (2 years).

CHAPTER 3

EXPERIMENTAL PAPER 2

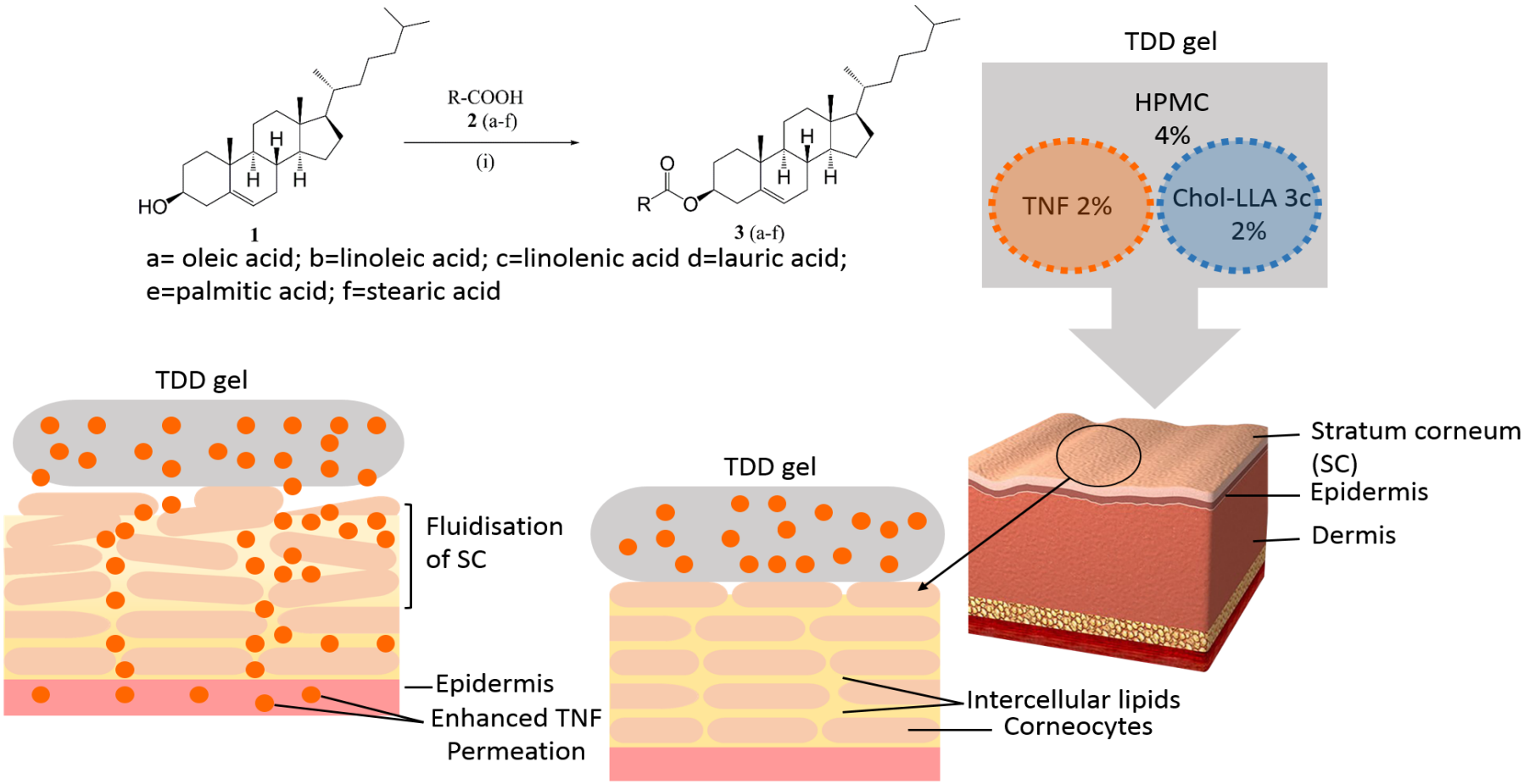
CHAPTER 3
EXPERIMENTAL PAPER 2

3.1 Introduction

This chapter addresses aim 1.4.2 and is a first authored experimental article published in an ISI international journal i.e. Drug Delivery and Translational Research (Impact Factor = 1.887). This article reports the synthesis, characterization and novel TDD application of a series of UFA esters of cholesterol using TNF as the model drug.

Mr. S Rambharose contributed to the design of the project, modification and optimisation of methods as well as synthesis and characterisation of UFAs and SFAs esters of cholesterol. Mr. S. Rambharose conducted all experimental work including rat skin tissue harvesting, *in vitro* transdermal permeation experiments, TEER experiments and light microscopy evaluations. Mr. S. Rambharose also wrote the paper. Dr. R.S. Kalhapure and Dr. M. Jadhav assisted with the overall design of the project and the methods of preparation and characterisation as well as editing of the paper. Prof. T. Govender served as supervisor and was responsible for project conceptualisation, editing of paper and abstract and general supervision of the study.

3.2 Graphical abstract



Exploring unsaturated fatty acid cholesteryl esters as transdermal permeation enhancers

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Abstract

The intrinsic protective barrier property of skin, one of the major challenges in the design of transdermal drug delivery systems, can be overcome through the use of chemical permeation enhancers (CPEs). Herein we explore the potential of unsaturated fatty acid (UFA) esters of Cholesterol (Chol) viz., oleate, linoleate and linolenate, as transdermal CPEs using tenofovir (TNF) as a model drug. All Chol UFA esters at 1% w/w were found to be more effective enhancers when compared to their respective parent FAs and saturated FA counterparts. Cholesteryl linolenate (Chol-LLA) showed the most superior performance (ER = 3.71). The greatest ER for Chol-LLA (5.93) was achieved at a concentration of 2% w/w. The histomorphological and transepithelial electrical resistance (TEER) evaluations supported the results of the permeability studies. These findings showed no significant loss in the integrity of the epidermis, with drug and enhancer treatment having temporary effects on the barrier property of the epidermis. Chol UFA esters can therefore be considered as new CPEs for exploitation in topical formulations for various classes of drugs.

Keywords: unsaturated fatty acids; cholesterol; esterification; chemical permeation enhancers; tenofovir; transdermal.

1. Introduction

Transdermal drug delivery (TDD) offers numerous advantages over the oral route by delivering the drug directly into systemic circulation. The major advantages are avoiding of first-pass metabolism, resulting in higher bioavailability of a drug and decreased GIT associated adverse effects [1, 2]. The administration of long acting TDD formulations allows for decreased dosing frequency and maintenance of stable plasma levels, which is essential for drugs that have a narrow therapeutic index. This lends to the decrease in the dose dependent side effects. Furthermore, the application of transdermal dosage forms is convenient and can be easily removed if there are any adverse reactions. These advantages should result in an increase in patient compliance [3, 2]. TDD therefore offers an attractive alternative for several classes of drugs as it avoids several limitations of current oral drug delivery [4, 5].

Tenofovir (TNF), an antiviral reverse transcriptase inhibitor, is used to prevent and treat HIV and AIDS. The oral administration of TNF has numerous limitations such as poor intestinal permeability, which necessitates its current administration as an ester prodrug, tenofovir disoproxil fumarate. Due to its low bioavailability, it is administered in high doses which results in several GIT associated adverse effects which lead to a decrease in patient compliance [6, 7]. Despite the current limitations of its oral delivery, TNF is still the most successful ARV drug to HIV infection [8]. With TNF being on the world health organization's (WHO) list of essential medicines [9] and HIV and AIDS continuing as one of the most serious, prevalent public health diseases globally [10] there is a justifiable need to explore alternate strategies to improve the systemic delivery of TNF. To overcome the limitations of current TNF oral administration, the buccal, rectal, vaginal and more recently the transdermal route have been explored for the delivery of TNF [11-13]. Our recent report showed that TNF in its basic to native form could be delivered in therapeutically relevant quantities via the transdermal route using a lower loading dose [12]. This route of administration has potential benefits for both the patient and the product manufacturer as there is no need to administer the ester prodrug thereby decreasing manufacturing costs and as the drug no longer passes through the GIT there is no risk of GIT associated adverse effects [12]. There is therefore a need to identify strategies for potentiating the transdermal delivery of TNF.

Although TDD offers promising benefits, a major drawback is the natural barrier property of the skin. One of the skin's main physiological functions is to regulate what enters and exits the body via this route. The skin's significant barrier property is largely due to the stratum corneum

(SC) which makes up the upper most layer of the epidermis. The SC is made up of corneocytes that are surrounded by extracellular lipid domains. These structured lipids such as ceramides, Chol and free fatty acids of the SC function to prevent excessive water loss from the body and prevent the entry of topically applied substances [3, 5, 2, 14] This barrier poses a significant challenge to the topical administration of drugs, unless they are lipid soluble and of low molecular weight.

Despite the formidable barrier presented by the skin, drug delivery via the transdermal route is a very attractive option and is extensively utilized for both local and systemic therapy [15, 16, 3]. A temporary reduction in the barrier properties of the skin is desirable in order to increase the number of drugs that can be administered via this route [17]. Significant efforts have therefore been expended on the development of new approaches to enhance TDD. These strategies can be broadly subdivided into chemical, biochemical and physical approaches [3]. Chemical permeation enhancers (CPEs) include compounds that interact with the lipid matrix of the SC to alter its nanostructure and thereby increasing permeability [18, 19, 17]. Although the corneocytes forms a scaffold for the extracellular lipid matrix, the main focus of drug delivery strategies has been on the manipulations of the extracellular lipid environment. In addition to the ability of lipid-soluble drugs being able to accumulate in the extracellular matrix enabling slow release, this region also provides additional opportunities for novel drug delivery strategies due to the presence of aqueous pores within the extracellular matrix [3, 2]. Fatty acids (FAs) are integral components of skin and have emerged as one of the most attractive CPEs employed for TDD. FAs have the ability to reduce the barrier properties of the SC and increase its fluidity by extracting extracellular lipids and altering SC lipid organization thereby increasing transdermal delivery by expanding intercellular domains [20-22].

Chol is an amphiphilic biomolecule that contains both a polar hydroxyl group and a non-polar tetracylic steroidal ring structure with a branched hydrocarbon tail. Chol has the property of orientational ordering and displays liquid crystalline behavior capable of binding with amphiphilic lipid membranes [23]. Chol has several effects on membrane properties which include membrane fluidity, phase transition and permeation of polar and non-polar molecules [24, 25]. Chol is regularly employed to modify the physicochemical properties of lipid bilayers and has been widely used to stabilize liposomes for their application in transdermal drug delivery [23, 26]. Regulating the Chol content of liposomes has been one of the crucial ways to control the permeability to solutes both *in vivo* and *in vitro* [23]. Chol has been shown to

alter the order and movement of the phospholipids in the bilayer and additional reports specify that the amount of Chol is important for the effective delivery of transdermal liposomes [27]. A study by Coderch *et al* reported an increase in the transdermal transport of a liposome entrapped substance with the addition of 20% Chol [28]. The conjugation of Chol onto molecules has been used for several biomedical applications such as a variety of Chol containing polymers for different micelles, nanoparticles, polymersomes, nanotubes, or nanogels [23]. Saturated (SFA) and unsaturated (UFA) fatty acids/FA esters have been reported as effective permeation enhancers for a variety of drugs [29]. Recent studies show that the application of ester derivatives or chemical modifications to the structure of the parent FAs are able to produce new chemical entities that display superior permeation enhancement capabilities [21, 30, 12, 31, 32].

Chol SFAs esters ranging from C_6 to C_{18} have been previously studied as transdermal permeation enhancers for phenazepam with promising results [33, 34]. However, the Chol UFA esters, although commercially available, have not been explored as permeation enhancers for any route. Literature reports that UFAs have superior permeation enhancement efficacy when compared to their SFA counterparts [35, 36, 22]. Further, UFAs with C_{18} have been identified as the optimal chain length lipids for superior permeation enhancement efficacy [17]. Considering the applicability of C_{18} UFAs as CPEs and the potential shown by the Chol SFA esters as transdermal permeation enhancers for phenazepam [33, 34], this study aimed to explore the use of Chol C_{18} UFA esters as transdermal permeation enhancers using TNF as the model drug. Chol SFA esters were used in the study for comparison in order to establish transdermal permeation enhancement potential of Chol UFA esters. The intention of this study was to identify chemical entities which can act as new efficient CPEs, thus widening the pool of available CPEs for transdermal drug delivery.

Materials and methods

1.1. Materials

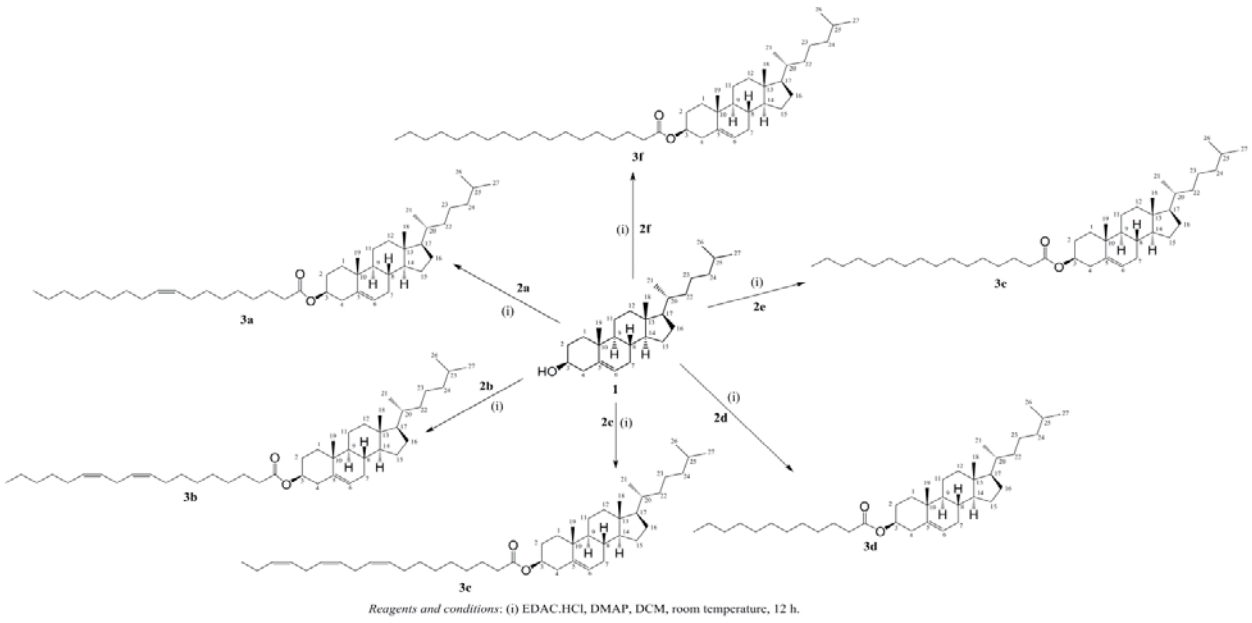
TNF was procured from Sinobright Pharmaceutical Co. Ltd (China). Oleic Acid, Linoleic Acid, Linolenic Acid, Lauric Acid, Palmitic Acid, Stearic Acid, N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDAC.HCl), p-dimethylaminopyridine (DMAP) and precoated Silica-gel 60F254 plates and hydroxypropyl methyl cellulose (HPMC) was

purchased from Sigma-Aldrich Co. Ltd., (USA). 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and all other reagents and solvents used for synthesis and purification were of analytical grade and were purchased from Merck Chemicals (Germany). Purified water was acquired from a Milli-Q water purification system (Millipore corp., USA). 200-250 g Wistar rats (Male) were acquired from the Biomedical Resource Unit (BRU), University of KwaZulu Natal (UKZN).

1.2. Methods

1.2.1. General procedure for synthesis of Chol-FA esters (Scheme 1)

To a stirred mixture of Chol (1 mmol), EDAC.HCl (1.01 mmol) and DMAP (0.5 mmol) in dry DCM (20 mL), FA (1.01 mmol) under a nitrogen atmosphere at room temperature (RT) was added. The resulting reaction mixture was further stirred at RT for 12 h, solvent was removed in vacuo and the crude residue was purified by column chromatography (silica gel #70-230 and 2-5% ethyl acetate in hexane as eluent) to obtain the final product (3a-f)**.**



Scheme 1. Synthetic scheme for Chol esters.

1.2.2. Structural characterization of Chol 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.

1.2.3. Calculation of log P values

In order to determine their lipophilicities, an online tool (chemicalize) was used to calculate the log *P* values of the Chol esters and their respective parent FAs [37].

1.2.4. Formulation of transdermal gels for in vitro permeation studies

The transdermal permeation enhancement efficacy of the FAs (**2a-f**) or their respective Chol esters (**3a-f**) was evaluated by formulating their gels. The composition of the gel was 1% w/w FA or Chol FA derivative, 4% w/w HPMC and 2% w/w TNF. A gel without enhancer was used as the control. To determine the optimal concentration of most promising enhancer gels containing 0.5, 2, 4, 6% w/w of either LLA **2a** or Chol-LLA **3a** were prepared and evaluated. [30, 12].

1.2.5. Transdermal permeation studies

The animal research ethics committee from the University of KwaZulu-Natal granted ethical clearance for this study in 2014 (054/14/Animal), and was subsequently renewed (015/15/Animal). The rats used in this study were contained at the BRU (UKZN) under controlled light, temperature and humidity conditions. The animals were fed a standard rodent diet and received water *ad libitum.* The animals were euthanized using CO_2 once they were at the desired weight range and body hair was removed through shaving. Abdominal skin was harvested and subcutaneous fat was removed with surgical scissors. The excised skin samples were stored at -20 ˚C and utilised within three months [21, 12]. The samples were defrosted in PBS (pH 7.4) at room temperature before its use in permeation studies.

Vertical Franz diffusion cells (PermeGear, Inc., Bethlehem, USA) with a diffusional area of 0.786 cm^2 was used to study the *in vitro* permeation at 37 ± 1˚C [38, 12]. The donor chamber contained either drug loaded gel or drug loaded gel in combination with the parent FAs or their respective Chol esters (Chol-OA **3a**, Chol-LA **3b**, Chol-LLA **3c**, Chol-LuA **3d**, Chol-PA **3e**, and Chol-SA **3f**) at a concentration of 1% w/w. The receptor compartments contained 25 ml of PBS (stirred with a teflon-coated magnetic bar). Skin samples were mounted between the donor and receptor chambers, and were equilibrated with PBS (pH 7.4) at 37 °C for 30 minutes. During the experiment, the receptor chamber was sampled at pre-set intervals and replenished with an equivalent amount of fresh PBS. The experiments were performed with a minimum of n = 6. The drug was measured by a UV spectrophotometry method at a λ_{max} of 262 nm using UV Spectrophotometer 1650 (Shimadzu, Japan). Once the optimal enhancer (Chol-LLA **3c**)

was established, the concentration effect studies were conducted on TNF gels that contained 0.5, 2, 4, 6% w/w of either Chol-LLA **3c** or the parent FA LLA **2c** [12, 30].

The cumulative amount of TNF permeated per unit surface area was plotted against time. Linear regression analysis was used to determine the steady state flux (Jss) from the linear part of the permeability curve (Microsoft Excel 2010, USA). The permeability coefficient (P) was calculated as follows [13, 39, 30]:

$P = (dQ/dT)/A \times Cd = Jss/Cd$

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 [13, 39]:

ER = P of the drug in the presence of enhancer/P of the drug in the absence of enhancer

1.2.6. Transepithelial electrical resistance (TEER) studies

The integrity of the treated skin tissue sections (TEER measurements) was determined using a Millicell ERS meter (Millipore, USA) connected to a pair of chopstick electrodes (STX01). The original integrity of the skin was recorded by obtaining TEER values before the addition of the test samples. This represented time zero and served as the control. Further measurements were made at the end of the 6h experiment to establish the change in the integrity of the skin samples by the drug treatment. Subsequently, the drug treatment was removed from the donor chamber and replenished with fresh PBS for a period of 2 h. The TEER values recorded at this point would be indicative of the rebound effect of the skin's barrier properties 2 h post drug treatment [40, 12, 13].

1.2.7. Histomorphological evaluation

Morphological assessments were conducted on control and treated skin samples. Freshly excised skins sections served as the control skin samples. These samples were fixed in 10% buffered formalin directly after skin harvesting and excision. Skin samples that were used during the 6h *in vitro* permeation study for either the enhancer free TNF gel or TNF loaded gels containing either 2% w/w LLA **2a** or Chol-LLA **3a** served as the treated samples. At the end of the permeation experiment, the treated skin samples were removed from the Franz diffusion cells and fixed in 10% buffered formalin. After 7 days, the skin samples were removed from fixative and dehydrated using an ethanol gradient from 50 to 96% followed by embedding in paraffin wax. The tissue samples were sectioned using a microtome (Leica

RM2235, Leica Biosystems Germany) and skin sections were collected on slides, dried and stained with hematoxylin and eosin (H&E). Sections were examined with Leica slide scanner (SCN 400, Leica Biosystems Germany). All experiments were performed in triplicate [12, 13, 30, 40].

1.2.8. Statistical Analysis

The results are expressed as mean ± standard deviation (SD). Data was statistically analysed using GraphPad Prism® (Graph Pad Software Inc., Version 5. USA). The data was analysed by either one-way analysis of variance (ANOVA) followed by the non-parametric Kruskal-Wallis test or Dunn's multiple comparison test or *t* tests were performed followed by the non-parametric Mann-Whitney test. A *p* value of less than 0.05 was considered statistically significant.

2. Results and discussion

2.1. Characterization of Chol esters

FT-IR, ^{1}H NMR and ^{13}C NMR were used to characterize the synthesized Chol esters. Transformation of acid function of FA into ester was easily monitored using FT-IR by observing the peak around 1732-1739 cm^{-1}.

Cholesteryl oleate (Chol-OA) **3a**

FT-IR: 2923.16, 2853.06, 1737.03, 1462.24, 1247.49, 1170.49, 1011.14 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ_H (ppm): 0.61 (s; 3H; C_{13}-CH_3). 0.80 (dd; 6H; C_{25}-$(CH_3)_2$), 0.82 (s; 3H; C_{18}-CH_3), 0.85 (d; 3H; C_{20}-CH_3), 0.95 (s; 3H; C_{10}-CH_3), 1.19 – 2.23 (m; 56H), 2.26 (t; 2H; -CH_2COO-), 4.54 (m; 1H; -C_3-H), 5.26 (m; 2H; -CH=CH-), 5.31 (d; 1H; C_6-H). ^{13}C NMR ($CDCl_3$) δ_C (ppm): 11.86, 14.12, 18.72, 19.32, 21.04, 22.56, 22.70, 22.82, 23.84, 24.29, 25.06, 27.17 - 29.78, 31.87, 31.91, 34.72, 35.80, 36.20, 37.01, 38.17, 39.52, 42.32, 50.04, 56.14, 56.70, 73.69, 122.58, 129.77, 129.99, 139.73, 173.30.

Cholesteryl linoleate (Chol-LA) **3b**

FT-IR: 2959.46, 2927.02, 2853.94, 1733.55, 1462.73, 1257.87, 1169.83, 1009.12 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ_H (ppm): 0.61 (s; 3H; C_{13}-CH_3). 0.79 (dd; 6H; C_{25}-$(CH_3)_2$), 0.82 (s; 3H; $C_{18'}$-CH_3), 0.85 (d; 3H; C_{20}-CH_3), 0.95 (s; 3H; C_{10}-CH_3), 1.03 – 2.24 (m; 52H), 2.69 (t; 2H; -CH_2COO-), 4.54 (m; 1H; -C_3-H), 5.25 (m; 4H; -CH=CH-), 5.32 (d; 1H; C_6-H). ^{13}C NMR ($CDCl_3$) δ_C (ppm): 11.86, 14.08, 18.72, 19.32, 21.04, 22.58, 22.70, 22.82, 23.83, 24.29, 25.06,

27.20 - 29.70, 31.53, 31.87, 34.72, 35.80, 36.18, 36.60, 37.00, 38.17, 39.52, 42.32, 50.04, 56.14, 56.70, 73.70, 122.59, 127.92, 128.04, 130.07, 130.22, 139.73, 173.30.

Cholesteryl linolenate Chol-LLA **3c**

FT-IR: 2927.12, 2853.46, 1733.98, 1462.84, 1247.00, 1171.09, 1083.77 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ_H (ppm): 0.61 (s; 3H; C_{13}-CH_3). 0.78 (dd; 6H; C_{25}-$(CH_3)_2$), 0.80 (s; 3H; $C_{18'}$-CH_3), 0.85 (d; 3H; C_{20}-CH_3), 0.95 (s; 3H; C_{10}-CH_3), 1.03 – 2.25 (m; 48H), 2.69 (t; 2H; -CH_2COO-), 4.54 (m; 1H; -C_3-H), 5.27 (m; 6H; -CH=CH-), 5.30 (d; 1H; C_6-H). ^{13}C NMR ($CDCl_3$) δ_C (ppm): 11.86, 14.12, 18.72, 19.32, 21.04, 22.56, 22.70, 22.82, 23.83, 24.29, 25.06, 27.20 - 29.70, 31.87, 31.91, 34.71, 35.80, 36.19, 36.60, 37.00, 38.17, 39.74, 42.32, 50.04, 56.14, 56.70, 73.70, 122.59, 127.12, 127.72, 128.26, 128.28, 130.28, 131.96, 139.72, 173.29.

Cholesteryl laurate (Chol-LuA) **3d**

FT-IR: 2923.09, 2852.13, 1732.03, 1463.03, 1165.93 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ_H (ppm): 0.61 (s; 3H; C_{13}-CH_3). 0.78 (dd; 6H; C_{25}-$(CH_3)_2$), 0.81 (s; 3H; $C_{12'}$-CH_3), 0.85 (d; 3H; C_{20}-CH_3), 0.95 (s; 3H; C_{10}-CH_3), 1.05 – 2.20 (m; 48H), 2.25 (t; 2H; -CH_2COO-), 4.53 (m; 1H; -C_3-H), 5.31 (d; 1H; C_6-H). ^{13}C NMR ($CDCl_3$) δ_C (ppm): 11.86, 14.17, 18.72, 19.32, 22.56, 23.84, 24.29, 27.82 - 29.60, 31.87, 34.73, 35.80, 36.60, 37.01, 38.17, 39.74, 42.32, 50.04, 56.70, 73.67, 122.57, 139.73, 173.32.

Cholesteryl palmitate (Chol-PA) **3e**

FT-IR: 2914.33, 2848.65, 1738.64, 1463.66, 1172.02 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ_H (ppm): 0.61 (s; 3H; C_{13}-CH_3). 0.79 (dd; 6H; C_{25}-$(CH_3)_2$), 0.81 (s; 3H; $C_{16'}$-CH_3), 0.85 (d; 3H; C_{20}-CH_3), 0.95 (s; 3H; C_{10}-CH_3), 1.03 – 2.22 (m; 56H), 2.25 (t; 2H; -CH_2COO-), 4.53 (m; 1H; -C_3-H), 5.30 (d; 1H; C_6-H). ^{13}C NMR ($CDCl_3$) δ_C (ppm): 11.57, 14.12, 18.72, 19.32, 22.57, 23.84, 24.29, 27.82 - 29.70, 31.87, 31.94, 34.73, 35.80, 36.60, 37.01, 38.17, 39.52, 42.32, 50.04, 56.14, 56.70, 73.67, 122.58, 139.73, 173.32.

Cholesteryl stearate (Chol-SA) **3f**

FT-IR: 2914.43, 2848.73, 1739.09, 1463.91, 1172.36 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ_H (ppm): 0.61 (s; 3H; C_{13}-CH_3). 0.79 (dd; 6H; C_{25}-$(CH_3)_2$), 0.81 (s; 3H; $C_{18'}$-CH_3), 0.85 (d; 3H; C_{20}-CH_3), 0.95 (s; 3H; C_{10}-CH_3), 1.03 – 2.17 (m; 60H), 2.20 (t; 2H; -CH_2COO-), 4.54 (m; 1H; -C_3-H), 5.31 (d; 1H; C_6-H). ^{13}C NMR ($CDCl_3$) δ_C (ppm): 11.86, 14.12, 18.72, 19.33, 22.70, 22.82,

23.83, 24.29, 27.82 - 29.71, 31.87, 31.94, 34.73, 35.80, 36.60, 37.01, 38.17, 39.52, 42.32, 50.04, 56.14, 56.70, 73.67, 122.58, 139.73, 173.33.

2.2. Transdermal permeation studies

The six h *in vitro* transdermal permeation experiments showed that the cumulative amount of TNF that penetrated through the skin was 253.1 ± 23.84 µg/cm^2 (Table 1, Fig. 1). The steady state flux was 40.91 ± 4.93 µg/cm^2 h (Table 1). These findings correlate well with our previous report for the transdermal permeability of TNF [12]. The study provided evidence suggesting that TNF permeates the skin via both the intercellular or the paracellular route, or both routes simultaneously, to reach the blood vessels located in the dermis [12]. The transdermal permeability potential displayed by TNF therefore provides an attractive alternate drug delivery route for this drug, as compared to conventional delivery routes.

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

Treatment	Amount permeated (µg.cm $^{-2}$)	Jss (flux) (µg.cm $^{-2}$.h^{-1})	Permeability (Px10^{-2})	ER
TNF (Control)	253.10 ± 23.84	40.91 ± 4.93	0.204 ± 0.02	**1**
Chol	270.40 ± 18.92	45.44 ± 3.13	0.227 ± 0.01	**1.11**
Chol-OA **3a**	389.75 ± 47.66	62.19 ± 7.87	0.310 ± 0.03	**1.52**
Chol-LA **3b***	424.42 ± 24.42	69.23 ± 3.01	0.346 ± 0.01	**1.69**
Chol-LLA **3c***	909.57 ± 63.51	152.1 ± 13.11	0.760 ± 0.06	**3.71**
Chol-LuA **3d**	298.01 ± 21.63	50.01 ± 3.65	0.250 ± 0.01	**1.22**
Chol-PA **3e**	347.99 ± 15.78	57.54 ± 2.98	0.287 ± 0.01	**1.4**
Chol-SA **3f**	363.53 ± 19.02	60.18 ± 3.60	0.300 ± 0.01	**1.47**

Only a few drugs have sufficient unaided transdermal permeability which enables therapeutically relevant amounts of the drug reaching systemic circulation. One of the approaches to aid the transport of drugs across the SC would be the addition of a substance that is able to temporarily decrease the skin barrier properties thereby delivering necessary quantities of the drug across the skin [17]. The addition of 1% w/w Chol to the gel displayed a slight enhancement in TNF permeability; however, the increases were not statistically significant ($p > 0.05$). The cumulative amount and steady state flux increased from 253.1 ±

23.84 to 270.40 ± 18.92 µg/cm^2 and 40.91 ± 4.93 to 45.44 ± 3.13 µg/cm^2 h respectively. This signified a 1.11-fold increase in the ER of TNF (Table 1, Fig. 1). Thus we further explored the potential of synthesized Chol esters (Chol-OA **3a**, Chol-LA **3b**, Chol-LLA **3c**, Chol-LuA **3d**, Chol-PA **3e** and Chol-SA **3f**) as transdermal permeation enhancers for TNF (Table 2).

Table 2. Molecular formula, number of unsaturation's and molecular weight of the Chol-FAs studied.

FA-Derivative	Molecular formula	Molecular weight (g mol^{-1})	No. of unsaturations
CHOL-OA **3a**	$C_{45}H_{78}O_2$	651.12	01
CHOL-LA **3b**	$C_{45}H_{76}O_2$	649.10	02
CHOL-LLA **3c**	$C_{45}H_{74}O_2$	647.09	03
CHOL-LuA **3d**	$C_{39}H_{68}O_2$	568.97	00
CHOL-PA **3e**	$C_{43}H_{76}O_2$	625.08	00
CHOL-SA **3f**	$C_{45}H_{80}O_2$	653.13	00

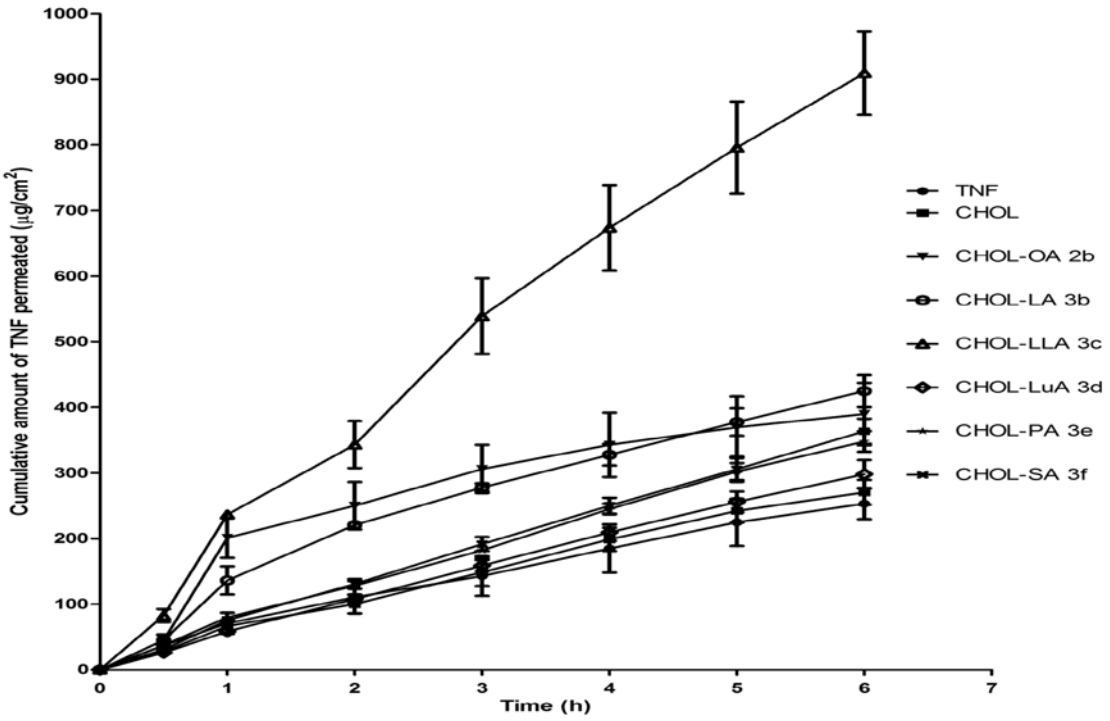


Fig. 1 The effect of synthesized Chol esters on the cumulative amount of TNF permeated across the skin. Results are presented as mean ± SD. (n = 6).

2.2.1. Effects of Chol esters on TNF permeability

This investigation evaluated the permeation enhancement efficiency of the synthesized Chol FA esters. The lipophilicity of all the synthesized Chol esters was greater than their respective parent FA as shown by the log *P* values represented in Table 3. This is a favourable attribute as enhancers with higher lipophilicities are able to cause greater disruption of the organised lipids in the SC. This allows larger quantities of drug to permeate as there is a greater reduction in the barrier properties [21, 41, 42]. The permeation results confirmed that all of the Chol esters were able to enhance the permeation of TNF to a greater extent than Chol at similar concentrations (Table 1, Fig. 1). However, the Chol esters containing unsaturated aliphatic chain (Chol-OA **3a**, Chol-LA **3b** and Chol-LLA **3c**) were able to achieve a higher permeation enhancement effect as compared to those containing saturated aliphatic chain (Chol-LuA **3d**, Chol-PA **3e** and Chol-SA **3f)**. This is in good agreement with literature which reports that UFAs have better permeation enhancement potentials than saturated fatty acids SFAs [35, 36, 22]. Although all of the Chol esters with saturated aliphatic chain enhanced the transdermal permeation of TNF, there were no statistically significantly increases ($p > 0.05$) in the permeation of TNF across the skin from these esters (Table 1). Chol-PA **3e** increased the cumulative amount permeated and steady state flux from 253.1 ± 23.84 and 40.91 ± 4.93 to 347.99 ± 15.78 μg/cm^2 h and 57.54 ± 2.98 μg/cm^2 respectively, with an ER of 1.4 (Table 1, Fig. 1). Chol-SA **3f** displayed the greatest enhancement potential with an ER of 1.47 displaying a cumulative amount permeated of 363.53 ± 19.02 μg/cm^2 h and a steady state flux of 60.18 ± 3.60 μg/cm^2 (Table 1, Fig. 1). Chol-LuA **3d** achieved an ER of 1.22 and marginally increased the cumulative amount permeated to 298.01 ± 21.63 μg/cm^2 h and the steady state flux to 50.01 ± 3.65 μg/cm^2 (Table 1, Fig. 1). Increase in carbon chain length from 12 to 18 resulted in increases in transdermal permeation of TNF. The order of enhancement was Chol-SA **3f** > Chol-PA **3e** > Chol-LuA **3d**. In contrast to the SFAs, the UFA esters displayed greater ER and permeability parameters. When compared to the control, Chol-OA **3a** increased the steady state flux and cumulative amount from 253.1 ± 23.84 and 40.91 ± 4.93 to 389.75 ± 47.66 μg/cm^2 and 62.19 ± 7.87 μg/cm^2 h respectively with an ER of 1.52 (Table 1, Fig. 1). However, the increases displayed by Chol-OA **3a** were not statistically significant when compared to the control ($p > 0.05$) (Table 1). Chol-LA **3b** increased the steady state flux and cumulative amount permeated to 69.23 ± 3.01 μg/cm^2 h and 424.42 ± 24.42 μg/cm^2 respectively and displayed an ER of 1.69 (Table 1, Fig. 1). The increase in permeability due to Chol-LA **3b** was statistically significant when compared to the control ($p < 0.05$) (Table 1). Amongst all the synthesized

Chol FA esters, Chol-LLA **3c** exhibited the highest increases in the permeability parameters of TNF. Chol-LLA **3c** significantly increased both the steady state flux and cumulative amount to 152.1 ± 13.11 μg/cm^2 h and 909.57 ± 63.51 μg/cm^2 respectively ($p < 0.05$), and displayed an ER of 3.71 (Table 1, Fig. 1). The Chol-LLA **3c** derivative displayed an ER of 1.51-fold higher than LLA **2c**.

Table 3. Log *P* values of FAs and their respective synthesized Chol derivatives.

FA/Derivative	**Log *P***
Chol	7.11
OA **2a**	6.78
Chol-OA **3a**	14.56
LA **2b**	6.42
Chol-LA **3b**	14.20
LLA **2c**	6.06
Chol-LLA **3c**	13.84
LuA **2d**	4.48
Chol-LuA **3d**	12.26
PA **2e**	6.26
Chol-PA **3e**	14.03
SA **2f**	7.15
Chol-SA **3f**	14.92

It should be noted that the ER range acquired by all the Chol esters in our study (1.22 to 3.71) was greater than the ER (1.15 to 2.81) of previously reported FA esters of isopulegol (ISO) as transdermal permeation enhancers for the delivery of flurbiprofen (FP) and amlodipine (AM) [43]. That reported study revealed that the chemical linkage of the FAs with ISO was critical for the enhancement and the ester derivatives displayed superior enhancement of FP and AM. The study by these researches suggested that the different effects of the FAs and their esters derivatives would be due to their respective effect on the different lipid domains. They indicated that the FAs could possibly exist in the ordered lipid domain, whereas the esters derivatives could partition into the less ordered lipid domain to produce the permeation effect [43]. In another study, FA esters of propylene glycol (PG) were explored as transdermal permeation enhancers for the delivery of lidocaine [44]. The results showed that only SA and

LA PG derivatives were able to enhance the transdermal permeation of lidocaine across the skin as compared to their parent lipid, whilst the OA and LLA PG derivatives displayed a lower ER than their respective parent lipid. The addition of the enhancers at a concentration of 1% w/w showed an ER of 0.93, 1.91, 1.68 and 1.70 fold for the SA, OA, LA and LLA PG ester derivatives. In our case, with the exception of Chol-OA **3a** the other respective Chol esters (Chol-SA **3f**, Chol-LA **3b** and Chol-LLA **3c**) were able to achieve superior permeation enhancement than the PG ester derivatives counterparts [44].

To assess whether the superior enhancement effect observed with the Chol esters were due to the structural modifications of Chol with FAs, we evaluated the parent FAs (OA **2a**, LA 2**b**, LLA **2c**, LuA **2d**, PA **2e** and SA **2f**) as transdermal permeation enhancers of TNF. The results indicated that the enhancement by these acids was statistically significantly lower than their Chol esters ($p < 0.05$)

All of the FAs studied at a concentration of 1% w/w; enhanced the permeability of TNF across the skin (Table S1 of Supporting Information). The UFAs displayed better permeation enhancement than SFAs. Similarly, those Chol esters containing unsaturation in their aliphatic chain were better than those having saturation. Of all the FA studied LLA **2c** displayed the highest ER (2.2) for TNF (Table S1 of Supporting Information). The increases in the permeability parameters due to LLA **2c** were statistically significant ($p < 0.05$) (Table S1 of Supporting Information). The ER displayed by LLA **2c** in this study is comparable to other reports that used rat skin to evaluate various other CPEs for transdermal delivery. In a similar study, the enhancing effect of UFAs (OA, LA and LLA) on flurbiprofen permeation from CMCNa hydrogels was determined. The results displayed that LLA showed greater permeation than OA and LA. At an enhancer concentration of 5%, the permeation studies through rat skin reported an ER of 1.55, 1.66 and 1.83 for OA, LA and LLA respectively [45]. Significantly, in our study LLA **2c** was able to surpass this ER at much lower concentration. In another report, the CPE myrcene was used as transdermal permeation enhancer for the delivery of piroxicam [46]. The study revealed that the addition of myrcene as a transdermal permeation enhancer at a concentration of 5, 10 and 15% (w/v) displayed an ER of 2.28, 2.36 and 2.45 respectively [46]. LLA **2c** was able to achieve the similar ER as Myrcene but at a significantly lower concentration (Table S1 of Supporting Information).

Superior transdermal penetration enhancement using UFAs has been linked to carbon chain length and the degree of unsaturation of the UFA [47, 48, 16, 49, 17]. Extensive experimental

reports in the literature agree that a carbon length of C_{18} is ideal for a UFA permeation enhancer [17]. The UFAs studied in this report have 18 carbons in their structures but their degree of unsaturation is different. Additionally, UFAs are able to increase the fluidity of the skin as their bent *cis*-configuration interrupts the intercellular lipid assembly. This creates mobile free volumes which allow molecules to enter at the kinks of the double bonds and migrate across the skin more readily. The number of kinks formed is directly proportional to the number of double bonds in the UFA. Therefore, as the number of double bonds increase in a UFA, the permeation enhancement effect is also expected to increase [47-49, 17]. Although OA **2a**, LA **2b** and LLA **2c** have the same carbon chain length of C_{18}, LLA **2c** possesses one more double bond than LA **2b** and two more than OA **2a** (Table 2). LLA **2c** therefore retains a combination of the most desirable characteristics of a UFA permeation enhancer and displayed greatest ER for TNF.

The efficacy of an enhancer is influenced by its physicochemical characteristics. This study shows that condensation of Chol with FAs to form Chol esters amplifies the enhancement potential of the parent FAs. The increase in the lipophilicity of the esters due to the presence of Chol moiety (Table 3) could have resulted in the increase in transdermal permeation of TNF [41, 21, 30, 12, 42]. This interaction could justify the increase in permeation activity observed for all the Chol esters. Interestingly Chol UFAs esters displayed a greater degree of permeation enhancement when compared to their SFAs counterparts. (Table 1, Fig. 1). For the Chol UFA esters, the increase in lipophilicity (Table 3) has improved the inherent superior transdermal permeation enhancement capabilities shown by their respective UFAs. The findings of this study show that Chol-LLA **3c** possesses all the ideal features of an effective UFA permeation enhancer, such as lipophilicity, optimal FA chain length (C_{18}) and number of unsaturations (3). Consequently, additional concentration effects on transdermal permeation enhancement efficacy were performed on Chol-LLA **3c** using LLA **2c** as a control and are reported here under.

*2.2.2. Concentration effect of Chol-LLA **3c***

The concentration effect of Chol-LLA **3c** on the transdermal permeability parameters of TNF were explored (Table 4, Fig. 2) to establish the optimal concentrations of Chol-LLA **3c** to be potentially employed as a CPE into a future TDD system for TNF. The findings displayed that Chol-LLA **3c** was able to significantly ($p < 0.05$) enhance the permeability of TNF as compared to the control, at all of the concentrations studied (0.5, 1, 2, 4, 6 % w/w) (Table 4, Fig. 2). Chol-

LLA **3c** also exhibited statistically significant ($p < 0.05$) increases in the enhancement of TNF permeation at similar concentrations to its parent material LLA **2c** (Table 4, Fig. 2). Both LLA **2c** and Chol-LLA **3c** displayed a similar concentration dependant permeation enhancement profile (Fig. 2). The greatest ER was obtained at 2% w/w, with a subsequent decrease in ER with further increases in enhancer concentration (Fig. 2).

Table 4. Effect of the various concentrations of LLA **2c** and Chol-LLA **3c** on the transdermal permeability properties of TNF. *Indicates significant difference i.e. $p < 0.05$ (all values compared to control), #Indicates significant difference when compared to LLA **2c** at similar concentration.

	Amount permeated (µg.cm $^{-2}$)		**Jss (flux) (µg.cm $^{-2}$.h^{-1})**		**ER**	
Control	253.10 ± 23.84		40.91 ± 4.93		**1**	
Concentrations [% w/w]	LLA **2c**	Chol-LLA **3c**	LLA **2c**	Chol-LLA **3c**	LLA **2c**	Chol-LLA **3c**
0.5	395.08 ± 20.26	485.17 ± 42.15	66.31 ± 3.24*	82.30 ± 7.72*#	**1.62**	**2.01**
1	531.96 ± 51.84	909.57 ± 63.51	90.15 ± 9.39*	152.1 ± 13.11*#	**2.20**	**3.71**
2	656.04 ± 72.18	1479.53 ± 79.10	110.52 ± 12.49*	242.64 ± 13.47*#	**2.70**	**5.93**
4	598.84 ± 53.64	809.57 ± 49.30	100.76 ± 9.63*	134.47 ± 7.89*#	**2.46**	**3.28**
6	484.55 ± 40.63	513.12 ± 49.96	82.19 ± 6.54*	84.80 ± 8.22*#	**2.00**	**2.07**

Chol-LLA **3c** at the optimal concentration of 2% w/w increased the steady state flux of the control to 242.64 ± 13.47 µg/cm^2 h and the cumulative amount to 1479.53 ± 79.10 µg/cm^2 with an ER of 5.93 compared to the control (Table 4, Fig. 2), interestingly this signified a 2.2-fold increase in the permeability of TNF as compared to the parent FA at the same concentration.

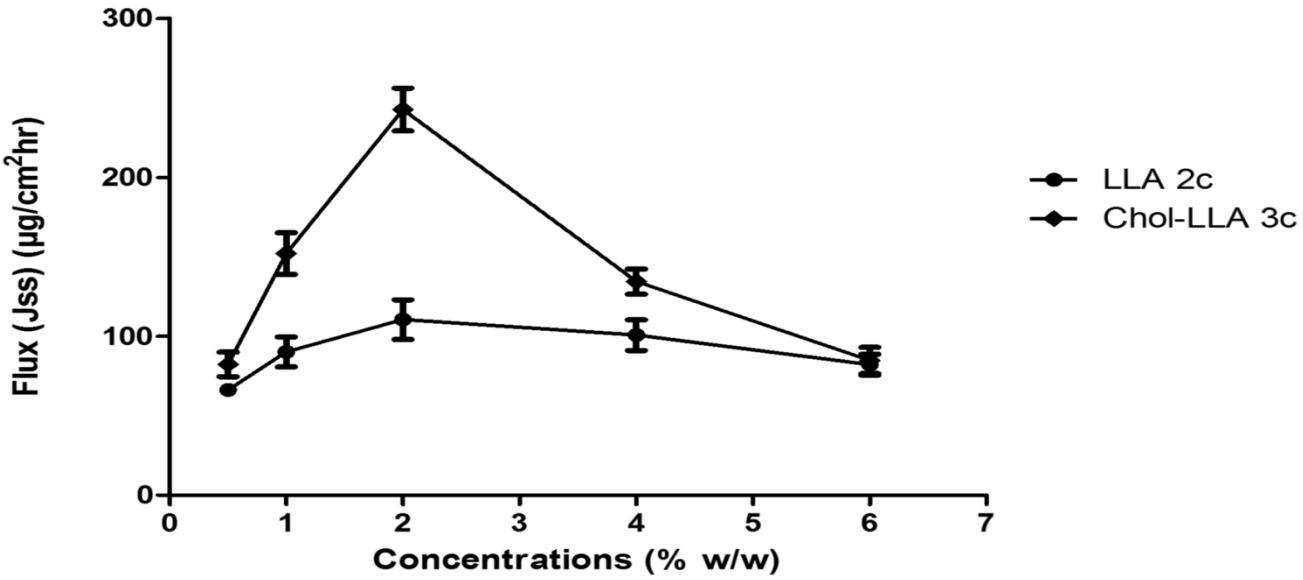


Fig. 2 Concentration effects of LLA **2c** and Chol-LLA **3c** on the flux of TNF (n = 6).

The IC_{50} range for TNF is between 0.55 – 2.2 µM [50], at approximately 158 – 632 µg respectively [12]. This study shows that the Chol esters able to deliver therapeutically relevant doses of TNF via the transdermal route as the cumulative amount of TNF permeated per cm^2 in this report is between 485.17 – 1479.53 µg.

The enhancer concentration verses ER trend observed in this study is similar to other reports, where an initial increase in ER was reported for lower enhancer concentrations, reaching a maximum as the concentration is increased and then a subsequent decrease in ER at higher enhancer concentrations [51-53, 30, 12]. In an independent report, the concentration effect (0.5, 1, 2.5 and 5% w/w) of tween 80 on the enhancement of lorazepam through rat skin was investigated. The study revealed that there was an initial increase at the lower concentration, reaching a maximum ER (3.75) at 1% w/w and further increase in tween 80 concentration resulted in a decrease in the ER [53]. In the present report, Chol-LLA **3c** was able to achieve a comparable ER (3.71) at the same concentration (1% w/w), however a further increase in Chol-LLA **3c** concentration elicited a higher ER (value) than tween 80 (Table 4, Fig. 2). A study by H. Chaudary *et al*. explored the effects of varying concentrations (5, 10, 12.5 and 15% w/v) of 3-Carene as a transdermal permeation enhancer for the delivery of piroxicam across rat skin [46]. The results displayed that there was an initial increase in the permeation of piroxicam up until 12.5%, however increasing the concentration to 15% decreased the permeability [46]. The ER achieved in that study ranged from 2.85 at 5% to a maximum of 4.55 at 12.5%.

Interestingly, Chol-LLA **3c** displays similar or higher ER at significantly lower concentrations to other reported promising transdermal permeation enhancers.

The initial increase in ER observed in this study from 0.5% to 2% w/w could be due to the increase in the lipophilicity of the formulation due to the increase in the concentration of the lipophilic enhancer. This would enable a greater disruption of the lipids in the SC, thereby facilitating higher quantities of the drug to cross the skin. The progressive decline in the ER at concentrations greater that 2% w/w could be attributed to the increased viscosity at the epidermal surface as the concentration of the enhancer was increased in the gel formulations [12, 30]. This, in combination with a decrease in the partitioning of the drug from the gel formulation as the FA concentration is increased, could have resulted in the decreased movement of TNF across the skin [12, 30, 52].

2.3. TEER studies

TEER evaluations are critical parameters used in assessing the integrity status of cellular barriers. TEER measurements can be used to quantitatively determine the integrity of the barrier properties of the skin. TEER measurements across biological barriers of the skin recorded prior to their utilisation for the transport of molecules can be indicative of its original integrity [12, 38, 54]. A decrease in the TEER would suggest an increase in the transport pathways across the skin, thereby causing a resultant decline in the barrier function. The change in resistance values pre and post experiments allows for quantifiable determination of the changes in the barrier properties brought on by the drug/enhancer treatment [12, 38, 54].

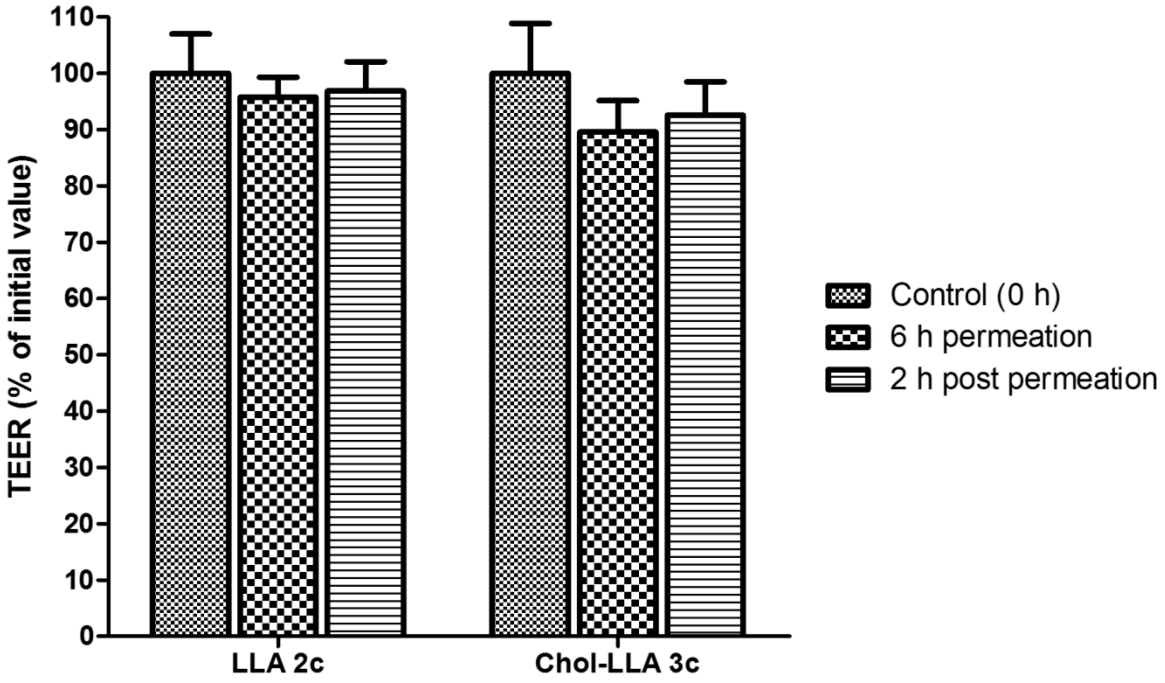


Fig. 3 % Change in TEER values after TNF permeation with either LLA **2c** or Chol-LLA **3c** at a concentration of 2% w/w (n = 6).

In order to establish if the optimal permeation enhancers did not irreversibly affect the barrier property/ integrity of the skin, TEER measurements were recorded on skin samples that were treated with TNF gel formulations containing either Chol-LLA **3c** or its parent material LLA **2c** at 2% w/w. The findings showed that both LLA **2c** and Chol-LLA **3c** at 2% w/w were able to decrease the TEER after the 6 h *in vitro* permeation study as compared to the control (TEER at 0 h) (Fig. 3). After 6h the LLA **2c** treatment reduced the skin's resistance from 480 ± 34 to 460 ± 17 Ω /cm^2, which symbolises a 4.2% decline in the resistance of the barrier. The Chol-LLA **3c** treatment was able to decrease the TEER of from 479 ± 42 to 429 ± 26 Ω /cm^2, which signifies a 10.4% decline in the barrier of the skin after 6 h (Fig. 3). This indicates that Chol-LLA **3c** reduces the TEER values 2.47-fold more than LLA **2c**. These findings support the data from the permeability studies which displayed that Chol-LLA **3c** had a 2.2-fold greater permeability enhancement than LLA **2c** at 2% w/w (Table 4).

Ideally the barrier properties of the skin should be restored upon removal of the permeation enhancer i.e. the disruption or fluidisation of the SC should be reversible [18, 20, 19, 17]. In order to establish if the skin regained its barrier properties, TEER measurements were recorded 2 h post removal of the gel formulations. The measurements revealed that there was a 1.11 % increase in TEER 2 h post experiment for the LLA **2c** treated samples (Fig 4). This shows 26.22

% retrieval of the original barrier function. In the Chol-LLA **3c** study, there was a 2.99% increase in the TEER 2 h post experiment (Fig. 3). This shows that the skin regained 28.66 % of its barrier function. These findings suggest that after the removal of the drug treatment, there is a rebound of the skin's barrier property/integrity. These findings therefore indicate that the barrier properties/skin integrity was only temporarily affected by the drug formulations [40, 12]. The results also showed that Chol-LLA **3c** did not cause any permanent changes in the epidermal layer.

2.4. Histomorphological analysis

The skin samples treated with the drug and enhancer formulation were assessed histomorphologically to establish whether there were any adverse effects after treatment for 6 h. The light microscopy (LM) analysis was performed on the skin samples exposed to enhancer free TNF gel, and on TNF gel containing either LLA **2c** or Chol-LLA **3c** respectively at a concentration of 2% w/w. From the morphological changes in the SC and the epidermis it may be possible to make inferences on the mode of action of the enhancers [55-58]. In order to deliver drugs into the systemic circulation located in the dermis, topically applied permeants need to cross the epidermal layer of the skin. The SC is the primary obstacle to the movement of drugs across the epidermis. The SC is flattened and primarily contains fibrous protein and lipids, with the intracellular keratin having an ordered pattern [59, 60]. Skin from the abdominal region of rats is frequently used for transdermal permeation [21, 61-63]. Skin is comprised of four layers with the stratum corneum (SC) as the most superficial layer followed by the stratum granulosum (SG), the stratum spinosum (SS) and terminates at the stratum basale (SB) which interfaces with the dermis (D). The control image (Fig 4a) closely resembles this description indicating that the tissue used in the study was healthy, normal skin.

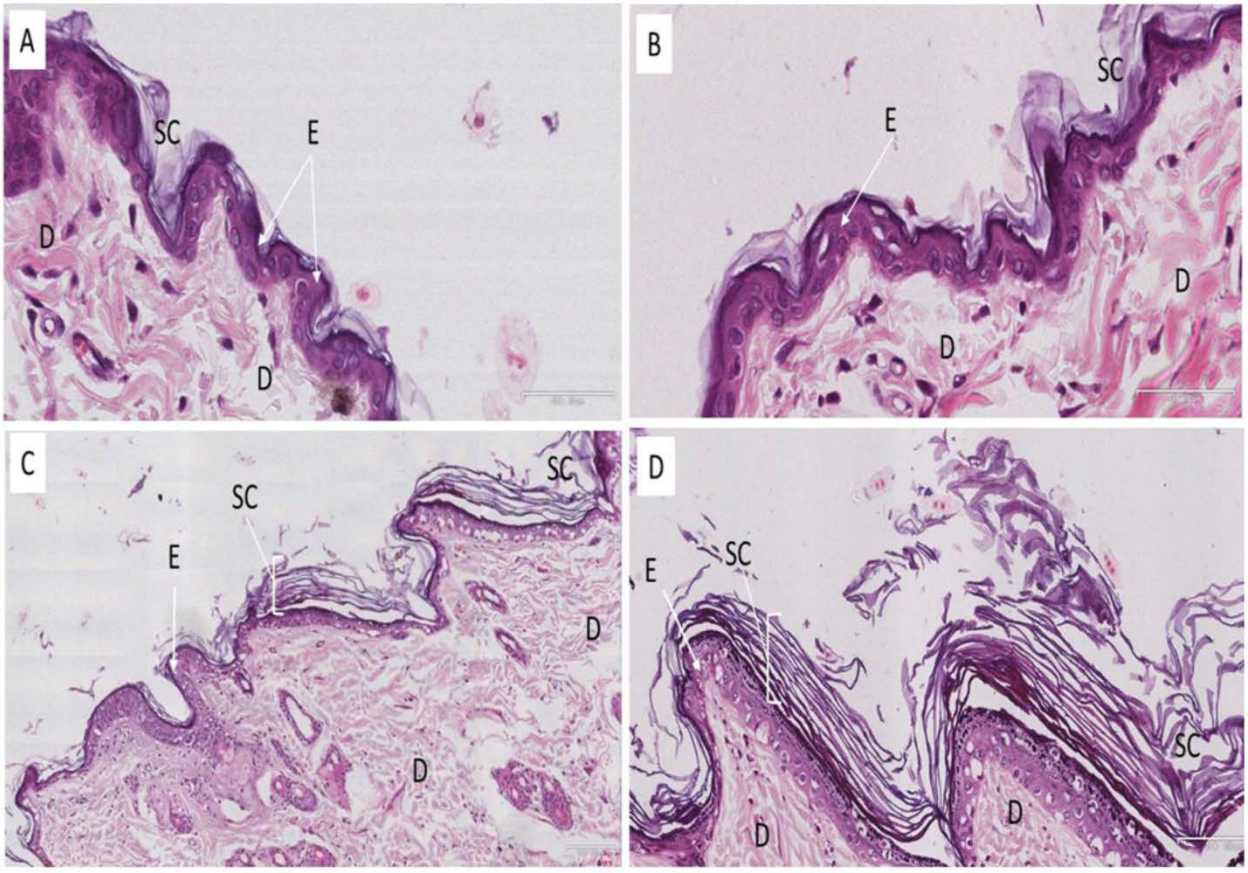


Fig. 4 Photomicrographs of the control and the treated skin selections for LM stained with H&E; (A) control/untreated, (B) treated with TNF gel, (C) treated with 2% w/w LLA **2c** TNF gel, (D) treated with 2% w/w Chol-LLA **3c** TNF gel. (D: dermis; E: epidermis; SC: stratum corneum).

The epidermal layers were clearly visible and distinguishable in the H&E images. The TNF gel treatment (Fig 4b) is morphologically similar to the control (Fig 4a), without any visible indication of adverse effects by the enhancer free TNF gel treatment. The LLA **2c** and Chol-LLA **3c** enhancer treatments (Fig 4c and 4d) displayed comparable morphology to the control image with regards to the underlying cell layers. The SG, SS and SB layers displayed normal morphology with no observable cellular distortions. There were no observable changes in the SB layer which suggests that the formulations did not induce any permanent changes. The SB layer is the regenerative layer made up of keratinocytes that divide to form the cells of the SS which migrate superficially [60]. The SG appeared darkly stained which suggest that there is no change in the functionality of these cells in comparison to the control. However, there was clearly noticeable distortions/disruption of the SC in both the LLA **2c** (Fig 4c) and the Chol-LLA **3c** (Fig 4d) treated images. The Chol-LLA **3c** samples displayed a greater degree of SC

disruption as compared to LLA **2c** (Fig 4c and 4d). These changes are indicative of the disruption of the lipids in the SC by LLA **2c** or Chol-LLA **3c** respectively. These disruptions cause fluidization of the SC, thus decreasing the natural barrier property of the SC and increasing the transport of TNF across the skin. Interestingly, the histomorphological observations directly correlate with the data from the *in vitro* permeation and TEER evaluations, which showed a significant increase in the permeation TNF (Table 4) and a decrease in the TEER of the skin (Fig. 3) in the presence of these enhancers.

3. Conclusions

In the present paper we have successfully demonstrated the potential of UFA esters of Chol as promising transdermal permeation enhancers for the delivery of small drug molecules using TNF as a model. Chol-LLA **3c** was identified as the best transdermal permeation enhancer with an ER of 3.71 at 1% w/w concentration. Attributes such as increased lipophilicity, optimal carbon chain length and the optimum number of double bonds favor Chol-LLA **3c** as the superior permeation enhancer. Additionally, the concentration dependent study showed that Chol-LLA **3c** at 2% w/w displayed the highest ER of 5.93. The permeation enhancement effects of the Chol esters of UFAs were superior to their respective parent UFAs and Chol esters of SFAs. The histomorphological studies revealed that the disruption and fluidization of lipids in the SC was responsible for the permeability enhancement of TNF by Chol-LLA **3c (**2% w/w). The LM assessments revealed that the 2% w/w treatment of Chol-LLA **3c** displayed no adverse effects on the skin's integrity.

This study has therefore identified Chol-UFA esters as transdermal permeation enhancers for active pharmaceutical ingredients using TNF as a model drug. In future these Chol-UFA esters can be evaluated as dual functional materials, as CPEs and providers of membrane integrity, in transdermal liposomal formulations.

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Animal studies

All institutional and national guidelines for the care and use of laboratory animals were followed.

Conflict of Interest

The authors declare that they have no conflict of interest

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Supplementary Material

Exploring unsaturated fatty acid cholesteryl esters as transdermal permeation enhancers

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Table S1. Effect of the various FAs 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) ($\mu g.cm^{-2}.h^{-1}$)**	**Permeability ($Px10^{-2}$)**	**ER**
TNF (Control)	253.10 ± 23.84	40.91 ± 4.93	0.204 ± 0.02	**1**
Chol	270.40 ± 18.92	45.44 ± 3.13	0.227 ± 0.01	**1.11**
OA **2a**	318.06 ± 37.57	52.25 ± 5.85	0.261 ± 0.02	**1.27**
LA **2b**	256.69 ± 28.77	41.73 ± 4.07	0.208 ± 0.02	**1.02**
LLA **2c***	531.96 ± 51.84	90.15 ± 9.39	0.450 ± 0.04	**2.2**
LuA **2d**	265.63 ± 29.29	43.81 ± 4.58	0.219 ± 0.02	**1.07**
PA **2e**	297.88 ± 14.94	48.65 ± 1.65	0.243 ± 0.008	**1.19**
SA **2f**	296.59 ± 12.31	47.37 ± 2.35	0.236 ± 0.011	**1.16**

CHAPTER 4

EXPERIMENTAL PAPER 3

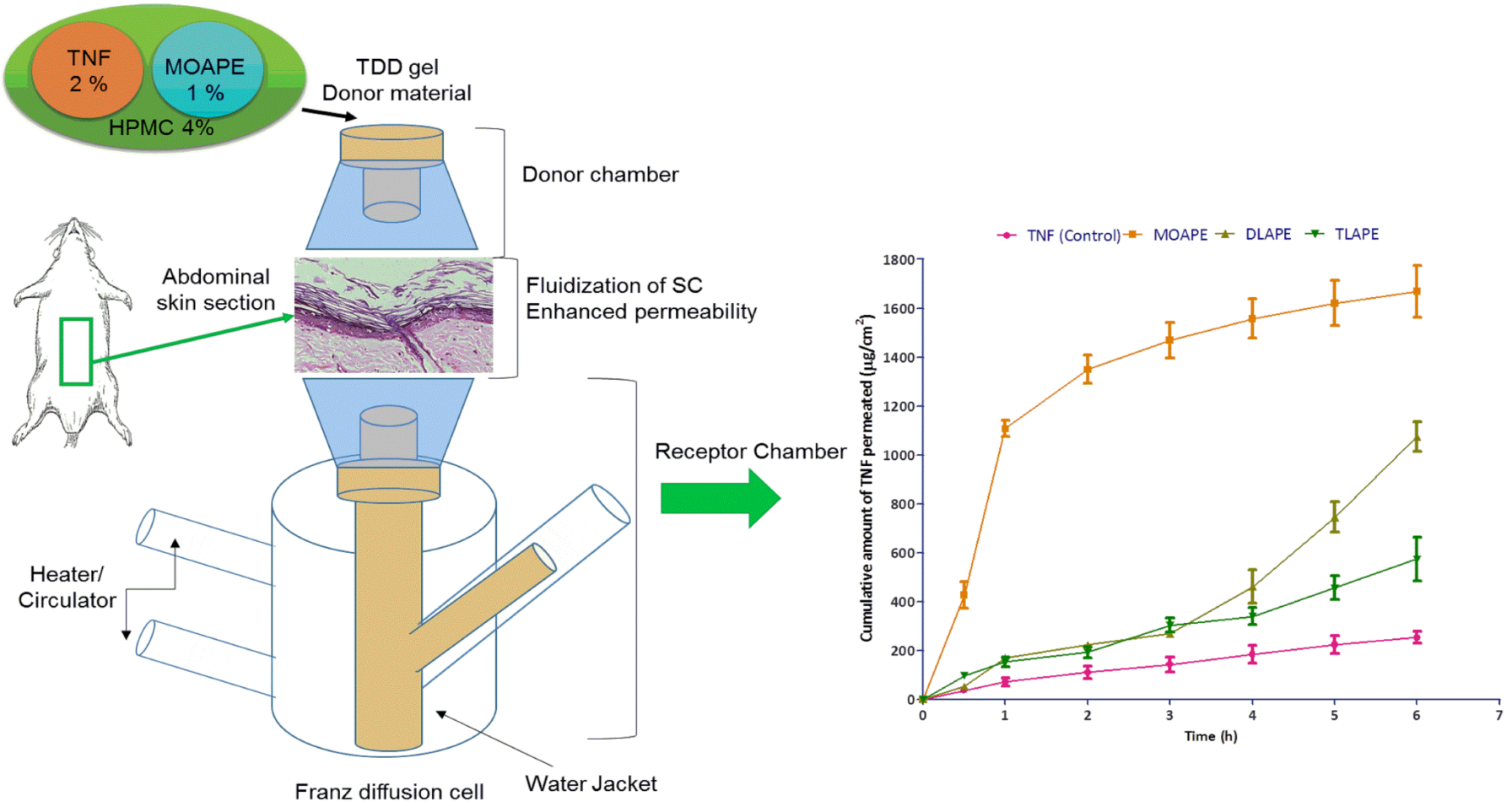
CHAPTER 4
EXPERIMENTAL PAPER 3

4.1 Introduction

This chapter addresses aim 1.4.3 and is a first authored experimental article manuscript submitted to the European Journal of Pharmaceutical Sciences (Impact Factor = 3.773) which is an ISI international journal, manuscript number: EJPS-D-16-01525. This manuscript reports the synthesis, characterization, biosafety and in vitro permeation enhancement effect of a novel series of mono, di and tri- ester derivatives of FAs bearing β-alanine t-butyl ester head group for the transdermal delivery of TNF.

Mr. S. Rambharose contributed to the design of the project, modification and optimisation of methods as well as synthesis and characterisation of all the novel mono, di and tri-ester derivatives of FAs. Mr. S. Rambharose conducted all experimental work including *in vitro* cytotoxicity, rat skin tissue harvesting, *in vitro* transdermal permeation experiments, TEER experiments and light microscopy evaluations. Mr. S. Rambharose also wrote the paper. Dr. R.S. Kalhapure and Dr. M. Jadhav assisted with the overall design of the project and the methods of preparation and characterisation as well as editing of the paper. Prof. T. Govender served as supervisor and was responsible for project conceptualisation, editing of paper and abstract and general supervision of the study.

4.2 Graphical abstract



Novel mono, di and tri- fatty acid esters bearing secondary amino acid ester head group as transdermal permeation enhancers

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Abstract

The use of chemical permeation enhancers (CPEs) has widened the pool of drugs that can be delivered via the transdermal route. This study explored the synthesis and characterization of novel mono, di and tri-fatty acid (FA) esters bearing β-alanine *t*-butyl ester head group as transdermal permeation enhancers using tenofovir (TNF) as a model drug. The synthesized compounds were non-toxic to mammalian cells confirming their safety for pharmaceutical applications. All the synthesized derivatives displayed better transdermal permeation enhancement capabilities as compared to their respective individual FAs. The results showed that there was no correlation between number of aliphatic carbon chains and enhancement ratio (ER). The mono oleate derivative (MOAPE) displayed the greatest ER for TNF (5.87) at 1% w/w. Histological investigations of the rat skin treated with MOAPE revealed fluidization of the stratum corneum. Histological and transepithelial electrical resistance (TEER) studies corroborated with the findings of the *in vitro* permeation experiments and revealed that there was no significant change to the viable epidermis of the skin after 1% MOAPE exposure. The TEER findings also suggested that the permeation enhancement effects of MOAPE were not permanent and the results indicated a return towards original skin integrity after removal of the enhancer formulation.

Keywords: fatty acids; esterification; synthesis; chemical permeation enhancers; tenofovir; transdermal

1. Introduction

There is increasing interest in the skin as an alternative site for the delivery of drugs to the systemic circulation. The numerous advantages of the transdermal route make the skin an attractive alternative to conventional oral dosage forms (Bavaskar et al., 2015; Vavrova et al., 2005) . However, the stratum corneum (SC) layer presents a challenging obstruction to drug permeation which reduces its bioavailability. Most drugs are not able to permeate the skin in therapeutically relevant quantities, which has led to the development of strategies to improve their delivery and increase the range of drugs that can be delivered via this route (Ham and Buckheit Jr, 2015; Prausnitz and Langer, 2008).

One approach in improving transdermal drug delivery is by reversibly decreasing the barrier property by the use of chemical penetration enhancers (CPEs). CPEs are chemicals which interact with the constituents of the SC and promote the passage of drug across the skin (Hussain et al., 2014; Lane, 2013; Saini et al., 2014) Fatty acids (FAs) have been widely used as CPEs due to their interactions with the SC and their ability to increase the fluidity by disrupting the packing of the lipids, thereby modifying the skin's barrier (Chantasart et al., 2004; Williams and Barry, 2012). In general FAs display greater permeability as compared to other classes of transdermal penetration enhancers (Babu et al., 2015). Although both unsaturated FAs (UFAs) and saturated FAs (SFAs) have been used as penetration enhancers for hydrophilic and lipophilic drugs (Williams and Barry, 2012), UFAs are well recognized as being more effective permeation enhancers (Babu et al., 2015; Oh et al., 2001). There are several reports on the effect of carbon chain length of FAs on the skin permeation enhancement of drugs; with the optimal chain length identified for UFAs being C18. Moreover, *cis*-configuration UFAs are more effective penetration enhancers than *trans*-configuration. Cis-configuration FAs are more effective in disrupting the lipid packing order within the bilayers (Choi et al., 2012; Hussain et al., 2014; Williams and Barry, 2012). The C18 UFAs oleic acid (OA), linoleic acid (LA) and linolenic acid (LLA) have been shown to be superior transdermal permeation enhancers for numerous drugs (Babu et al., 2015; Chi et al., 1995; Fang et al., 2003; Gwak and Chun, 2002; Gwak et al., 2004) with oleic acid showing the greatest promise (Baek et al., 2012; Dimas et al., 2004; Shah et al., 2012). These *cis*-confirmation UFAs have been shown to form separate domains within the SC lipids which effectively decreases either resistance or the length of the diffusional path (Babu et al., 2015; Ongpipattanakul et al., 1991).

OA in particular has been shown to be readily absorbed into the SC causing a decrease in the order of the lipid bilayer and increasing the fluidity. Due to its *cis* double bond, it is suggested that OA disrupts the packed structure of the intercellular lipids; in contrast the C18 SFA counterpart [stearic acid (SA)] is not as efficient in increasing the fluidity of the skin's membrane (Babu et al., 2015; Neubert et al., 1997; Oh et al., 2001).

Literature reports greater permeation enhancement using derivatives as compared to their parent material (Kalhapure and Akamanchi, 2013; Ojewole et al., 2014; Rambharose et al., 2015; Sinha and Kaur, 2000; Takahashi et al., 2002; Williams and Barry, 2012; Zhao et al., 2008), this therefore drives the need to modify existing CPEs to obtain novel CPEs with better performance. Recent studies suggest that ester derivatives of FAs have a greater transdermal enhancement potential than their respective parent FAs (Kalhapure and Akamanchi, 2013; Ojewole et al., 2014; Rambharose et al., 2015). FA esters such as isopropyl myristate, isopropyl decanoate, isopropyl palmitate, sucrose laurate, methyl laurate, and ethyl oleate among others have been widely explored as transdermal permeation enhancers (Babu et al., 2015). FA esters have been shown to exhibit good enhancing effect on the transdermal absorption of numerous hydrophilic and hydrophobic permeants such as 6-carboxyfluorescein, hydrocortisone, 5-fluorouracil, diclofenac, metoprolol, lidocaine, ketoprofen, indomethacin and isosorbide dinitrate (Babu et al., 2015; Chen et al., 2014). FA esters usually decrease the skin's barrier by partitioning themselves in the ordered lipid domains of the SC (Babu et al., 2015). Recent reports have indicated that esters increase the partition coefficient to enhance the permeation of drugs. Additionally, the physicochemical properties such as the lipophilicity (log *P*) of a permeant significantly determines its ability to passage through the skin (Patel et al., 2002; Potts and Guy, 1992; Xie et al., 2016). Therefore, the chemical modification of FAs to obtain more lipophilic CPEs which can promote permeation of hydrophilic as well as hydrophobic drugs through the skin by reversible disruption and fluidization of the SC is a promising strategy.

Tenofovir (TNF) is an antiretroviral (ARV) drug classified by its ability to specifically inhibit the enzyme reverse transcriptase and causing the process of viral replication to be terminated or blocked. In the present situation, TNF is a drug which is considered to be most successful to manage HIV and AIDS (Cressey et al., 2015; Diblíková et al., 2014). The metabolic release of TNF occurs within the gastrointestinal tract (GIT), resulting in the onset of several GIT associated adverse side effects such as nausea, diarrhea, inflammation and irritation of the

intestinal lining (Chapman et al., 2003; Fernandez-Fernandez et al., 2011). Formulation scientists have explored various target sites such as vaginal, buccal, rectal and dermal routes for the alternative administration of TNF to by-pass its side effects (Rambharose et al., 2015). The delivery of TNF through skin could be the most attractive route of administration as it presents several advantages that contribute to increasing patient compliance.

Although transdermal drug delivery (TDD) is an attractive alternate route of administration, the number of ARV drugs explored for this route is limited (Rambharose et al., 2015). The natural barrier properties of the skin combined with the low number of CPE's limit the applicability of this route for many drugs. While new chemical entities are being discovered at a rapid rate, the number of new CPE molecules has plateaued (Karande et al., 2005). The identification of new CPEs is critical in achieving the full potential of these materials in TDD. Therefore, the aim of our investigation was to explore the use of novel mono, di and tri-ester derivatives of FAs (SA, OA, LA and LLA) as prospective penetration enhancers for the TDD of TNF. Such derivatives have not been reported as CPEs for any route.

2. Materials and methods

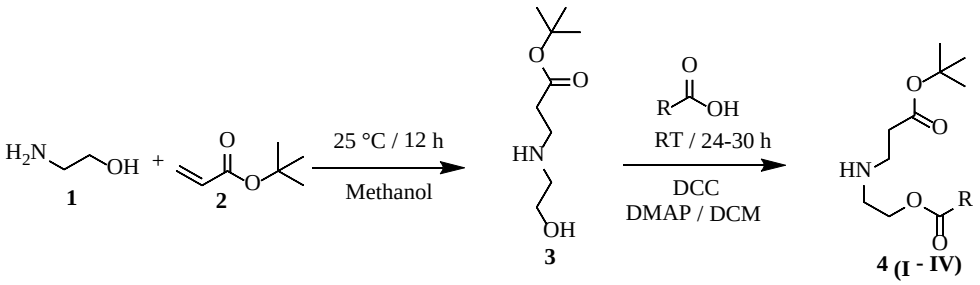
2.1 Materials

TNF was obtained from Sinobright Pharmaceutical Co. Ltd (China). Oleic Acid, Linoleic Acid, Linolenic Acid, Stearic Acid, N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDAC.HCl), p-dimethylaminopyridine (DMAP) and precoated Silica-gel 60F254 plates and hydroxypropyl methyl cellulose (HPMC) was purchased from Sigma-Aldrich Co. Ltd., (USA). 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and all other reagents and solvents used for synthesis and purification procured from Merck Chemicals (Germany) were of analytical grade. Purified water was obtained in our laboratories, from the water purification system (Millipore corp., USA).

2.2 Methods

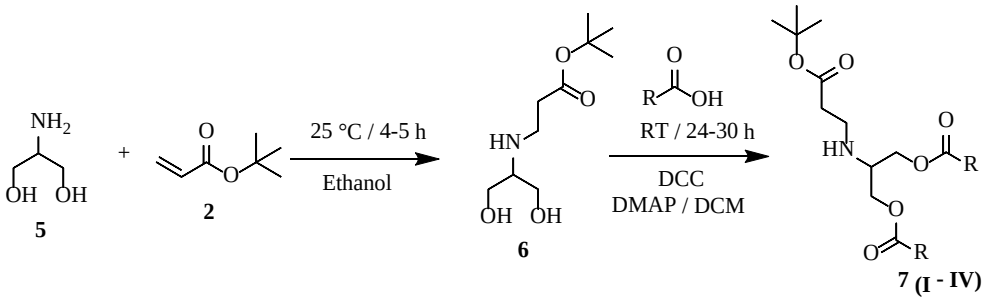
2.2.1 Synthesis and characterization

Scheme 1. Synthesis of mono-ester fatty acid derivatives



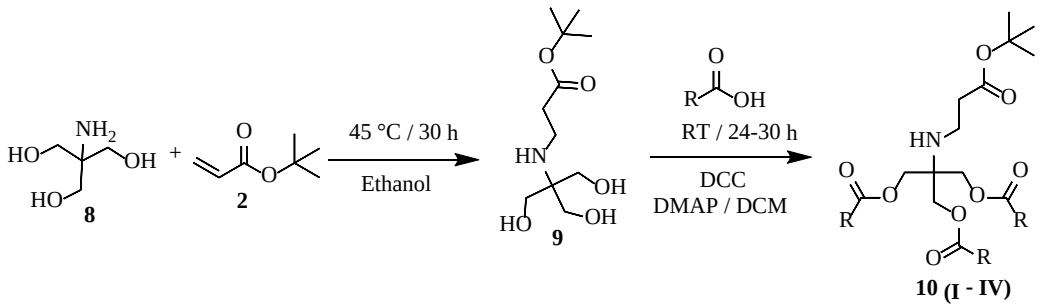
R : $-(CH_2)_{16}-CH_3$ (Stearic acid; I), $-(CH_2)_7-CH=CH-(CH_2)_7-CH_3$ (Oleic acid; II),
$-(CH_2)_7-CH=CH-CH_2-CH=CH-(CH_2)_4-CH_3$ (Linoleic acid; III) and
$-(CH_2)_7-CH=CH-CH_2-CH=CH-CH_2-CH=CH-CH_2-CH_3$ (Linolenic acid; IV)

Scheme 2. Synthesis of di-ester fatty acid derivatives



R : $-(CH_2)_{16}-CH_3$ (Stearic acid; I), $-(CH_2)_7-CH=CH-(CH_2)_7-CH_3$ (Oleic acid; II),
$-(CH_2)_7-CH=CH-CH_2-CH=CH-(CH_2)_4-CH_3$ (Linoleic acid; III) and
$-(CH_2)_7-CH=CH-CH_2-CH=CH-CH_2-CH=CH-CH_2-CH_3$ (Linolenic acid; IV)

Scheme 3. Synthesis of tri-ester fatty acid derivatives



R : $-(CH_2)_{16}-CH_3$ (Stearic acid; I), $-(CH_2)_7-CH=CH-(CH_2)_7-CH_3$ (Oleic acid; II),
$-(CH_2)_7-CH=CH-CH_2-CH=CH-(CH_2)_4-CH_3$ (Linoleic acid; III) and
$-(CH_2)_7-CH=CH-CH_2-CH=CH-CH_2-CH=CH-CH_2-CH_3$ (Linolenic acid; IV)

A two-step synthetic route was employed to synthesize the amino ester derivatives with different alkyl chains (Scheme 1-3). Key intermediates, *tert*-butyl 3-((2-hydroxyethyl) amino) propanoate (compound **3**; Scheme 1); *tert*-butyl 3-((1,3-dihydroxypropan-2-yl) amino) propanoate (compound **6**; Scheme 2); and *tert*-butyl 3-((1,3-dihydroxy-2-(hydroxymethyl) propan-2-yl) amino) propanoate (compound **9**; Scheme 3) were obtained quantitatively via single Michael addition reaction between amine **(1, 5 or 8)** and *tert*-butyl acrylate (**2)**. The intermediate (**3, 6 or 9)** was coupled to stearic acid (SA), oleic acid (OA), Linoleic acid (LA) and Linolenic acid (LLA) by Steglich esterification using *N*,*N'*-di cyclohexyl carbodiimide (DCC) as a coupling reagent to obtain mono- (Scheme 1), di-(Scheme 2) or tri-(Scheme-3) substituted ester derivatives **(4, 7 or 10)** with good yield (70-83%) (Supplementary Information).

2.2.2 Calculation of lipophilicity

In order to determine lipophilicity an online tool (*chemicalize*) was used to calculate the log *P* values of the derivatives and their respective parent FAs(chemicalize.org, 2016)

2.2.3 In vitro cytotoxicity

MTT cytotoxicity evaluation of the fatty acid derivatives were investigated using the human breast adenocarcinoma (MCF 7), liver hepatocellular carcinoma (Hep G2), and human lung carcinoma (A549) (Supplementary Information).

2.2.4 In vitro permeation studies

2.2.4.1 Preparation of gels

The permeability enhancement properties of the FA esters were evaluated by preparing gels that contained 4% w/w HPMC and 2% w/w TNF as the control. The test formulations were made up using control gels with the addition of enhancers (FA or FA derivative) at 1% w/w. To determine the optimal concentration of most promising enhancer gels containing 0.5, 2, 4, 6% w/w of the parent FA or the respective FA derivative from the different groups were prepared and evaluated.

2.2.4.2 Harvesting rat skin tissue

Wistar rats (male) weighing 200-250 g were used in the study. The University of KwaZulu-Natal (UKZN) Biomedical Research Ethics Committee granted ethical clearance for this study in 2014 (054/14/Animal), and was renewed (015/15/Animal). The animals used for

experiments were housed at the Biomedical Resource Unit (BRU) of UKZN under controlled 12-h light/dark cycles, temperature and humidity conditions. The rats were fed and received water *ad libitum* on a daily basis. After CO_2 euthanasia, the body fur was carefully removed using standard shaving procedures. The skin located around the abdominal region was harvested and excised to remove any subcutaneous fat. Skin samples were stored (-20˚C) and utilized within 3 months (Kalhapure and Akamanchi, 2013; Rambharose et al., 2015). The skin was thawed using a solution of pH 7.4 PBS at room temperature prior to permeation experiments.

2.2.4.5 In vitro permeation studies

The *in vitro* permeation experiments were performed using a modified vertical Franz type diffusion cells system (PermeGear, Inc., Bethlehem, USA) with a diffusional area of 0.786 cm^2 at a temperature of 37 ± 1˚C (Rambharose et al., 2015). The donor compartment of the modified vertical Franz diffusion cells contained either control or sample gels and the receptor compartment contained 25ml PBS (stirred with a teflon-coated magnetic bar). The central diffusional area contained a round section of rat skin tissue mounted directly between the donor and receptor cells. The skin section was initially equilibrated with PBS (pH 7.4) at 37 °C for 30 minutes. Thereafter samples were removed from the receptor compartments at predetermined time intervals and replenished with the equal volume of PBS. Each experiment represents a minimum of six replicates. UV spectrophotometry analysis (UV Spectrophotometer 1650, Shimadzu, Japan) was used to measure the amount of drug permeating through the skin tissues at λ_{max} of 262 nm (Rambharose et al., 2015). After establishing the optimal FA derivative enhancer in each series, the concentration effect studies were performed using the identified derivative and its respective parent FA gel for each series (Ojewole et al., 2014; Rambharose et al., 2015).

2.2.4.6 Permeability data analysis

The permeation percentage of drug (TNF) that entered or permeated per unit surface area of the skin tissue was plotted against time. The linear part of the permeability curve was used to determine the steady state flux (Jss) by linear regression analysis (Microsoft Excel 2010, USA). The permeability coefficient (P) was calculated using the following equation (Ojewole et al., 2014; Rambharose et al., 2015; Rambharose et al., 2014a).

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

A, is the diffusion area and Cd is the drug concentration in the donor compartment and dQ/dt is the cumulative amount permeated per unit time. The enhancement ratio (ER) was calculated as follows (Ojewole et al., 2014; Rambharose et al., 2015).

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

2.2.5 Transepithelial electrical resistance (TEER) evaluations

A Millicell ERS meter (Millipore, USA) connected to a pair of chopstick electrodes (STX01) was used to establish the integrity of the treated skin tissue sections. TEER values of the skin were recorded before and after of the permeation experiment. TEER reading taken before the experiment (0 h) was representative of 100% skin integrity i.e. before exposure to the gel treatments to the skin. 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 hours and replacing it with fresh PBS for a period of 2 hours followed by subsequent TEER measurements (Jones et al., 2013; Rambharose et al., 2015).

2.2.6 Light Microscopy (LM)

The control tissues used in this study originated from histological evaluations of freshly harvested excised skin tissue sections. After careful tissue removal, the control skin tissue was fixed in 10 % buffered formalin, whereas treated tissue samples comprised of skin tissues that were exposed to either OA or MOAPE at a concentration of 1% w/w. The treated skin tissues were removed from the Franz diffusion cells at the end of the permeation study, and fixed in 10 % buffered formalin. The control and enhancer treated skin samples were fixed using formalin for seven days at room temperature. Thereafter, an ethanol gradient ranging from 50 % up to 96 % was used to gradually dehydrate the skin tissue sections. Subsequently, the skin tissue sections were embedded within paraffin wax blocks. The tissue samples were sectioned using a microtome (Leica RM2235, Leica Biosystems Germany) and skin sections were collected on slides, dried and stained with hematoxylin and eosin (H&E). The images from the stained slides were captured using a Leica slide scanner (SCN 400, Leica Biosystems Germany). All experiments were performed in triplicate (Jones et al., 2013; Rambharose et al., 2015; Rambharose et al., 2014b).

2.2.7 Statistical Analysis

The data was analysed using GraphPad Prism® (Graph Pad Software Inc., Version 5., USA) and presented as mean ± standard deviation (SD). The results were analysed by one-way analysis of variance (ANOVA) followed by the non-parametric Kruskal-Wallis test or t tests followed by the non-parametric Mann-Whitney test. $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1 Cytotoxicity evaluation

An *in vitro* cell culture system using MCF-7, Hep G2 and A549 cells with the MTT assay was employed to evaluate the biosafety of the synthesized ester derivatives. The MTT assay findings displayed non-toxicity for all of the synthesized derivatives against all cell lines studied. The cell viability was between 76.55 to 87.39 % for the single chain derivatives, 77.05 to 90.79 % for the double chain derivatives and 76.76 to 92.17 % for the triple chain derivatives (Fig. 1). A one-way ANOVA, with non-parametric Kruskal-Wallis and Dunn's multiple comparison tests showed no statistically significant ($p > 0.05$) differences between the novel ester derivatives and their respective controls in all the cell lines tested. The results indicated that these novel mono, di and tri-ester FA derivatives can be safely used for biological studies.

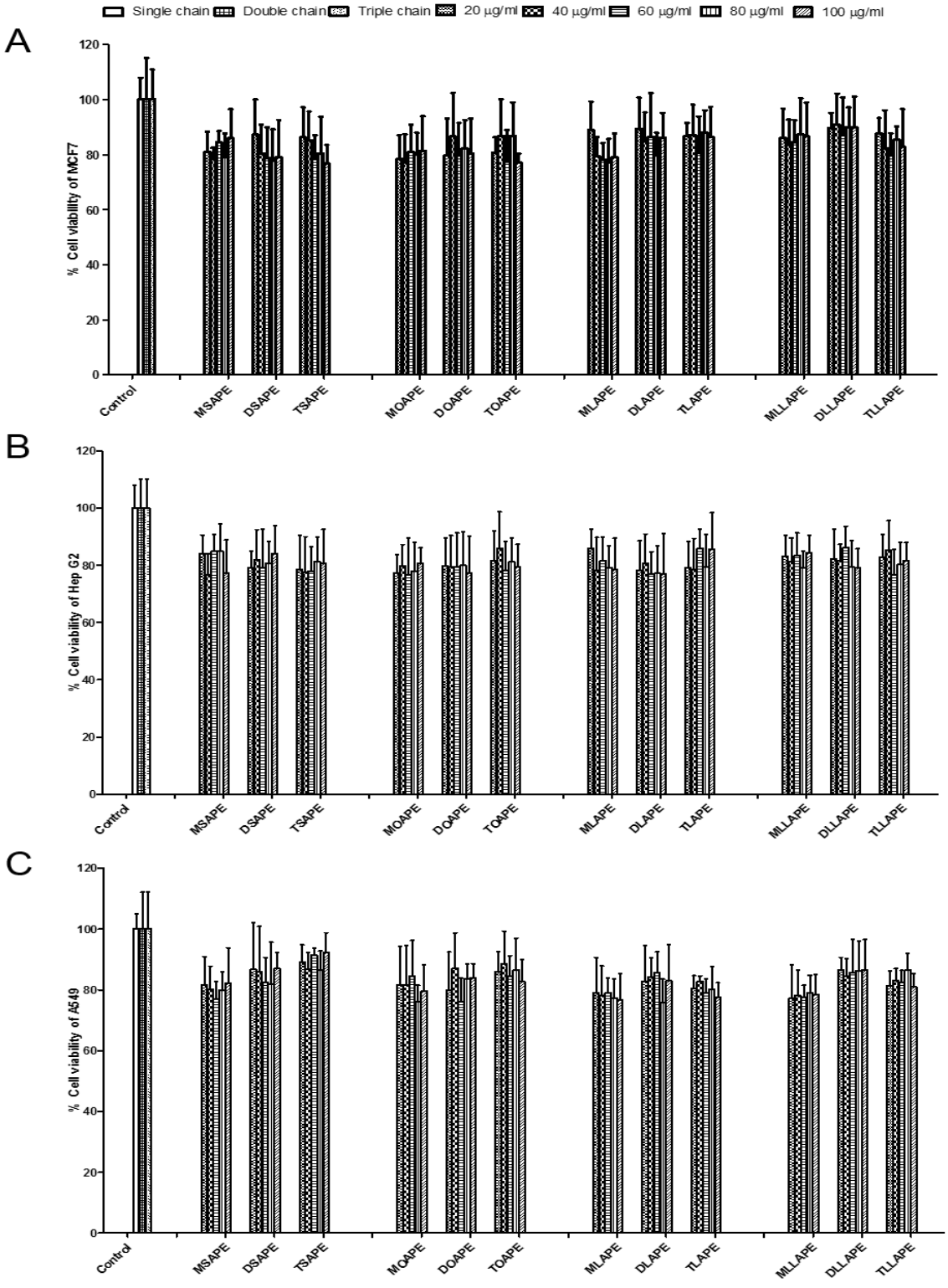


Fig. 1 Cytotoxicity assay displaying percentage cell viability after exposure to various concentrations of the FA derivatives on (A) MCF – 7, (B) Hep G2 and (C) A549 cells. Results are presented as mean ± SD (n = 6).

3.2 In vitro transdermal permeation

In order to ascertain the permeation enhancement effects of the newly synthesized mono (series 1), di (series 2) and tri-ester (series 3) derivatives, the transdermal penetration potential of TNF in the absence of enhancers was also determined. The results displayed that bare TNF was able to penetrate the skin with a cumulative amount of 253.1 ± 23.84 µg/cm^2 and steady state flux of 40.91 ± 4.93 µg/cm^2 h (Table 1). These findings were in good agreement with previous a paper on TNF's transdermal permeation potential (Rambharose et al., 2015). The results showed that TNF was able to permeate the skin unaided. However, to ensure that therapeutically relevant doses of TNF reach the systemic circulation we explored the enhancement potential of the novel single, double and triple chain FA ester derivatives for the delivery of TNF.

Table 1 Effect of the novel groups of FA ester derivatives on the permeability parameters of TNF. *Indicates statistically significant difference i.e. $p < 0.05$ (compared to TNF (control)). #indicates statistically significant difference as compared to its respective parent FA.

Treatment	**Amount permeated (µg.cm $^{-2}$)**	**Jss (flux) (µg.cm $^{-2}$.h^{-1})**	**Permeability (Px10^{-2})**	**ER**
TNF (Control)	253.10 ± 23.84	40.91 ± 4.93	0.204 ± 0.02	**1**
OA	334.75 ± 28.29	54.65 ± 4.22	0.273 ± 0.02	**1.33**
MOAPE	1668.75 ± 105.30	240.55 ± 21.06*#	1.20 ± 0.10	**5.87**
DOAPE	424.95 ± 59.84	70.07 ± 10.89*#	0.350 ± 0.05	**1.71**
TOAPE	342.3 ± 26.94	61.39 ± 4.52*	0.306 ± 0.02	**1.5**
SA	296.59 ± 12.31	47.37 ± 2.35	0.236 ± 0.011	**1.16**
MSAPE	407.12 ± 66.51	63.48 ± 12.41*#	0.317 ± 0.06	**1.55**
DSAPE	336.1 ± 25.67	57.33 ± 5.64*#	0.286 ± 0.02	**1.4**
TSAPE	531.70 ± 23.87	85.76 ± 3.69*#	0.428 ± 0.018	**2.09**
LA	369.02 ± 25.45	60.46 ± 4.32*	0.302 ± 0.02	**1.47**
MLAPE	574.97 ± 74.09	92.07 ± 13.72*#	0.460 ± 0.06	**2.25**
DLAPE	1074.62 ± 61.12	161.87 ± 11.32*#	0.809 ± 0.05	**3.95**
TLAPE	574.39 ± 88.70	86.47 ± 11.54*#	0.432 ± 0.05	**2.11**
LLA	531.96 ± 51.84	90.15 ± 9.39*	0.450 ± 0.04	**2.2**
MLLAPE	945.88 ± 44.93	151.58 ± 8.04*#	0.7579 ± 0.04	**3.7**
DLLAPE	400.37 ± 18.92	67.58 ± 2.53*	0.337 ± 0.01	**1.65**
TLLAPE	453.53 ± 37.43	77.80 ± 8.07*	0.389 ± 0.04	**1.9**

Table 2 Lipophilicity of novel series 1, 2 and 3 derivatives as represented by their respective log *P* values.

	Group 1	**Log *P***	**Group 2**	**Log *P***	**Group 3**	**Log *P***	**Group 4**	**Log *P***
Series	OA	6.78	SA	7.15	LA	6.42	LLA	6.06
1	MOAPE	7.48	MSAPE	7.84	MLAPE	7.12	MLLAPE	6.76
2	DOAPE	14.30	DSAPE	15.02	DLAPE	13.57	DLLAPE	12.85
3	TOAPE	20.98	TSAPE	22.07	TLAPE	19.89	TLLAPE	18.81

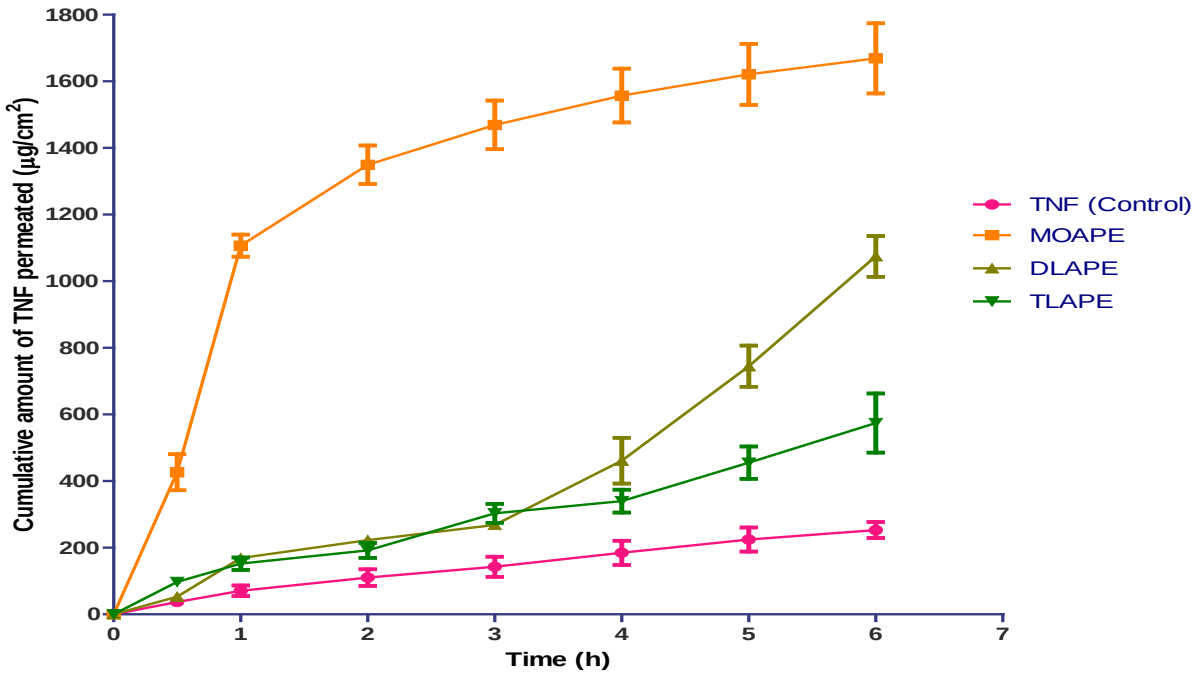


Fig. 2 The effect of the optimal derivatives from the different ester series on the cumulative amount of TNF permeated. Results are presented as mean ± SD (n = 6).

The results showed that the chemical modification to the parent FAs yielded chemical entities with greater lipophilicity characteristics (Table 2). The lipophilicities of the derivatives increased as the number of chains were increased from single to double to triple (Table 2). The *in vitro* transdermal permeation studies revealed that all the modified FA derivatives were able to significantly enhance the permeation of TNF ($p < 0.05$), however there was no clear trend observed between FA chain number and the ER. Being the only SFA, SA displayed the lowest ER of all the FAs investigated (Table 1). Likewise, the SA derivatives also achieved the lowest ERs of all the ester derivatives studied with an enhancement range of 1.4 to 2.09–fold, with the tri-ester TSAPE displaying the best ER which was 0.93-fold higher than the parent FA (SA)

(Table 1). The comparative results between the SFA and the UFAs in this study are reflective of literature that reports SFAs being less effective permeation enhancers than their unsaturated counterparts (Babu et al., 2015; Oh et al., 2001). In a related report the use of propylene glycol (PG) conjugates of SA, OA, LA and LLA in the mono and di-ester form at a concentration of 1% w/w for the transdermal delivery of lidocaine was investigated (Ben-Shabat et al., 2007). The current findings correlate with the trends observed by the Ben-Shabat study (Ben-Shabat et al., 2007). Their study showed that the UFA, SA and its mono and di-ester derivative displayed the lowest permeability as compared to the UFAs (OA, LA and LLA) and their respective mono and di-ester derivatives respectively (Ben-Shabat et al., 2007). That study also revealed that neither SA nor its mono and di-ester derivatives at a concentration of 1% w/w were able to enhance the permeability of lidocaine. However, both the mono and di-ester SA derivatives showed a slightly better efficacy than their parent FA (SA)(Ben-Shabat et al., 2007). Comparatively, at a similar concentration, our study showed that SA and its ester derivatives at were able to enhance the permeability of TNF and the ester derivatives displayed a statistically significant increase in permeability as compared to SA (Table 1).

Literature reports that as the number of unsaturations of a UFA increases, the enhancement potential of the UFA also increases (Raut et al., 2014). LLA had the advantage of having the highest number of unsaturations of all the UFAs studied and therefore achieved the highest ER of the parent FAs studied (Table 1). Conversely, permeation experiments of the LLA group of ester derivatives displayed the lowest ER when compared to the other UFA derivative groups. The LLA derivatives displayed an ER range of 1.65 to 3.7-fold, with the highest increase being only 1.5-fold greater than the parent FA (LLA) (Table 1). Interestingly, with the LLA ester group, the greatest ER was achieved by the mono-ester MLLAPE, with decreases in ER observed with the di-ester (DLLAPE) and tri-ester (TLLAPE) derivatives to a level below that of the parent FA (LLA) (Table 1). The findings of this study are similar to that of Novotny´ et al, who reported that single chain dicarboxylic esters were more effective permeation enhancers than their double chain counterparts (Novotný et al., 2009). The ERs obtained in our study for LLA and its derivatives are superior to those achieved in the Ben-Shabat study for the delivery of lidocaine at similar enhancer concentrations (Ben-Shabat et al., 2007). Their study showed that although the mono and di-ester derivatives of LLA were able to enhance the permeability of lidocaine, the ER achieved by those derivatives were significantly lower than the parent FA (Ben-Shabat et al., 2007). These findings of the current study suggest that only single chain

LLA offers the optimal capabilities and performance in its group (Table 1). Additional conjugations of aliphatic chains do not elicit an increment of the permeation enhancement capabilities of this FA, rather a significant decrease is noted (Table 1). As LLA possesses the highest number of unsaturations, it will therefore have the most number of "kink" isomers in its structure. Additionally, the bent *cis*-configuration of LLA is anticipated to increase the fluidity and create free volumes by interrupting the packing of the intercellular lipids, which allows drug molecules to enter the free volumes of the kinks and move across the epithelium thereby allowing the drug to transverse the skin (Choi et al., 2012; Harrison et al., 1996; Rambharose et al., 2015; Tanojo et al., 1997; Williams and Barry, 2012). The addition of more than one aliphatic chain could cause possible hindrances to the bent-*cis* configuration of LLA and therefore decrease its ability to interrupt the lipid matrix of the SC, which could possibly decrease its ability to cause membrane fluidisation.

The permeation results displayed showed that the LA derivatives were able to enhance the permeability of TNF within an ER range of 2.11 to 3.95-fold (Table 1). The highest ER signifies a statistically significant 2.48-fold increase over the parent FA (LA) ($p < 0.05$). The results also showed that as compared to the parent FA (LA) there was an increase in ER with the mono-ester MLAPE and reached a maximum with the di-ester DLAPE, with a subsequent decrease with the addition of a third aliphatic chain (TLAPE) (Table 1). Interestingly, DLAPE and TLAPE were able to achieve the highest ER of all the di-ester (series 2) and tri-ester (series 3) derivatives respectively (Table 1, Fig. 2). The results of this LA group display greater potential than the PG LA mono and di-esters, as the ERs for LLA mono and di-esters in the present study are superior to the ERs achieved by the PG LA derivatives at similar concentrations (Ben-Shabat et al., 2007).

The results show that with the exception of di- and tri-ester derivatives from LLA group, all of the synthesized derivatives displayed greater ER as compared to their respective parent FAs at similar concentrations (Table 1). At a concentration of 1% w/w, LLA displayed a significantly greater permeability potential compared to DLLAPE and TLLAPE respectively (Table 1). On the other hand, MLLAPE was able to achieve a significantly higher ER than LLA ($p < 0.05$). The ER of MLLAPE was 1.5-fold greater than LA. The findings of the LLA group in this study show significantly higher ER when compare to the PG LLA series (Ben-Shabat et al., 2007). The ERs of the derivatives and the differences between the derivatives and the parent FA in

this study were significantly greater than that achieved by PG mono and di-ester derivatives of LLA (Ben-Shabat et al., 2007).

However, the greatest overall permeation enhancement potential was displayed by OA derivatives, which exhibited an ER range between 1.5 to 5.87-fold (Table 1, Fig. 2). The superiority of OA as a FA transdermal permeation enhancer is well documented in the literature (Babu et al., 2015). OA's ability to enhance transdermal permeation has been attributed to its ability to modify the organisation of the highly ordered lipids in the SC and cause fluidisation of this layer (Babu et al., 2015; Neubert et al., 1997). The resultant formation of such pools causes permeability defects within the lipid bilayer and therefore promotes the movement of drugs across the epidermis. The results show that the chemical modification of OA to produce the mono, di and tri esters displayed a greater transdermal permeation enhancement than the parent lipid (Table 1). MOAPE was able to achieve the greatest ER (5.87) of the OA derivative group. The ER of PG mono and di-ester derivatives of OA did not show improved efficacy when compared to its parent OA (Ben-Shabat et al., 2007). However, significantly the mono LLA ester in the present report displayed a 4.54-fold increase in the permeation of TNF when compared to the efficacy of its respective parent OA. These findings are also similar to a previous study where the mono dicarboxylic esters displayed the greatest permeation enhancement of theophylline (Novotný et al., 2009).

Interestingly MOAPE, DLAPE and TLAPE showed the most promise as transdermal permeation enhancers (Table 1, Fig. 2) within their respective mono (series 1), di (series 2) or tri-ester (series 3) categories. Establishing their concentration effect on the permeability parameters of TNF will provide valuable information that will aid in the optimal formulation of these enhancers to be incorporated into a future TDD system.

3.2.2 Concentration effect of optimal permeation enhancer per series

The concentration effect profile of identified enhancers in each series was evaluated and compared against its respective parent FA. The results revealed that MOAPE was able to enhance the permeation of TNF from an initial concentration of 0.5 to 1% w/w. A 2% w/w increase of the enhancer concentration resulted in a statistically significant ($p < 0.05$) decrease in the ER of TNF (Table 3, Fig. 3). Any further enhancer concentration increases (from 4 to 6% w/w) did not elicit any significant changes ($p > 0.05$) in the permeability of TNF as the ER remained almost constant for 4% and 6% w/w concentrations. Interestingly, the parent FA

(OA) did not display any significant changes in the permeability of TNF across the concentration range studied (Table 3, Fig. 3). OA displayed a slight increase in the ER from 0.5 to 1% w/w, however further increases in the concentration from 2 to 6% failed to significantly increase the permeability parameters of TNF (Table 3, Fig. 3). With the exception of the sharp decline in ER observed with MOAPE at 2% w/w, the concentration enhancement trend for both the parent FA and the novel derivative were very similar (Table 3, Fig. 3). Although both the parent FA and derivative displayed a similar concentration enhancement profile, the ERs obtained by the derivative were statistically significantly ($p < 0.05$) different from that of the parent FA at all concentrations (Table 3). These observations exhibit the superiority of the novel OA ester derivative (MOAPE) over the parent FA (OA). The ester derivative ethyl oleate (10%) was only able to enhance the permeability of cyclosporine A by 1.43-fold (Cui et al., 2013), comparatively MOAPE (1% w/w) in the current study was able to achieve a 4.44-fold greater enhancement at a 10 times lower enhancer concentration than ethyl oleate. This displays MOAPE's ability to significantly enhance transdermal drug delivery at substantially lower enhancer concentrations.

Table 3 Concentrations effect of OA and MOAPE on the transdermal permeability parameters of TNF. *Indicates a statistically significant difference i.e. $p < 0.05$ (compared to control), #Indicates a statistically significant difference when compared to OA at similar concentration.

	Amount permeated (µg.cm $^{-2}$)		**Jss (flux) (µg.cm $^{-2}$.h^{-1})**		**ER**	
Control	253.10 ± 23.84		40.91 ± 4.93		**1**	
Concentrations [% w/w]	OA	MOAPE	OA	MOAPE	OA	MOAPE
0.5	256.92 ± 35.78	521.14 ± 55.22	41.47 ± 6.56	85.05 ± 11.16*#	**1.01**	**2.07**
1	334.75 ± 28.29	1668.75 ± 105.30	54.65 ± 4.22*	240.55 ± 21.06*#	**1.33**	**5.87**
2	328.38 ± 45.32	751.45 ± 37.82	54.04 ± 7.00*	130.43 ± 6.13*#	**1.32**	**3.18**
4	318.06 ± 37.57	733.38 ± 38.50	52.25 ± 5.85	122.87 ± 6.26*#	**1.27**	**3**
6	308.06 ± 36.80	718.39 ± 35.87	50.37 ± 6.60	121.34 ± 7.47*#	**1.23**	**2.96**

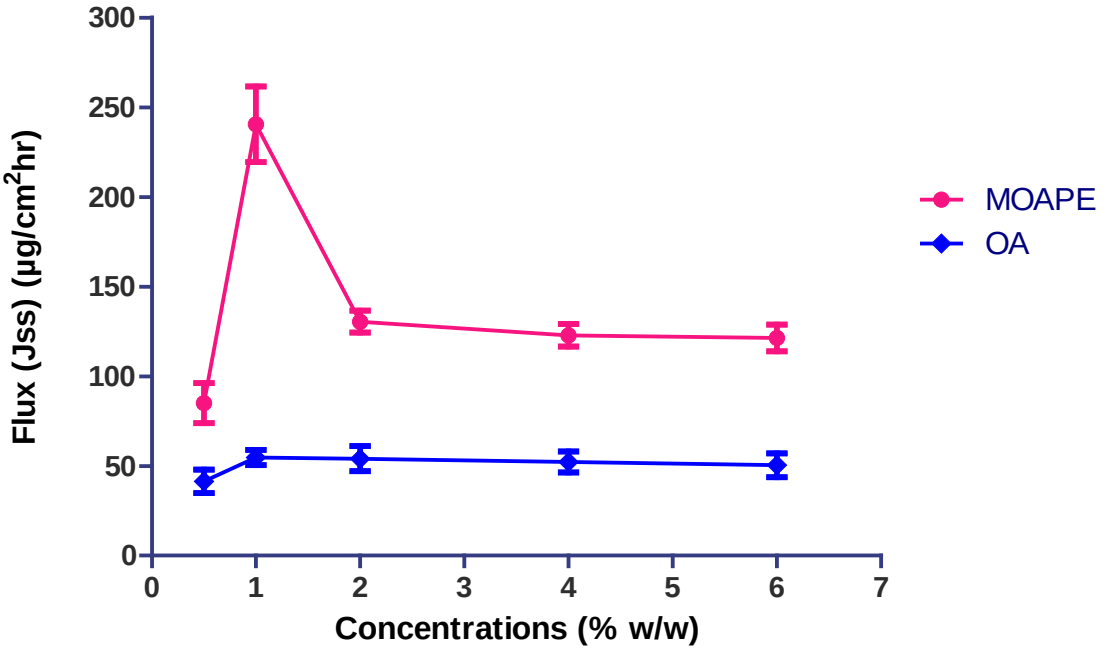


Fig. 3 Concentration effects of OA and the mono-ester OA derivative (MOAPE) on the flux of TNF (n = 6).

The results for the double chain FA derivatives showed that DLAPE displayed a similar enhancement profile to that of MOAPE, where there was an initial increase in ER from 0.5% obtaining a maximum ER of 3.95 at 1% w/w (Table 4, Fig. 4). As the enhancer concentration increased to 2% w/w there was also a statistically significant ($p < 0.05$) decrease in the permeability of TNF, and further increments in the enhancer concentration from 2 to 6% w/w resulted in a decrease in the permeability of the TNF. Comparatively, the parent FA, LA displayed an increase in the permeation of TNF from 0.5% to 2% w/w and a subsequent decrease in the permeation from 4 to 6% w/w (Table 4, Fig. 4). The differences between the derivatives and the parent FA at 0.5 and 1% w/w were statistically significant ($p < 0.05$). Interestingly, at concentrations above 2% w/w the parent FA (LA) displayed a marginally higher ER than the di-ester LA derivative (DLAPE). These results reveal that the di-ester LA (DLAPE) derivative is only able to display superior permeation than its parent FA at concentrations below 2% w/w (Table 4, Fig. 4).

Table 4 Concentrations effect of LA and DLAPE on the transdermal permeability parameters of TNF. *Indicates a statistically significant difference i.e. $p < 0.05$ (compared to control), #Indicates a statistically significant difference when compared to LA at similar concentration.

	Amount permeated (µg.cm $^{-2}$)		**Jss (flux) (µg.cm $^{-2}$.h^{-1})**		**ER**	
Control	253.10 ± 23.84		40.91 ± 4.93		**1**	
Concentrations [% w/w]	LA	DLAPE	LA	DLAPE	LA	DLAPE
0.5	256.69 ± 28.77	432.83 ± 36.18	41.73 ± 4.07	73.36 ± 5.90*#	**1.02**	**1.79**
1	369.02 ± 25.45	1074.62 ± 61.12	60.46 ± 4.32*	161.87 ± 11.32*#	**1.47**	**3.95**
2	576.20 ± 50.23	548.83 ± 26.30	94.73 ± 8.68*	90.82 ± 4.33*	**2.31**	**2.21**
4	527.02 ± 39.54	469.02 ± 49.20	81.82 ± 8.46*	79.65 ± 9.05*	**2**	**1.94**
6	461.38 ± 10.59	405.31 ± 64.12	77.08 ± 1.86*	67.66 ± 11.89*	**1.88**	**1.65**

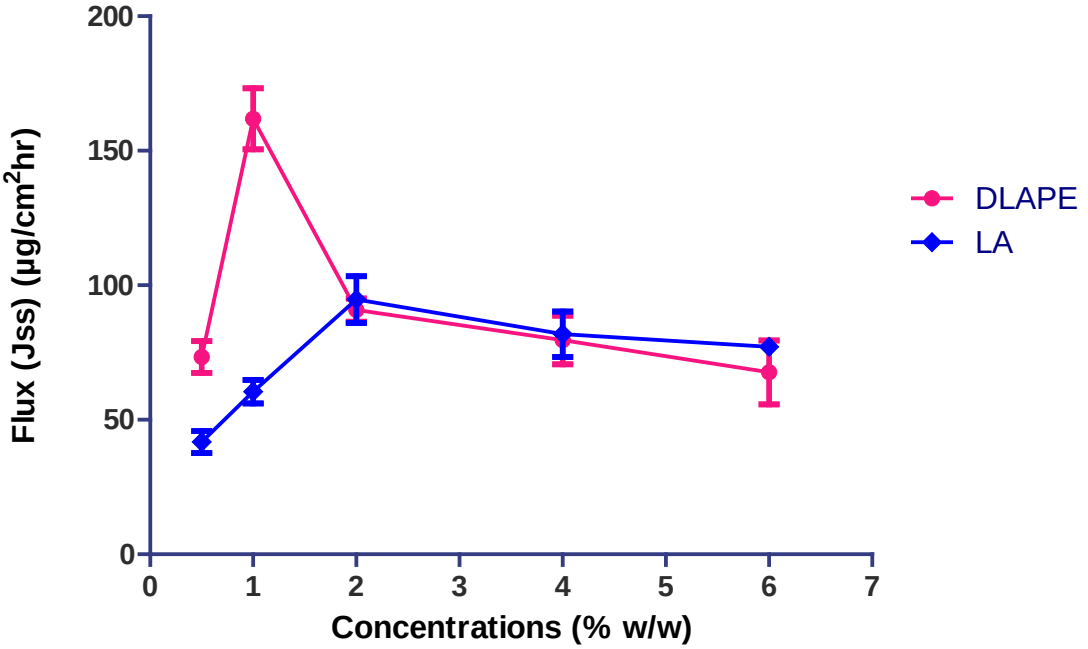


Fig. 4 Concentration effects of LA and the di-ester LA derivative (DLAPE) on the flux of TNF (n = 6).

The concentration range studied was increased to include 0.125 and 0.25% w/w in the case of TLAPE as this derivative showed the most promising enhancement potential at the initial lowest concentration of 0.5% w/w and therefore warranted further investigation at lower concentrations. The extended concentration effect of TLAPE showed that the derivative was able to proportionally increase the ER as the enhancer concentration was increased from 0.125 to 0.5% w/w (Table 5, Fig. 5). At a concentration of 0.5% there was a 4-fold increase in the permeation of TNF (Table 5, Fig. 5). The increases in TLAPE concentrations from 1 to 6% displayed a decrease in the permeation of TNF (Table 5, Fig. 5). Comparatively, the parent FA (LA) displayed an increase in the permeability of TNF from 0.125 to 2% and further increases in concentration from 4 to 6% elicited a decrease in its permeation enhancement effects (Table 5, Fig. 5). Interestingly, from 2 to 6% the ER displayed by the parent FA (LA) was greater than the ER obtained with the tri-ester derivative TLAPE (Table 5, Fig. 5). A similar trend was observed in the series 2 (di-ester DLAPE) derivatives, which reinforces the notion that LA derivatives are only able to display greater permeation enhancement efficacies at lower concentrations with the parent FA (LA) surpassing its derivatives enhancement effect at concentrations greater than 2%.

Table 5 Concentrations effect of LA and TLAPE on the transdermal permeability parameters of TNF. *Indicates a statistically significant difference i.e. $p < 0.05$ (compared to control), $^{\#}$Indicates a statistically significant difference when compared to LA at similar concentration.

	Amount permeated (µg.cm $^{-2}$)		**Jss (flux) (µg.cm $^{-2}$.h^{-1})**		**ER**	
Control	253.10 ± 23.84		40.91 ± 4.93		**1**	
Concentrations [% w/w]	LA	TLAPE	LA	TLAPE	LA	TLAPE
0.125	255.14 ± 22.56	279.60 ± 34.31	41.22 ± 2.68	46.88 ± 5.45	**1.01**	**1.14**
0.25	243.58 ± 21.44	401.15 ± 47.53	39.86 ± 3.09	61.39 ± 8.61*$^{\#}$	**0.97**	**1.5**
0.5	256.69 ± 28.77	1024.96 ± 35.20	41.73 ± 4.07	163.77 ± 4.26*$^{\#}$	**1.02**	**4**
1	369.02 ± 25.45	574.39 ± 88.70	60.46 ± 4.32*	86.47 ± 11.54*$^{\#}$	**1.47**	**2.11**
2	576.20 ± 50.23	436.08 ± 48.47	94.73 ± 8.68*	67.56 ± 9.20*$^{\#}$	**2.31**	**1.65**
4	527.02 ± 39.54	350.78 ± 14.15	81.82 ± 8.46*	55.22 ± 2.036*$^{\#}$	**2**	**1.35**
6	461.38 ± 10.59	316.68 ± 33.80	77.08 ± 1.86*	53.11 ± 5.37*$^{\#}$	**1.88**	**1.29**

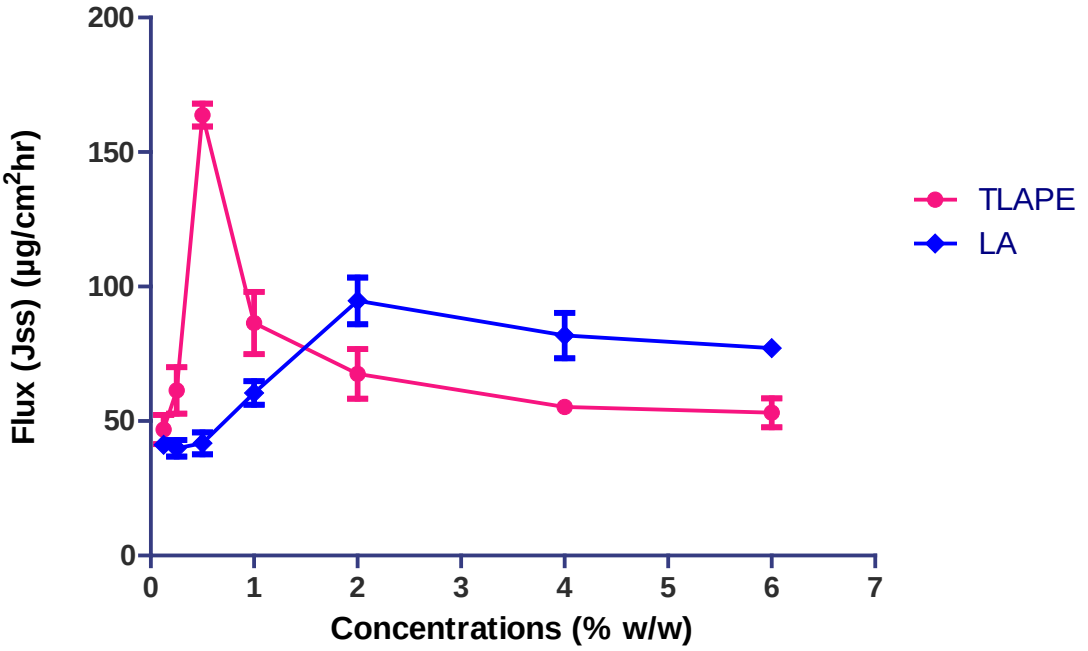


Fig. 5 Concentration effects of LA and the tri-ester derivative (TLAPE) on the flux of TNF (n = 6).

The results of the concentration effect study clearly show the mono-ester OA derivative (MOAPE) at a concentration of 1% w/w as the superior transdermal permeation enhancer of all three series and increased TNF steady state flux to 240.55 ± 21.06 µg/cm^2 and cumulative amount to 1668.75 ± 105.30µg/cm^2 h (Table 3, Fig. 3). Further histomorphological and TEER studies were therefore conducted only on MOAPE, the optimal permeation enhancer, and its respective parent FA (OA) at a concentration of 1% w/w.

3.3 TEER studies

TEER studies were performed to determine the change in the skin's integrity during the 6 h permeation studies. Changes in the TEER measurements are indicative of changes in the barrier properties of the skin (Bartosova and Bajgar, 2012; Rambharose et al., 2015; Srinivasan et al., 2015). TEER values at the end of the experiment are compared to the values recorded prior to the addition of the test material to ascertain the change in the barrier property of the skin due to the interaction of the drug treatment. A decrease in the TEER values is suggestive of the decrease in the barrier properties of the skin, which therefore implies that more transport pathways are available through the skin (Rambharose et al., 2015).

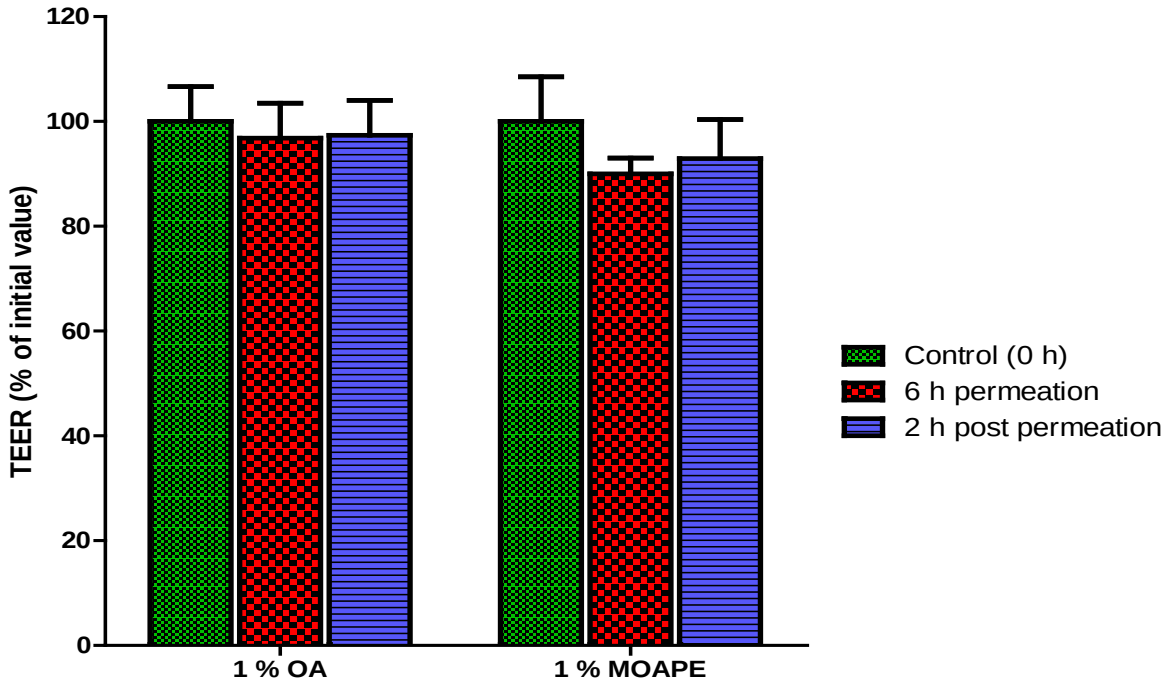


Fig. 6 TEER measurements (%) recorded after TNF permeation with either 1% w/w of OA or MOAPE (n = 6).

The results showed that the skin samples intended for use in both OA and MOAPE displayed similar 0 h TEER values. This confirms that the skin integrity of both test groups were equivalent prior to the commencement of the permeation experiment and the results obtained from these studies would therefore be able to provide reliable comparisons between the effects of both treatments. The skin samples used for the OA and MOAPE study presented an initial resistance of 479.67 ± 42.67 Ω /cm^2 and 488.33 ± 32.53 Ω /cm^2 respectively. These values were used as the respective controls (i.e. 100% TEER value) and compared to the recordings taken after the 6 h permeation as well as to the values obtained 2 h post permeation (Fig. 6). At the end of the 6 h permeation the skin samples exposed to the 1% w/w OA treatment had a TEER value of 472.67 ± 32.65 Ω /cm^2 which signified a 3.2% decline in the resistance of the skin as compared to its respective 0 h recordings (Fig 6). Comparatively, the 1% w/w MOAPE treated skin displayed a TEER value of 390 ± 14.93 Ω /cm^2 which is indicative of a 9.63% decrease of its 0 h recordings (Fig. 6). These findings indicate that the 1% w/w MOAPE treatment is able to decrease the resistance of the skin by 3-fold than the 1% w/w OA treatment. These findings correlate well with the findings from the *in vitro* permeation studies which showed that the MOAPE treatment achieves a significantly greater ER than the OA treatment at 1% w/w (Table 3, Fig. 3). The 2 h post permeation recordings indicated that the removal of

the OA and MOAPE treatments resulted in an increase in the TEER values (Fig. 6). Two hours after the removal of the OA treatment the TEER value was recorded at 475.33 ± 32.72 Ω /cm^2. As compared to the change noted from the differences between the 0 h and 6 h TEER recordings, the 2 h post TEER values therefore show a 17.02% recovery in the resistance of the skin after removal of the OA treatment (Fig. 6). Similarly, the MOAPE treated skin also displayed a recovery in resistance after the 2 h post recordings, however to a greater extent. The 2 h post TEER values of the MOAPE skin samples were recorded at 401 ± 32.19 Ω /cm^2, which indicate a 26.4% recovery in skin resistance (Fig. 6). The results therefore indicate neither OA nor MOAPE at a concentration of 1% w/w irreversibly change the integrity of the skin, as the TEER evaluations show a return towards the original measured resistance of the skin after removal of the enhancer formulations (Fig. 6). These findings confirm that both OA and MOAPE at a concentration of 1% w/w were able to display reversible effects on the barrier properties of the skin, which is one of the key attributes of ideal permeation enhancers.

3.4 Light microscopy

The assessment of skin samples after the permeation study is critical to observe the histomorphological changes brought on by the exposure to the drug treatments. Histological assessments would enable the evaluation of any adverse effects to the individual epithelial layers of the skin by the drug and enhancer treatment (Rambharose et al., 2015). H&E stained sections of skin were evaluated using light microscopy (LM) to visualise the effects of the various treatments on the skin's morphology. As the SC is the major hindrance to the movement of drugs across the epithelium, histological assessments on this region would provide insight to the effects that the enhancers had on this layer to enable larger quantities of the drug to pass through the epidermal layer to the blood vessels in the underlying dermis (D).

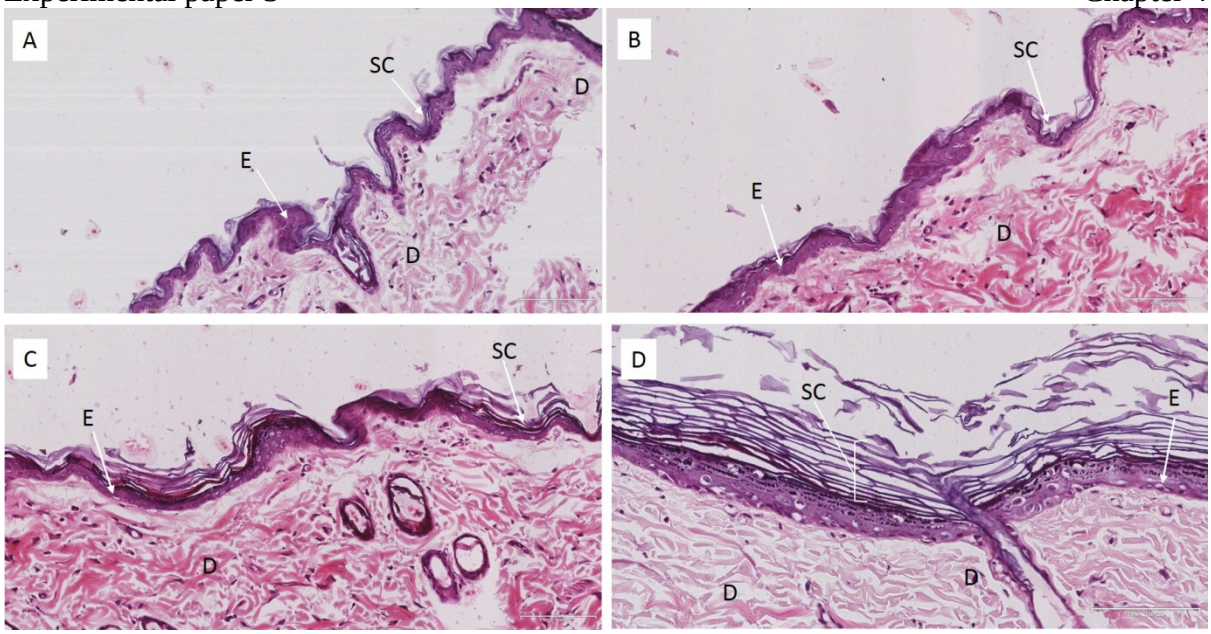


Fig. 7 LM images of H&E stained samples of the control and the treated skin; (A) untreated skin (control), (B) samples exposed to TNF gel, (C) skin exposed to 1% w/w OA TNF gel, (D) skin samples exposed to 1% w/w MOAPE TNF gel (D: dermis; E: epidermis; SC: stratum corneum).

The control image (Fig. 7A) shows normal thin skin histomorphology, with the SC clearly visible and intact. The enhancer free TNF image (Fig. 7B) displayed a very similar morphological appearance to that of the control sample. Similar to the control image, the SC in this treatment also appeared undamaged and the layers of the epidermis didn't display any indication of changes when compared to the control image. In the OA treated (Fig. 7C) and MOAPE treated (Fig. 7D) samples, there were observable changes in the appearance and the morphology of the SC layer. The 1% w/w OA treatment resulted in the disruption of the SC layer, which is clearly identified by the increase in thickness of this layer which is a result of the disruption/fluidisation of the highly ordered lipids found in this layer (Fig. 7C). This disruption/fluidisation causes this layer to appear loosely packed (Liu and Chang, 2011) and thereby looks thicker than the SC of the control sample. There were also intercellular spaces observed in SC of the OA sample which was a further confirmation of the disruption/fluidisation of this layer. Interestingly, the MOAPE treated sample also displayed these characteristics of a disrupted/ fluidised SC. However, these observations were significantly more prominent in the MOAPE treated sample (Fig. 7D). The fluidisation of the SC in the MOAPE sample (Fig. 7D) was much greater, and the intercellular spaces were larger than that observed in the OA sample (Fig. 7C). These observations are indicative of a greater

degree of disruption/fluidisation of the SC in the MOAPE treated sample. This disruption/fluidisation of the SC would decrease the natural barrier property of the skin and promote the movement of drugs across the epidermis. As the MOAPE sample displayed evidence of more prominent disruptions/fluidisation than the OA image, therefore there would be a resultant increase in the amount of drug that would be able to permeate through MOAPE treated skin. These observations correlate directly with the findings of the *in vitro* permeation study which showed that MOAPE caused a significantly higher quantity of TNF to permeate the skin as compared to OA at 1% w/w (Table 3, Fig. 3). Although there are noticeable changes in the uppermost layer (SC) of the epidermis in both the OA and MOAPE treated skin (Fig. 7C and 7D), the underlying layers of the epidermis appear unchanged, with normal histomorphology comparable to that of the control image. The basal layer of all the treated samples (Fig. 7B, 7C and 7D) also appear normal, with characteristics of regular cellular arrangement and morphology which are therefore suggestive that this regenerative layer is unharmed by the drug or enhancer treatments. These observations therefore suggest that the drug and enhancer treatments would not have permanent effects of the epidermal layer of the skin.

4. Conclusions

Our study reports the successful synthesis and characterization of novel mono, di and tri ester FA derivatives. *In vitro* biosafety testing revealed their non-toxic effects on human cell lines which confirm their applicability for biomedical applications. The evaluation of their permeation enhancement efficacies were successfully performed using the model drug TNF. The results showed that all the novel ester derivatives were able to enhance the permeability of TNF at a concentration of 1% w/w; however, there was no direct correlation between FA chain number and the permeation enhancement efficacy of the derivatives. The results revealed that all the FA esters displayed greater permeation enhancement activity as compared to their parent FAs. These findings confirm the superiority of these novel ester derivatives as compared to their respective parent material. The UFAs and their respective derivatives in this study showed a greater increase in ER when compared to the SFA (SA) and its derivatives. Within the three different series MOPE, DLAPE and TLAPE displayed the greatest enhancement potential for the mono, di and tri-ester derivatives respectively. A concentration affect study on the optimal enhancer from each series revealed that MOAPE at 1% w/w from the mono ester derivative series achieved the highest permeation enhancement of TNF with an ER of 5.87. MOAPE

displayed superior permeation enhancement at all concentrations when compared to OA, a parent lipid. Further histomorphological and TEER investigations displayed that 1% w/w MOAPE caused no adverse effects to the skin and the skin's integrity, and that the effects of the enhancer treatment were temporary. In conclusion, this study reported novel FA esters showing promise as effective permeation enhancers. These findings show that the novel mono ester derivative of OA (MOAPE) adds to the pool of CPEs available to formulation scientists and can be safely incorporated into TDD systems for several classes of drugs.

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Supplementary Information

Novel mono, di and tri- fatty acid esters bearing secondary amino acid ester head group as transdermal permeation enhancers

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1. Synthesis and characterisation

*General procedure for mono Michael addition **I***

To a solution of *tert*-butyl acrylate **2** in alcohol**,** an amine **1, 5 or 8** was added at room temperature and stirred for 4 - 30 h at 25 to 45 °C. Alcohol and excess *tert*-butyl acrylate were evaporated in vacuo and the resulting residue was recrystallized or column purified using hexane and ethyl acetate (3:1) to yield the mono Michael addition product (**3, 6 and 9)**.

*General procedure (esterification) for synthesis of amino ester derivatives **II***

Fatty acid was added to a stirred mixture of mono Michael adduct (compound **3**, **6** or **9**), DCC, and DMAP in dry DCM under a nitrogen atmosphere at room temperature (RT). The resulting reaction mixture was further stirred RT for 18 - 24 h. From the reaction mass, precipitated dicyclohexylurea was removed by filtration. The organic layer (filtrate) was evaporated under reduced pressure and the obtained residue was purified by column chromatography (silica gel #70-230 and 10-15% ethyl acetate in hexane as an eluent) to yield the ester derivative.

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).

*Synthesis of tert-butyl 3-((2-hydroxyethyl) amino) propanoate (compound **3**)*

To a solution of *tert*-butyl acrylate **2** (135.0 g, 1.05 mol) in methanol (500 ml**),** 2-aminoethanol **1** (61.0 g, 1.0 mol) was added at room temperature and stirred for 24 h at the same temperature. Methanol and excess *tert*-butyl acrylate were evaporated in vacuo and the resulting residue was purified by column chromatography using hexane and ethyl acetate (3:1) to yield compound **3**

as a thick oil (151.0 g, 80%). ^{1}H NMR ($CDCl_3$) δ (ppm): 1.38 (s; 9H; -C(<u>CH_3</u>)$_3$), 2.30 (t; 2H; -NHCH$_2$<u>CH_2</u>-), 2.39-2.43 (m; 4H; –<u>CH_2</u>NH<u>CH_2</u>-), 3.59 (m; 2H; -<u>CH_2</u>OH). ^{13}C NMR ($CDCl_3$) δ (ppm): 28.08, 35.46, 44.65, 50.90, 60.43, 80.73, 172.01.

*Synthesis of tert-butyl 3-((1,3-dihydroxypropan-2-yl) amino) propanoate (compound **6**)*

To a solution of *tert*-butyl acrylate **2** (141.0 g, 1.10 mol) in ethanol (300 ml**),** 2-amino-1,3-propanediol **5** (91.11 g, 1.0 mol) was added at room temperature and stirred for 4 h at the same temperature. Ethanol and excess *tert*-butyl acrylate were evaporated in vacuo and the resulting residue was recrystallized using hexane and ethyl acetate (3:1) to yield compound **6** as a white solid (201.74 g, 92%). M.p. 69 °C. ^{1}H NMR ($CDCl_3$) δ (ppm): 1.39 (s; 9H; -C(<u>CH_3</u>)$_3$), 2.30 (t; 2H; -NHCH$_2$<u>CH_2</u>-), 2.47 (m; 1H; –NH<u>CH</u>(CH_2-)$_2$), 2.74 (t; 2H; -NH<u>CH_2</u>CH_2;), 3.25 - 3.38 (m; 4H; -<u>CH_2</u>OH). ^{13}C NMR ($CDCl_3$) δ (ppm): 28.09, 35.84, 36.41, 60.18, 62.83, 81.40, 172.84. ESI-TOF MS *m/z*: $[M + Na]^+$ - calculated 242.1367 found 242.1368.

*Synthesis of tert-butyl 3-((1, 3-dihydroxy-2-(hydroxymethyl) propan-2-yl) amino) propanoate (compound **9**)*

To a solution of *tert*-butyl acrylate **2** (128.17 g, 1.0 mol) in ethanol (250 ml**),** Trizma **8** (60.57 g, 0.5 mol) was added at 45 °C and stirred for 30 h at the same temperature. Ethanol and excess *tert*-butyl acrylate were evaporated in vacuo and the resulting residue was recrystallized using hexane and ethyl acetate (3:1) to yield compound **9** as a white solid (112.19 g, 90%). M.p. 69 °C; Rf = 0.55 ($CHCl_3$: MeOH; 9:1); FTIR: 3322.08, 3285.98, 2872.02, 1707.18, 1171.35, 1020.30 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ (ppm): 1.46 (s; 9H; -C(<u>CH_3</u>)$_3$), 2.44 (t; 2H; -NHCH$_2$<u>CH_2</u>-), 2.83 (t; 2H; -NH<u>CH_2</u>CH_2;), 3.18 (bs; 3H; -*OH*), 3.56 (s; 6H; -<u>CH_2</u>OH). ^{13}C NMR ($CDCl_3$) δ (ppm): 28.09, 35.84, 36.41, 60.18, 62.83, 81.40, 172.84. ESI-TOF MS *m/z*: $[M + Na]^+$ - calculated 272.15 found 272.10.

*2-((3-(tert-butoxy)-3-oxopropyl) amino) ethyl stearate (MSAPE) **4** (**I**)*

Stearic acid (14.51g, 0.051 mol) in DCM (50 ml) was added to a solution of compound **3** (9.50 g, 0.05 mol), DCC (10.52 g, 0.051 mol) and DMAP (1.22g, 0.01 mol) in DCM (50 ml) under stirring. The reaction mixture was stirred at room temperature for 18 h and worked up as per the given general procedure II to obtain MSAPE **4** (I) as thick liquid (20.280 g, 89% yield) after column chromatographic purification. FTIR: 2915.37, 2847.87, 1727.59, 1642.58, 1461.33, 1155.84 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ (ppm): 0.81 (t; 3H; -*CH*$_3$), 1.18 (m; 28H; -*CH*$_2$-), 1.38 (s; 9H; -C(*CH*$_3$)$_3$), 1.55 (m; 2H; -*CH*$_2$$CH_2COO$-), 2.29 (t; 2H; -*CH*$_2$$COO$-), 2.45 (t; 2H; -$NHCH_2$*CH*$_2$-), 3.51-3.57 (m; 4H; –*CH*$_2$$NH$*CH*$_2$-), 4.12 (m; 2H; -*CH*$_2$$OOC$-). ^{13}C NMR ($CDCl_3$) δ (ppm): 14.11, 22.69, 24.83, 25.43, 29.66, 31.93, 33.91, 34.12, 34.23, 35.18, 42.87, 44.70, 45.06, 47.27, 61.94, 81.46, 170.21, 173.50. ESI-TOF MS *m/z*: $[M + Na]^+$ - calculated 478.3872 found 478.3870.

*2-((3-(tert-butoxy)-3-oxopropyl) amino) ethyl oleate, (MOAPE) **4** (**II**)*

A solution of oleic acid (14.41g, 0.051 mol) in DCM (50 ml) was added to a mixture of compound **3** (9.50 g, 0.05 mol), DCC (10.52g, 0.051 mol) and DMAP (1.22g, 0.01 mol) in DCM (50 ml) under stirring. The reaction mixture was stirred at room temperature for 18 h and worked up as per the general procedure II to obtain MOAPE **4** (II) as thick liquid (20.73g, 91% yield) after column chromatographic purification. FTIR: 2922.54, 2852.98, 1730.35, 1653.61, 1457.99, 1151.46 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ (ppm): 0.81 (t; 3H; -*CH*$_3$), 1.19 (m; 20H; -*CH*$_2$-), 1.36 (s; 9H; -C(*CH*$_3$)$_3$), 1.55 (m; 2H; -*CH*$_2$$CH_2COO$-), 1.98 (m; 4H; -*CH*$_2$$CH=CH$*CH*$_2$-), 2.22 (t; 2H; -*CH*$_2$$COO$-), 2.45 (t; 2H; -$NHCH_2$*CH*$_2$-), 3.49-3.54 (m; 4H; –*CH*$_2$$NH$*CH*$_2$-), 4.12 (m; 2H; -*CH*$_2$$OOC$-), 5.28 (m; 4H; -*CH=CH*-). ^{13}C NMR ($CDCl_3$) δ (ppm): 14.10, 22.68, 25.29, 27.22, 28.05, 28.06, 29.76, 33.09, 33.98, 34.09, 34.20, 35.23, 42.71, 44.60, 44.90, 47.08, 61.73,

62.05, 81.36, 129.96, 129.99, 170.28, 171.48, 173.35, 173.45. ESI-TOF MS *m/z*: $[M + Na]^+$ - calculated 476.3716 found 476.3728.

2-((3-(tert-butoxy)-3-oxopropyl) amino) ethyl (9Z,12Z)-octadeca-9,12-dienoate, (MLAPE) ***4*** ***(III)***

A solution of linoleic acid (12.0 g, 0.043 mol) in DCM (25 ml) was added to a mixture of compound **3** (7.94 g, 0.042 mol), DCC (8.83 g, 0.043 mol) and DMAP (1.22 g, 0.01 mol) in DCM (25 ml) under stirring. The reaction mixture was stirred at room temperature for 22 h and worked up as per the given general procedure II to obtain MLAPE **4** (III) as thick liquid (17.05 g, 90% yield) after purification. FTIR: 2923.36, 2853.52, 1730.13, 1653.24, 1457.99, 1151.44 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ (ppm): 0.82 (t; 3H; -*CH*$_3$), 1.23 (m; 16H; -*CH*$_2$-), 1.38 (s; 9H; -C(*CH*$_3$)$_3$), 1.55 (m; 2H; -*CH*$_2$CH$_2$COO-), 1.98 (m; 4H; -*CH*$_2$CH=CH*CH*$_2$-), 2.25 (t; 2H; -*CH*$_2$COO-), 2.45 (t; 2H; -NHCH$_2$*CH*$_2$-), 3.49-3.54 (m; 4H; –*CH*$_2$NH*CH*$_2$-), 4.12 (m; 2H; -*CH*$_2$OOC-), 5.28 (m; 4H; -*CH=CH*-). ^{13}C NMR ($CDCl_3$) δ (ppm): 14.05, 22.55, 24.84, 25.28, 27.18, 28.04, 29.09-29.75, 31.50, 31.88, 33.072, 33.12, 34.08, 35.21, 47.08, 61.71, 62.03, 80.64.05, 81.35, 127.88, 130.18, 170.27, 171.47, 173.36, 173.43. ESI-TOF MS *m/z*: $[M + Na]^+$ - calculated 474.3559 found 474.3557.

2-((3-(tert-butoxy)-3-oxopropyl) amino) ethyl (9Z,12Z,15Z)-octadeca-9,12,15-trienoate (MLLAPE) ***4*** ***(IV)***

A solution of linolenic acid (7.50 g, 0.0269 mol) in DCM (25 ml) was added to a mixture of compound **3** (5.0 g, 0.0264 mol), DCC (5.45 g, 0.0269 mol) and DMAP (1.22g, 0.01mol) in DCM (25 ml) under stirring. The reaction mixture was stirred at room temperature for 24 h and worked up as per the given general procedure II to obtain MLLAPE **4** (IV) as thick liquid (10.095 g, 85% yield) after column chromatographic purification. FTIR: 2928.52, 2856.01,

1727.82, 1629.20, 1457.72, 1152.66 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ (ppm): 0.89 (t; 3H; -*CH*$_3$), 1.23 (m; 12H; -*CH*$_2$-), 1.36 (s; 9H; -C*(CH*$_3$*)*$_3$), 1.53 (m; 2H; -*CH*$_2$$CH_2COO$-), 1.98 (m; 4H; -*CH*$_2$CH=CH*CH*$_2$-), 2.24 (t; 2H; -*CH*$_2$COO-), 2.44 (t; 2H; -$NHCH_2$*CH*$_2$-), 3.49-3.53 (m; 4H; –*CH*$_2$NH*CH*$_2$-), 4.10 (m; 2H; -*CH*$_2$OOC-), 5.29 (m; 4H; -*CH=CH*-). ^{13}C NMR ($CDCl_3$) δ (ppm): 14.26, 20.53, 22.55, 25.28, 25.60, 31.50, 33.08, 33.12, 33.97, 34.08, 34.19, 42.71, 44.91, 47.78, 61.73, 62.04, 81.37, 127.09, 128.22, 130.20, 131.92, 170.28, 171.48, 173.37.

2-((3-(tert-butoxy)-3-oxopropyl) amino) propane-1,3-diyl distearate (DSAPE) ***7 (I)***

Stearic acid (13.10 g, 0.0461 mol) in DCM (50 ml) was added to a solution of compound 3 (5.0 g, 0.0228 mol), DCC (9.50 g, 0.0461 mol) and DMAP (2.44 g, 0.02 mol) in DCM (50 ml) under stirring. The reaction mixture was stirred at room temperature for 22 h and worked up following general procedure II to obtain DSAPE **7** (I) as thick liquid (15.78g, 92% yield) after purification by using column chromatography. FTIR: 2914.26, 2849.09, 1732.04, 1470.43, 1152.05 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ (ppm): 0. 81 (t; 6H; -*CH*$_3$), 1.19-1.23 (m; 56H; -*CH*$_2$-), 1.38 (s; 9H; -C*(CH3)*$_3$), 1.55 (q; 4H; -*CH*$_2$$CH_2COO$-), 1.95 (m; 8H; -*CH*$_2$CH=CH*CH*$_2$-), 2.25 (t; 4H; -*CH*$_2$COO-), 2.34 (t; 2H; -$NHCH_2$*CH*$_2$-), 2.83 (t; 2H; -NH*CH*$_2$$CH_2$-), 2.96 (m; 1H – NH*CH*$(CH_2$-$)_2$), 4.03 (s; 4H; -*CH*$_2$OOC-). ^{13}C NMR ($CDCl_3$) δ (ppm): 14.10, 22.67, 24.87, 27.16, 27.21, 28.10, 29.11 - 29.76, 31.90, 34.16, 43.02, 55.38, 63.38, 80.58, 171.84, 173.57. ESI-TOF MS, *m/z*: $[M + Na]^+$ - calculated 774.6588, found 774.6595.

2-((3-(tert-butoxy)-3-oxopropyl) amino) propane-1,3-diyl dioleate (DOAPE) ***7 (II)***

A solution of oleic acid (13.0 g, 0.0461 mol) in DCM (50 ml) was added to a solution of compound **3** (5.0 g, 0.0228 mol), DCC (9.50 g, 0.0461 mol) and DMAP (2.44 g, 0.02 mol) in DCM (50 ml) under stirring. The reaction mixture was stirred at room temperature for 22 h and worked up as per the given general procedure II to obtain DOAPE **7** (II) as thick liquid (15.87g,

93% yield) after column purification. FTIR: 2923.54, 2853.64, 1734.07, 1662.78, 1459.33, 1367.17, 1156.55 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ (ppm): 0. 81 (t; 6H; -CH_3), 1.19-1.23 (m; 40H; -CH_2-), 1.38 (s; 9H; -C$(CH3)_3$), 1.55 (q; 4H; -$CH_2$$CH_2$COO-), 1.95 (m; 8H; -$CH_2$CH=CH$CH_2$-), 2.25 (t; 4H; -$CH_2$COO-), 2.34 (t; 2H; -NHCH$_2$$CH_2$-), 2.83 (t; 2H; -NH$CH_2$$CH_2$-), 2.96 (m; 1H –NH$CH$($CH_2$-)$_2$), 4.03 (s; 4H; -$CH_2$OOC-), 5.27 (m; 4H; -$CH$=$CH$-). ^{13}C NMR ($CDCl_3$) δ (ppm): 14.10, 22.67, 24.87, 27.16, 27.21, 28.10, 29.11 - 29.76, 31.90, 34.16, 43.02, 55.38, 63.38, 80.58, 129.72, 129.98, 171.84, 173.57. ESI-TOF MS, *m/z*: $[M + Na]^+$ - calculated 770.6275, found 770.6281.

2-((3-(tert-butoxy)-3-oxopropyl) amino) propane-1,3-diyl (9Z,9'Z,12Z,12'Z)-bis(octadeca-9,12-dienoate) (DLAPE) ***7 (III)***

A solution of linoleic acid (6.46g, 0.023mol) in DCM (25ml) was added to a mixture of compound **6** (2.5 g, 0.0114 mol), DCC (4.75g, 0.023 mol) and DMAP (1.22 g, 0.01 mol) in DCM (25 ml) under stirring. The reaction mass was stirred at room temperature for 24 h and worked up following general procedure II to obtain DLAPE **7** (III) as thick liquid (7.64 g, 90% yield) after column chromatographic purification. FTIR: 2925.68, 2854.28, 1735.04, 1454.93, 1156.54 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ (ppm): 0. 82 (t; 6H; -CH_3), 1.23 (m; 32H; -CH_2-), 1.38 (s; 9H; -C$(CH3)_3$), 1.55 (q; 4H; -$CH_2$$CH_2$COO-), 1.98 (m; 8H; -$CH_2$CH=CH$CH_2$-), 2.25 (t; 4H; -$CH_2$COO-), 2.33 (t; 2H; -NHCH$_2$$CH_2$-), 2.70 (t; 2H; -NH$CH_2$$CH_2$-), 2.83 (m; 1H –NH$CH$($CH_2$-)$_2$), 4.03 (s; 4H; -$CH_2$OOC-), 5.28 (m; 8H; -$CH$=$CH$-). ^{13}C NMR ($CDCl_3$) δ (ppm): 14.10, 22.57, 24.88, 25.45, 25.62, 27.19, 28.11, 29.12 - 29.61, 31.52, 34.16, 34.92, 36.10, 43.05, 55.36, 63.46, 80.54, 127.90, 128.04, 130.02, 130.20, 171.84, 173.59. ESI-TOF MS, *m/z*: $[M + Na]^+$ - calculated 766.5962, found 766.5976.

2-((3-(tert-butoxy)-3-oxopropyl) amino) propane-1,3-diyl (9Z,9'Z,12Z,12'Z,15Z,15'Z)-bis(octadeca-9,12,15-trienoate) (DLLAPE) ***7 (IV)***

A solution of linolenic acid (6.41 g, 0.023 mol) in DCM (25 ml) was added to a mixture of compound 6 (2.5 g, 0.011 mol), DCC (4.75g, 0.023 mol) and DMAP (1.22 g, 0.01 mol) in DCM (25 ml) under stirring. The reaction mixture was stirred at room temperature for 24 h and worked up as per the given general procedure II to give DLLAPE **7** (IV) as thick liquid (7.26g, 86% yield) after purification. FTIR: 2926.91, 2854.49, 1734.86, 1456.85, 1154.96 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ (ppm): 0. 90 (t; 6H; -CH_3), 1.24 (m; 24H; -CH_2-), 1.38 (s; 9H; -C($CH3)_3$), 1.55 (q; 4H; -CH_2CH_2COO-), 1.98 (m; 8H; -$CH_2CH{=}CHCH_2$-), 2.25 (t; 4H; -CH_2COO-), 2.33 (t; 2H; -$NHCH_2CH_2$-), 2.73 (t; 2H; -$NHCH_2CH_2$-), 2.84 (m; 1H –$NHCH(CH_2$-$)_2$), 4.03 (s; 4H; -CH_2OOC-), 5.29 (m; 12H; -$CH{=}CH$-). ^{13}C NMR ($CDCl_3$) δ (ppm): 14.27, 20.54, 22.57, 24.86, 25.52, 25.61, 27.20, 28.10, 29.11 - 29.58, 31.52, 34.15, 43.00, 55.39, 63.33, 80.64, 127.11, 127.73, 128.24, 128.28, 130.20, 130.24, 131.94, 171.85, 173.56. ESI-TOF MS, *m/z*: $[M + Na]^+$ - calculated 762.5649, found 762.5663.

2-((3-(tert-butoxy)-3-oxopropyl) amino)-2-((stearoyloxy)methyl) propane-1,3-diyl distearate, (TSAPE) ***10 (I)***

A solution of stearic acid (14.37 g, 0.051 mol) in DCM (50 ml) was added to a mixture of compound 9 (2.0 g, 0.016 mol), DCC (10.42 g, 0.051 mol) and DMAP (2.44 g, 0.02 mol) in DCM (50 ml) under stirring. The reaction mixture was stirred at room temperature for 24 h and worked up as per the given general procedure II to obtain TSAPE **10** (I) as thick liquid (15.22 g, 88% yield) after column chromatographic purification. M.p. 58 °C; FTIR: 2916.07, 2849.18, 1752.83, 1718.20, 1609.02, 1468.45, 1195.72, 1154.03 - 1020.30 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ (ppm): 0. 81 (t; 9H; -CH_3), 1.19 (m; 84H; -CH_2-), 1.39 (s; 9H; -C($CH_3)_3$), 1.54 (q; 6H; -CH_2CH_2COO-), 2.25 (t; 6H; -CH_2COO-), 2.32 (t; 2H; -$NHCH_2CH_2$-), 2.77 (t; 2H; -$NHCH_2CH_2$-), 4.03 (s; 6H; -CH_2OOC-). ^{13}C NMR ($CDCl_3$) δ (ppm): 14.11, 22.67, 24.90,

28.11, 29.16, 29.26, 29.35, 29.69, 29.70, 31.92, 34.20, 36.10, 36.76, 60.51, 63.07, 81.06, 172.22, 173.45; ESI-TOF MS *m/z*: $[M + Na]^+$ - calculated 1070.93, found 1070.79.

2-((3-(tert-butoxy)-3-oxopropyl) amino)-2-((((Z)-octadec-9-enoyl) oxy) methyl) propane-1,3-diyl(9Z,9'Z)-bis(octadec-9-enoate) (TOAPE) ***10 (II)***

A solution of oleic acid (17.84 g, 0.063 mol) in DCM (50 ml) was added to a mixture of compound **9** (2.5 g, 0.021 mol), DCC (13.03 g, 0.063 mol) and DMAP (2.44 g, 0.02 mol) in DCM (25 ml) under stirring. The reaction mixture was stirred at room temperature for 24 h and general procedure II was followed for work up to obtain TOAPE **10** (II) as thick liquid (19.37 g, 90% yield) after column chromatographic purification. FTIR: 2923.46, 2855, 1746.15, 1662.78, 1459.29, 1379.17, 1157.32 cm^{-1}. ^1H NMR ($CDCl_3$) δ (ppm): 0. 81 (t; 9H; -CH_3), 1.19-1.23 (m; 60H; -CH_2-), 1.37 (s; 9H; -C$(CH3)_3$), 1.54 (q; 6H; -CH_2CH_2COO-), 1.93 (m; 12H; -CH_2CH=CHCH_2-), 2.25 (t; 6H; -CH_2COO-), 2.28 (t; 2H; -NHCH$_2CH_2$-), 2.75 (t; 2H; -NHCH_2-CH_2-), 4.04 (s; 6H; -CH_2OOC-), 5.27 (m; 6H; -CH=CH-). ^{13}C NMR ($CDCl_3$) δ (ppm): 14.11, 22.67, 24.86, 27.18, 27.22, 28.10, 29.14 - 29.77, 31.90, 34.16, 37.71, 57.27, 63.22, 80.57, 129.71, 129.98, 171.79, 173.26. ESI-TOF MS, *m/z*: $[M + Na]^+$ - calculated 1064.88, found 1064.76.

2-((3-(tert-butoxy)-3-oxopropyl) amino)-2-((((9Z,12Z)-octadeca-9,12-dienoyl) oxy) methyl) propane-1,3-diyl (9Z,9'Z,12Z,12'Z)-bis(octadeca-9,12-dienoate) (TLAPE) ***10 (III)***

Linoleic acid (7.08 g, 0.025 mol) in DCM (25 ml) was added to a mixture of compound **9** (1.0 g, 0.008 mol), DCC (5.12 g, 0.025 mol) and DMAP (1.22 g, 0.01 mol) in DCM (25 ml) under stirring. The reaction mass was stirred at room temperature for 24 h and worked up as per the given general procedure II to obtain TLAPE **10** (III) as thick liquid (7.36g, 86% yield) after column chromatographic purification. FTIR: 2922.84, 2854.77, 1744.70, 1666.39, 1457.92,

1378.45, 1150.55 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ (ppm): 0. 90 (t; 9H; -CH_3), 1.31 (m; 48H; -CH_2-), 1.45 (s; 9H; -C$(CH_3)_3$), 1.62 (m; 6H; -CH_2CH_2COO-), 2.04 (m; 12H; -CH_2CH=CHCH_2-), 2.33 (t; 6H; -CH_2COO-), 2.37 (t; 2H; -$NHCH_2CH_2$-), 2.83 (t; 2H; -$NHCH_2$-), 4.12 (s; 6H; -CH_2OOC-), 5.38 (m; 12H; -CH=CH-). ^{13}C NMR ($CDCl_3$) δ (ppm): 14.10, 22.57, 24.84, 25.53, 27.19, 27.52, 28.11, 29.14 - 29.62, 31.52, 34.15, 57.27, 63.21, 80.57, 127.90, 128.04, 130.21, 171.80, 173.27. ESI-TOF MS, *m/z*: $[M+Na]^+$ - calculated 1058.84, found 1058.77.

2-((3-(tert-butoxy)-3-oxopropyl) amino)-2-((((9Z,12Z,15Z)-octa -dec-9,12,15-trienoyl) oxy) methyl) propane-1,3-diyl (9Z,9'Z,12Z,12'Z,15Z, 15'Z) -bis(octadeca-9,12,15-trienoate) (TLLAPE) ***10*** *(**IV**)*

Linolenic acid (7.03 g, 0.025 mol) in DCM (25 ml) was added to a mixture of compound **9** (1.0 g, 0.008 mol), DCC (5.12 g, 0.025 mol) and DMAP (1.22 g, 0.01 mol) in DCM (25 ml) under stirring. The reaction mixture was stirred at room temperature for 24 h and worked up as per the given general procedure II to obtain TLLAPE **10** (IV) as thick liquid (7.15 g, 84% yield) after column chromatographic purification. FTIR: 2927.36, 2858.37, 1746.03, 1663.52, 1458.04, 1388.94, 1157.18 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ (ppm): 0. 90 (t; 9H; -CH_3), 1.23 (m; 36H; -CH_2-), 1.37 (s; 9H; -C$(CH3)_3$), 1.54 (m; 6H; -CH_2CH_2COO-), 1.99 (m; 12H; -CH_2CH=CHCH_2-), 2.25 (t; 6H; (-CH_2COO-), 2.29 (t; 2H; -$NHCH_2CH_2$-), 2.75 (t; 2H; -$NHCH_2$), 4.04 (s; 6H; -CH_2OOC-), 5.29 (m; 18H; -CH=CH-). ^{13}C NMR ($CDCl_3$) δ (ppm): 14.27, 22.58, 24.84, 25.60, 27.20, 28.10, 29.13, 29.33, 29.59, 31.52, 34.14, 37.69, 57.25, 63.21, 80.55, 127.11, 127.73, 128.23, 128.27, 130.22, 131.93, 171.79, 173.25. ESI-TOF MS, *m/z*: $[M + Na]^+$ - calculated 1052.79, found 1052.68.

2. *In vitro* cytotoxicity

Cell culture

Complete growth medium was prepared by supplementing EMEM cell culture media with streptomycin (100 mg/ml), 10 % bovine calf serum (10 %) and penicillin (100 units/ml). The resultant complete growth medium was used to sustain the growth of human breast adenocarcinoma (MCF 7), liver hepatocellular carcinoma (Hep G2), and human lung carcinoma (A549) cells using standard aseptic cell culture protocols. The cells were incubated at the following conditions (a temperature of 37 °C and humidified atmosphere of 5 % CO_2).

Solutions

Stock solutions of the different series were prepared by dissolving in appropriate volumes of DMSO and purified water. These stock solutions were further diluted in the growth media to attain the final concentrations of 20, 40, 60, 80 and 100 µg/ml (Rambharose et al., 2015).

MTT assay

MCF 7, A549 and Hep G2 cells were seeded in 96 well plates at an equivalent cell density of 4.4 $x10^3$ cells per well. Following 24 h incubation the initial culture medium was removed and each well was replaced with 100 µL of fresh growth medium containing the ester derivatives with final concentrations of 20, 40, 60, 80 and 100 µg/ml. The control consisted of cells and culture medium only, whilst wells containing medium only was used as the blank control. The treatments were incubated with the cells for 48 h. Subsequent to the 48 h incubation with the derivatives, the treatment mediums were removed and each well was supplemented with MTT reagent (100 µL of 5 mg/ml in PBS) and 100 µL of complete medium, then incubated for a further 4 h. Afterwards the media and MTT solution was removed and 100 µL of DMSO was added to each well, allowing the solubilisation the MTT formazan crystals. A microplate spectrophotometer (Spectrostar Nano) at a wavelength of 540 nm was used to detect the optical density of each well (Rambharose et al., 2015). All the cytotoxicity experiments were carried out with n = 6. The quantity of viable cells was calculated with the following equation:

$$\%\ Cell\ Viability\ = \frac{A_{540\ nm\ treated\ cells}}{A_{540\ nm\ untreated\ cells}}\ x100 \qquad eq.(1)$$

References

Rambharose, S., Kalhapure, R.S., Akamanchi, K.G., Govender, T., 2015. Novel dendritic derivatives of unsaturated fatty acids as promising transdermal permeation enhancers for tenofovir. J. Mater. Chem. B 3, 6662-6675.

CHAPTER 5
EXPERIMENTAL PAPER 4

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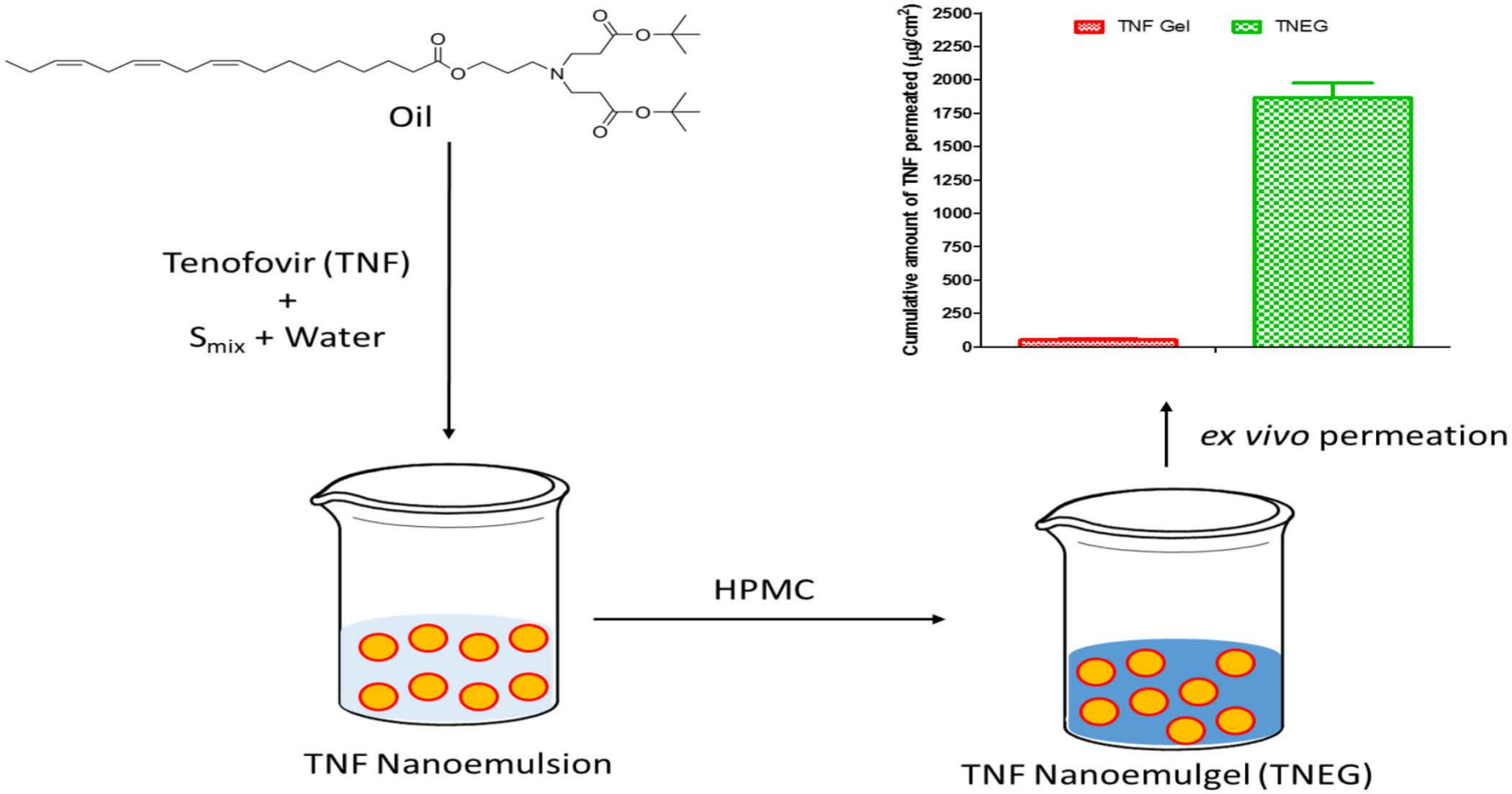
5.1 Introduction

This chapter addresses aim 1.4.4 and is a first authored experimental article published in Colloids and Surfaces B: Biointerfaces (Impact Factor = 3.902) which is an ISI international journal. This article reports the use of a novel linolenic acid based heterolipid, LLA1E (a novel transdermal permeation enhancer), as an oily phase in the development of a nanoemulgel for the transdermal drug delivery of tenofovir. LLA1E was selected as the oily phase for the formulation of the TNF nanoemulgel, as it exhibited the highest enhancement ratio (ER = 6.11) of all the novel FA derivatives synthesized in this PhD study.

Mr. S. Rambharose contributed to the design of the project, modification and optimisation of methods as well as synthesis of the linolenic acid ester dendritic derivative (LLA1E). Mr. S. Rambharose also conducted the experiments for the construction of the pseudo-ternary phase diagrams and prepared and characterized all NE and NEG formulations in terms of mean globule diameter, polydispersity index, zeta potential, rheology, morphology and stability. Mr. S. Rambharose conducted the incorporation efficiency studies and all transdermal permeation experimental work including, rat skin tissue harvesting, *in vitro* permeation experiments, TEER experiments and light microscopy evaluations. Mr. S. Rambharose also wrote the paper. Dr. R.S. Kalhapure assisted with the overall design of the project and the methods of preparation and characterisation as well as editing of the paper. Prof. T. Govender served as supervisor and was responsible for project conceptualisation, editing of paper and abstract and general supervision of the study.

5.2 Graphical abstract



Nanoemulgel using a bicephalous heteolipid as a novel approach to enhance transdermal permeation of tenofovir

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Abstract

Improvement in permeation enhancement strategies such as nanoemulsions (NEs) and nanoemulgels (NEGs) has led to a renewed interest in transdermal drug delivery (TDD). This study aimed to investigate the potential of LLA1E, a novel dendritic permeation enhancer, as an oily phase in the development of a NEG for the TDD of tenofovir (TNF). TNF loaded NEs (TNEs) were first prepared and analysed for mean globule diameter (MGD), polydispersity index (PDI), zeta potential (ZP) and morphology. NEGs of the TNEs (TNEGs) were prepared and evaluated for *ex vivo* transdermal permeation efficacy. The skin integrity before and after experiments was assessed using histology and transepithelial electrical resistance (TEER). TNEs had a MGD of 129.06 ± 3.35 nm, PDI of 0.192 ± 0.038 and ZP of 20.9 ± 2.02 mV with an incorporation efficiency of 91.94 ± 0.84%. There was no significant change is these properties after incorporation of TNEs into hydrogel as MGD, PDI and ZP of TNEGs were found to be 136.13 ± 5.21 nm, 0.182 ± 0.020 and -20.9 ± 2.08 mV respectively. *Ex vivo* permeation studies showed that the TNEG significantly enhanced the TNF permeation by 39.65-fold, with a cumulative amount of 1866.54 ± 108.62 µg.cm $^{-2}$. Histological and TEER assessments showed no permanent effects on the skin by TNEG. Therefore, this novel TNEG nanosystem has the potential of further translation into clinical trials for optimal treatment alternatives for HIV/AIDs patients.

Keywords: nanoemulsion; nanoemulgel; linolenic acid; heterolipid; transdermal permeation enhancer; tenofovir

1. Introduction

The global burden of HIV/AIDS shows a drastic increase in the spread of this disease each year, with a large number of HIV infected patients relying mainly on the use of antiretroviral drugs to help control the progression of this virus [1]. Although the World Health Organisation (WHO) has recommended the use of tenofovir (TNF) as an essential medicine for the treatment of HIV [2-4], its current oral dosing presents several undesired gastrointestinal tract (GIT) associated side effects [5]. Therefore, strategies to formulate TNF for alternative delivery routes which can bypass the GIT have shown promising outcomes. Various alternative routes such as vaginal, buccal, rectal and more recently the transdermal route [6-10] have been explored for the administration of TNF. Transdermal drug delivery (TDD) is well reported as a promising alternative route for the administration of drugs which create GIT discomfort in patients such as diclofenac sodium, peroxicam, ketoprofen and donepezil [11-14].

TDD systems offer numerous advantages over conventional oral drug therapy such as higher bioavailability, evasion of metabolic degradation pathways and avoidance of GIT associated side [15]. However, a key limitation in TDD applications is the protective barrier properties of the stratum corneum (SC) layer of the skin [16]. TDD systems are therefore carefully designed to reversibly interact with and alter the barrier properties of the SC layer [16] via mechanisms such as reversible disruption and fluidisation which results in an increase in the dermal uptake of the drug. [17-19]. Fatty acids (FAs) and their esters have evolved as the most attractive class of chemical permeation enhancers (CPEs) for the effective transdermal delivery of drugs [20, 19, 21, 10]. More recently scientists have developed strategies to enhance TDD through the use of nanomaterials, specifically lipid based nanoparticle formulations such as nano or microemulsions and nanoemulgels that could enhance the delivery of drugs across skin [22-26].

Nanoemulsions (NEs) have a size range of 20-500 nm and have been reported for the delivery of drugs via the transdermal route [27]. NE are composed an oil and water phase stabilized by an interfacial film of surfactants usually in combination with a co-surfactant [28, 29]. NEs offer numerous advantages such as lower formulation costs, thermodynamic stability, higher storage stability and the absence of organic solvents [30]. NEs have been advocated for the efficient delivery of drugs through the skin as the nano sized emulsion with large surface areas allows rapid penetration through the pores of the skin to reach the systemic circulation [28, 31]. Oil in water

(o/w) NEs have an oil core, with the interface stabilized by an appropriate combination of surfactants/co-surfactants [28, 29]. The oil phase of an o/w NE is a critically important due to its ability to solubilize lipophilic drug molecules and increase absorption through lipid membranes [31]. It has been reported that conventional transdermal formulations such as gels and emulsions have lower drug permeation than transdermal nanoemulsions [30, 28]. However, due to the inconvenience of its low viscosity, the clinical application of NE is restricted [32]. Literature reports that hydrogel thickened NEs serve as effective TDD systems, with improved rheological characteristics as well as enhanced permeability and long term stability [32]. When NEs are incorporated into a hydrogel matrix, it is termed nanoemulgels (NEGs). NEGs have further improved the transdermal applicability as they have the advantages of both NEs and gels and have therefore shown the ability to achieve superior permeation enhancement across the skin [33, 34]. Although these advantages have resulted in the recent development of NEGs for TDD of drugs from several classes [35-38, 34, 39, 40], there have been no reports on the investigation of TNF NEs or NEGs for transdermal application.

Although the literature is replete with preliminary studies on novel materials that improve the permeation of drug solutions via the transdermal route [41, 42, 18, 19, 43, 17], there is limited data on their translation into a dosage form or delivery systems for TDD, which is essential to realise their pharmaceutical applications. The identification of newly reported materials that can act as efficient oily cores of NEs can contribute to optimizing TDD via NEG systems. In a recent report, we synthesized and characterized a novel series of FA ester derivatives, dendritic heterolipids, that functioned as effective transdermal permeation enhancers of TNF drug solution and displayed greater permeation enhancement efficacies when compared to their respective parent FAs [10]. The optimal enhancer identified in that reported series was 2% w/w LLA1E, which displayed the greatest promise to be incorporated into a future TDD system [10]. Its applicability in an actual TDD formulation for drugs, which is essential for eventual patient administration, remain to be explored. In this study we now propose the use of LLA1E as the oily phase in the development of a NE for the TDD of TNF. In addition to this study reporting for the first time, the use of this novel heterolipid as an excipient in a nano delivery system, it also reports for the first time a NE and NEG system of TNF. Therefore, the aim of the present investigation was to identify the potential of LLA1E as an oily phase excipient in the preparation of an efficient TNF NEG in order to provide

a future TDD formulation for effective management of HIV/AIDS without compromising patient compliance.

2. Materials and methods

2.1 Materials

TNF was procured from Sinobright Pharmaceutical Co. Ltd., (China). Solutol HS 15®, PEG 400, triethylamine and hydroxypropyl methyl cellulose (HPMC) were purchased from Sigma-Aldrich Co. Ltd., (USA). Milli-Q water purification system (Millipore corp., USA) was used to obtain purified water for the preparation of formulations. The materials, methods and procedure for the synthesis of LLA1E are provided in the supporting information.

2.2 Methods

2.2.1 Construction of pseudo-ternary phase diagram

Pseudo-ternary phase diagrams were constructed by titrating homogenous mixtures of LLA1E as the oily phase, Solutol HS 15® and PEG 400 as the surfactant/co-surfactant mixture (S_{mix}) with water at room temperature. The phase diagrams were constructed for 1:1 (Km = 1) and 2:1 (Km = 2) ratio of surfactant: co-surfactant. For each phase diagram mixtures of LLA1E, surfactant and co-surfactant were prepared from weight ratios of 9:1 to 1:9. The LLA1E and S_{mix} mixtures were titrated drop-wise with water under vortex. After equilibration for 30 min at the room temperature, the systems were visually characterized. Single-phase, transparent mixtures were chosen as nanoemulsions with no attempts to completely identify the other regions of the phase diagram. Based on the findings of the phase diagrams, appropriate quantities of LLA1E and S_{mix} was selected and used for the preparation of NEs [44].

2.2.2 Formulation of NEs

NEs were prepared by an ultra-sonication method [45]. In short, the oily phase (LLA1E) and the S_{mix} were mixed, the required quantity of milli-Q water was added and sonicated [probeOmni Sonic Ruptor 400 Ultrasonic Homogenizer (Kennesaw, USA)] at 30% amplitude for 10 min (20 °C) to form the blank NE. The emulsification was conducted in a temperature controlled ice bath to regulate the heat generated in this high energy process. The drug loaded NE was prepared following the above mentioned method, with the exception of the addition of 0.25% TNF to the

oily phase prior to mixing with the S_{mix}. No phase change was noted after addition of the drug or after equilibration.

2.2.3 Incorporation efficiency

The TNEs were centrifuged at 4500 rpm and 4 °C for 40 min using a Hermle Z326k centrifuge (Germany) in order to separate the un-incorporated drug. The supernatant was analyzed at a λ_{max} of 262 nm using UV Spectrophotometer 1650 (Shimadzu, Japan) to determine the amount of un-incorporated drug (W_1) from total amount of drug used (W_2). Total drug content was estimated by dissolving the lipid emulsion in methanol. The percentage incorporation efficiency (% IE) was calculated using the following equation [46]:

$$\% IE = \frac{W2 - W1}{W2} X\ 100$$

2.2.4 Formulation of TNF nanoemulgel (TNEG)

To prepare the TNEG, 400mg (4% w/w) HPMC was added to the prepared TNF loaded NE under magnetic stirring for 24 h. The pH was adjusted to 7.4 using triethylamine. The physico-chemical characterization of the TNF NE and the TNEG was characterized as follows:

2.2.5 Characterization of TNF NE (TNE) and TNEG

The TNE and TNEG were characterized for mean globule diameter (MGD), polydispersity index (PDI) and zeta potential (ZP) using a Zetasizer Nano ZS90 (Malvern Instruments Ltd., Worcestershire, UK) after suitably diluting with milli-Q water. All measurements were performed in triplicate at 25 °C.

2.2.6 Rheology

A MCR 302 Rheometer® (Anton Parr, Graz, Austria) with a parallel plate (PP50) applying the shear rate in a linear manner from 0 to 100 s^{-1} at 25 °C was used to measure the viscosity of the prepared TNE and TNEG. The apparent viscosity was calculated at a shear rate 100 s^{-1}. n = 3 [45].

2.2.7 Morphology

Surface morphology of TNE was evaluated using a transmission electron microscope (TEM) (Jeol, JEM-1010, Tokyo, Japan). TEM images were obtained by diluting a drop of TNE and positioned on 3 mM forman coated copper grid and allowed to dry, it was subsequently stained with 1% w/v phosphotungstic acid and captured.

2.2.8 Ex vivo transdermal permeation studies

Ethical clearance was obtained from the University of KwaZulu-Natal (UKZN) Animal Research Ethics Committee in 2014 (AREC/054/14/Animal), and renewed (AREC/015/15/Animal). Wistar rats (Male) were acquired from the Biomedical Resource Unit (BRU), (UKZN). The experimental animals received water and food ad libitum and were maintained under controlled light, temperature and humidity in the animal housing facility at the BRU of UKZN. The euthanized animals were shaved and the abdominal skin was removed with surgical scissors. The skin was stored (-20 ˚C) and utilised within three months [12, 10].

Ex vivo permeation studies were conducted using vertical Franz diffusion cells (PermeGear, Inc., Bethlehem, USA) at 37 ± 1 ^{o}C [47, 10]. Prior to the experiments, skin samples were defrosted in PBS (pH 7.4) at room temperature. The receptor compartment was filled with PBS of pH 7.4 (25 ml) andthe skin was mounted on Franz diffusion cells with the SC facing the donor compartment. The skin samples were equilibrated with PBS (pH 7.4) at 37 °C for 30 minutes before conducting the permeation experiments.

The TNF gel or the TNEG (2 ml) was applied on the SC and receptor compartment was stirred with a teflon-coated magnetic stirrer bar at 37 ^{o}C. The receptor compartment was sampled and replenished with equal volumes of fresh PBS at predetermined intervals during the 6 h permeation study. The drug was quantified at a λ_{max} of 262 nm using UV Spectrophotometer 1650 (Shimadzu, Japan). The experiments were performed with a minimum of n = 6 [48, 10]. The gel prepared using only TNF and HPMC served as a control.

The cumulative amount of TNF permeated per unit surface area was plotted against time. Linear regression analysis was used to determine the steady state flux (J_{ss}) from the linear part of the permeability curve (Microsoft Excel 2010, USA). The permeability coefficient (P) was calculated as follows [10, 49]:

$P = (dQ/dT)/A \times Cd = Jss/Cd$

Where 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 [10]:

ER = P of the drug in the presence of enhancer/P of the drug in the absence of enhancer

2.2.9 Transepithelial electrical resistance (TEER) studies

A millicell ERS meter (Millipore USA) connected to a pair of chopstick electrodes (STX01) was used to measure changes in the integrity of skin during the permeation study. The TEER recordings before the addition of the formulation served as the original integrity values. TEER recordings at the end of the 6 h permeation would establish if the values changed from the original measured skin integrity during the permeation study. Two hours after removal of the formulation, further TEER measurements were taken to determine whether there was any rebound by the skin's barrier properties [50, 10].

2.2.10 Histological evaluation

Control and treated skin samples were assessed histologically to assess any tissue damage or morphological changes due to exposure to the formulations. Freshly excised skin was fixed in 10% buffered formalin and served as the control. The treated skin (TNF gel or TNEG) were removed from the Franz diffusion cells and fixed in 10% buffered formalin directly after the 6 h permeation study. After 7 days, skin samples were removed from the fixative and dehydrated in a 50 to 96% ethanol gradient. The samples were then embedded in paraffin wax and sectioned using a microtome (Leica RM2235, Leica Biosystems Germany). The skin sections were collected on slides, dried and stained with hematoxylin and eosin (H&E) for light microscopy (LM) evaluations. Sections were examined with Leica slide scanner (SCN 400, Leica Biosystems Germany). All experiments were performed in triplicate [50, 49, 51, 10].

2.2.11 Stability

The stability of the TNEG was evaluated at room temperature (RT) and at 4 °C over a period of 3 months. Physical appearance, MGD, PDI and ZP were used as assessment parameters for stability.

2.2.12 Statistical Analysis

The results are expressed as mean ± standard deviation (SD). Data was statistically analysed using GraphPad Prism® (Graph Pad Software Inc., Version 5., USA) by either one-way analysis of variance (ANOVA) followed by the non-parametric Kruskal-Wallis test or Dunn's multiple comparison test or *t* tests were performed followed by the non-parametric Mann-Whitney test. A *p* value of less than 0.05 was considered statistically significant.

3. Results and Discussion

3.1 Pseudo-ternary phase diagrams

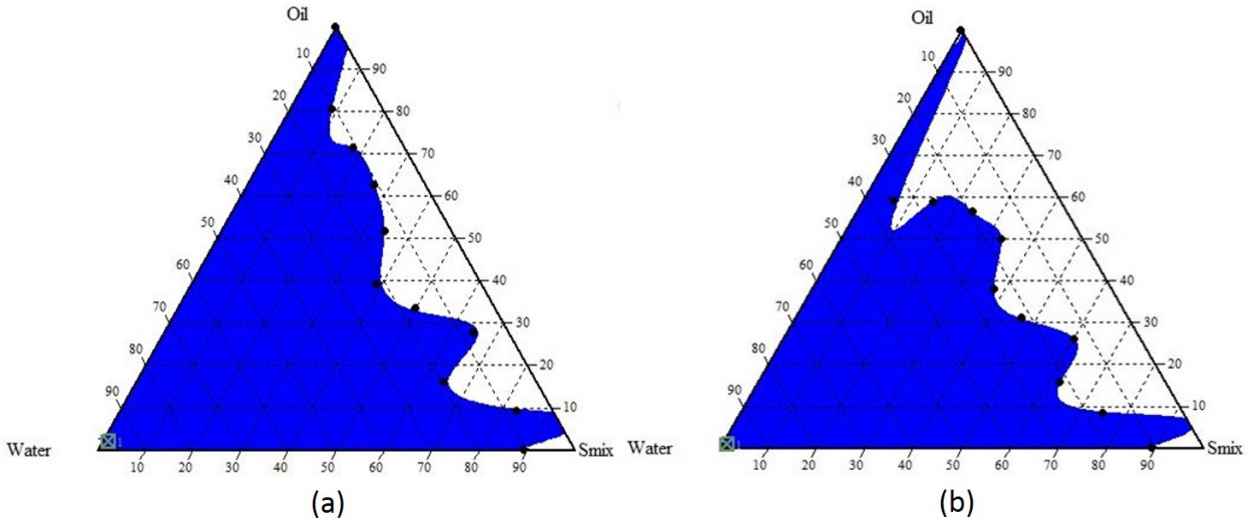

Fig. 1 Pseudoternary phase diagrams depicting the area of nanoemulsion existence. (a) K_m = 1, (b) K_m = 2. Oily phase = LLA1E, surfactant = Solutol HS 15®, co-surfactant = PEG 400, aqueous phase = milli-Q water.

Solutol HS 15® was chosen as a surfactant due its physiological compatibility and ability to decrease globule size of o/w NEs [52, 44]. PEG 400 was selected as a co-surfactant considering its low toxicity, miscibility with aqueous fluids at all concentrations and ability to dissolve several poorly aqueous soluble compounds [53]. In order to determine the nanoemulsion regions pseudo-ternary phase diagrams were constructed [44]. The shaded area of the graph represents the area of nanoemulsion existence (Fig. 1). The results showed that the 1:1 (Km = 1) S_{mix} displayed a larger region of nanoemulsion existence as compared to the 2:1 (Km = 2) S_{mix}. This demonstrated that

Solutol HS 15®/PEG 400 at 1:1 ratio was able to nanoemulsify LLA1E to a greater extent and was therefore selected for use in the NE formulation.

3.2 Formulation of NEs

In the previous report on LLA1E's transdermal permeation enhancement abilities, a concentration effect study revealed that LLA1E at a concentration of 2% w/w displayed the highest ER of TNF [10]. It was hypothesized that the incorporation of LLA1E at the same concentration would ensure that besides functioning as an oily phase of the NE it may also function as an effective transdermal permeation enhancer. Therefore, to maintain the ideal concentration of LLA1E (2% w/w) in the nano formulation, NEs were prepared with 2% w/w LLA1E as the oily phase. In order to establish the optimal concentration of S_{mix} (K_m = 1) to form NEs with low MGD, PDI and sufficient stearic stability different concentrations of S_{mix} with 2 % w/w LLA1E were screened (Table 1).

Table 1. MGD, PDI and ZP achieved using different ratios of LLA1E: S_{mix}

LLA1E:S_{mix} (K_m = 1)	**MGD (nm)**	**PDI**	**ZP (mV)**
1:1	131.5 ± 2.00	0.237 ± 0.02	-8.43 ± 0.52
1:2	116.5 ± 0.47	0.158 ± 0.009	-7.49 ± 1.59
1:3	106.83 ± 1.56	0.335 ± 0.029	-1.33 ± 0.16

The results displayed that all the ratios of LLA1E: S_{mix} (K_m = 1) formed NEs after sonication for 10 min at 30% amplitude (Table 1). The results showed that as the concentration of S_{mix} (K_m = 1) was increased there was a decrease in the MGD and an increase in the ZP (Table 1). Although the NEs at 1:3 ratio had a lower MGD, its PDI was more than 2-fold higher than the NEs at 1:2 ratio (Table 1). Therefore, NEs with 1:2 LLA1E to Smix (Km = 1) ratio which exhibited MGD close to the 100 nm range, with a good PDI and acceptable ZP (Table 1) were used in further studies [31].

Table 2. MGD, PDI and ZP of the prepared nano formulations (n = 3). *****Indicates statistically significant difference i.e. $p < 0.05$ (compared to blank NE).

Formulation	**MGD (nm)**	**PDI**	**ZP (mV)**
Blank NE	116.5 ± 0.47	0.158 ± 0.009	-7.49 ± 1.59
TNE	129.06 ± 3.35	0.192 ± 0.038	*20.9 ± 2.02
TNEG	136.13 ± 5.21	0.182 ± 0.020	-20.9 ± 2.08

After studying effect of drug loading on the characteristics of NEs, 0.25% w/w TNF was selected as an optimum concentration. The addition of 0.25% w/w TNF to the NE formulation resulted in a slight increase in the MGD and PDI whereas ZP value changed significantly (Table 2). The change in ZP could be attributed to un-entrapped TNF which bears 4-herocyclic and one primary nitrogen in its structure. The results indicated that the increase in MGD after the addition of 0.25% w/w TNF was not statistically significant ($p > 0.05$) (Table 2). The TNF loaded particle size (MGD) obtained in this study is similar to or better than particle sizes achieved by other studies that incorporated TNF in liposomes and polymeric nanoparticles respectively [54, 55]. The % IE of TNF in the TNE was calculated at 91.94 ± 0.84 % which is similar to other reports for % IE in NEs, as well as for the % IE of TNF in other nano formulations [54, 56, 57]. The ability of LLA1E to form NEs with good MGD, PDI and acceptable ZP in combination with the high drug loading of this o/w NE system is evidence of its potential to function as an effective oily phase. The characteristics of the formulated TNEs in this study are within the range of literature reported NEs for TDD of several classes of drugs [58, 57, 59, 60]. In order to thicken the TNE formulation to allow ease of topical application for transdermal permeation studies, the TNE formulation was converted into a hydrogel. The addition of 4% w/w HPMC to the TNE resulted in the formation of a hydrogel (TNEG) that contained TNE with a MGD of 136.13 ± 5.21 nm (Table 2). The shift in ZP value from positive to negative in TNEG could be attributed to the addition of HPMC to form the TNEG.

3.3 Rheology

Both the TNE and the TNEG exhibited Newtonian flow characteristics as the viscosity of the TNE and the TNEG remained constant with an increase in the rate of shear. The viscosity of the TNE

and the TNEG was calculated at 1.24 ± 0.01 and 147.67 ± 32.64 respectively, at the shear rate 100 s^{-1}. This shows that there was a 119-fold increase in the viscosity when TNE was converted to TNEG. The viscosity of the TNEG achieved in this study is suitable for a hydrogel thickened NEs intended for transdermal application [32].

3.4 Morphology

TEM images revealed that the TNE droplets were spherical in shape with MGD in the range of 86.51 to 137.83 nm (Fig. 2). The size range of TNE globules obtained by TEM analysis correlate with the MGD obtained using a dynamic light scattering with the zetasizer.

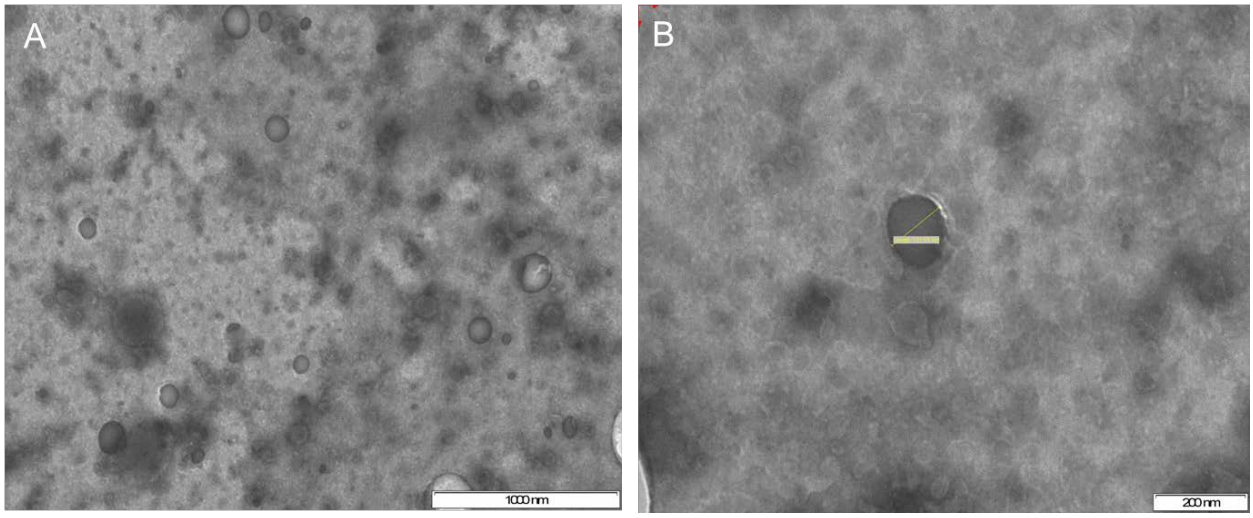

Fig. 2 TEM images of the optimized TNEs. A – Population of lipid globules; B – individual globule measuring 131.55 nm

3.5 Ex vivo permeation

Table 3. TNEG permeability parameters across rat skin. *****Indicates statistically significant difference i.e. $p < 0.05$ [compared to TNF gel (control)].

Treatment	**Amount permeated (µg.cm $^{-2}$)**	**Jss (flux) (µg.cm $^{-2}$.h^{-1})**	**Permeability (Px10^{-2})**	**ER**
TNF gel (Control)	50.88 ± 7.18	8.41 ± 1.17	0.336 ± 0.04	**1**
TNEG	1866.54 ± 108.62	*****333.49 ± 18.25	13.33 ± 0.73	**39.65**

The *ex vivo* permeation studies revealed that the control gel was able to permeate TNF through the skin. TNF from control gel permeated the skin with a cumulative amount of 50.88 ± 7.18 µg/cm^2 and steady state flux of 8.41 ± 1.17 µg/cm^2 h (Table 3). The reported IC_{50} value for TNF is between 0.55–2.2 µM (158–632 µg) [61, 10]. Therefore, the quantity of TNF permeated from TNF control gel was significantly lower than the required therapeutic dose of TNF. Comparatively, the TNEG was able to significantly ($p < 0.05$) enhance the permeability of TNF by 39.65-fold and deliver therapeutically relevant doses of TNF via the skin (Table 3). The TNEG displayed a permeated cumulative amount of 1866.54 ± 108.62 µg/cm^2 and steady state flux of 333.49 ± 18.25 µg/cm^2 h (Table 3). These permeation enhancement values are significantly higher than that achieved by 2% w/w LLA1E in the previous report [10]. In that study 2% LLA1E was able to achieve a cumulative amount of 1214.88 ± 87.33 µg/cm^2 and steady state flux of 192.37 ± 17.58 µg/cm^2 h with an ER of 6.11 [10]. Interestingly, the loading dose of TNF in that study was 2% w/w; however, in the present report TNF loading was 0.25% w/w which signifies an 8-fold lower TNF loading dose in the current study. These findings therefore display the superiority of the 0.25 % TNEG as it was able to achieve a significantly higher cumulative amount than the LLA1E TNF gel, however at a considerably lower loading dose. The use of NEs is proposed as a promising strategy for enhancing TDD without the use of CPEs, as the constituents such as the oily phase of the NEs can act as permeation enhancers themselves [39, 62]. Therefore, in addition to the permeation enhancement advantages that are characteristic of the NEG, the use of the reported superior transdermal permeation enhancer LLA1E as the oily phase could have added to the significantly higher ER obtained by the TNEG.

3.6 TEER

A change in the skin's integrity after exposure to the TNEG can be determined using TEER studies. The changes in the TEER recordings are suggestive of associated changes in the barrier properties of the skin [10]. In order to assess the influence of drug formulations on the skins integrity TEER measurements are recorded prior to, and after permeation experiments. The differences in these recorded values enable the quantification of the change in skin integrity after exposure to the drug formulation [10, 47, 63]. A reduction in the TEER values can be correlated to a decline in the barrier properties of the skin [10].

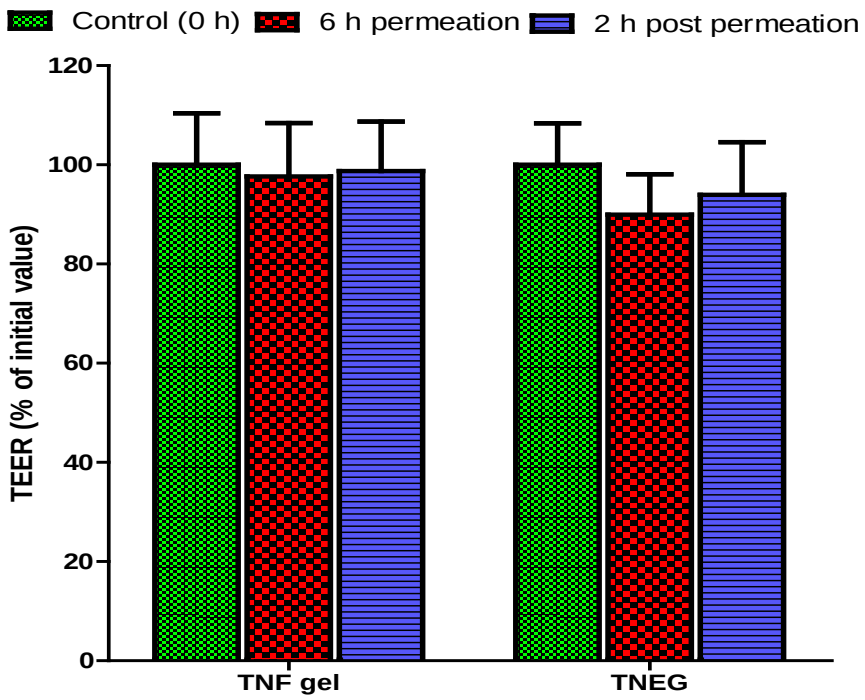

Fig. 3 TEER measurements (%) recorded after permeation studies with either TNF gel or TNEG (n = 6).

TEER recordings confirmed that the integrities of skin samples used in both experiments (TNF gel and TNEG) were similar in terms of their 0 h TEER measurements. The skin intended for use in the TNF gel and TNEG studies presented a 0 h recording of 444.00 ± 46.18 Ω /cm^2 and 423.33 ± 35.50 Ω /cm^2 respectively (Fig. 3). These 0 h values were used as the 100% TEER values for the respective experiments and were compared to the 6 h TEER recordings measured at the end of the permeation experiment to establish the change brought on by the drug formulation. The 2 h post permeation TEER measurements were also compared to the 0 h recordings to determine if there was any return towards the original recorded skin integrity. The TEER values recorded at the end of the permeation studies for the TNF gel and TNEG were 433.67 ± 47.72 Ω /cm^2 and 381.67 ± 34.56 Ω /cm^2 respectively (Fig. 3). This signified a 2.32% and 9.84 % decrease in the original measured (0 h) TEER values of the TNF gel and TNEG skin samples respectively (Fig. 3) after 6 h. Comparatively, 2% w/w LLA1E in the previous report was able to reduce the TEER values by 8.3 % when it was incorporated in a gel for transdermal delivery of TNF [10]. The assessments in the present study showed that the TNEG decreases the skins integrity 4.24-fold more than the TNF gel. The present study revealed that 2% w/w LLA1E incorporated as the oily phase in the TNEG

displays a greater decrease in the skins integrity. These findings correlate with the *ex vivo* transdermal permeation studies, which showed that the TNEG is able to achieve a significantly higher permeation of TNF compared to the TNF gel (Table 3). Interestingly, 2 h after the removal of the drug formulations there was increase in the TEER recordings from both experiments (Fig. 3). The 2 h post permeation TEER value of the TNF gel and TNEG were recorded as 435.33 ± 47.88 Ω /cm^2 and 397.67 ± 45.08 Ω /cm^2 respectively. When compared to the differences between the 0 h and 6 h TEER measurements, the 2 h post permeation TEER values showed a 16.12 % and 38.4 % recovery in the resistance values of the skin after removal of the TNF gel and TNEG respectively (Fig. 3). This indicates a return towards the original measured resistance of the skin (Fig. 3). These observations show that both drug formulations have reversible effects on the integrity of the skin, which is one of the key attributes of ideal permeation enhancers.

3.7 Histology

Histomorphological investigations allow for the assessment of morphological changes due to the exposure of the skin to the drug formulations. Observations made in the individual epithelial layers would enable evaluations of any harmful effects of the drug formulations on the treated skin samples. Histomorphological evaluation of the SC in particular would provide valuable information in determining the drug formulation's ability to decrease the integrity of the epidermis, as this layer provides the major barrier to the movement of drugs across the epithelium of skin [64, 19, 17].

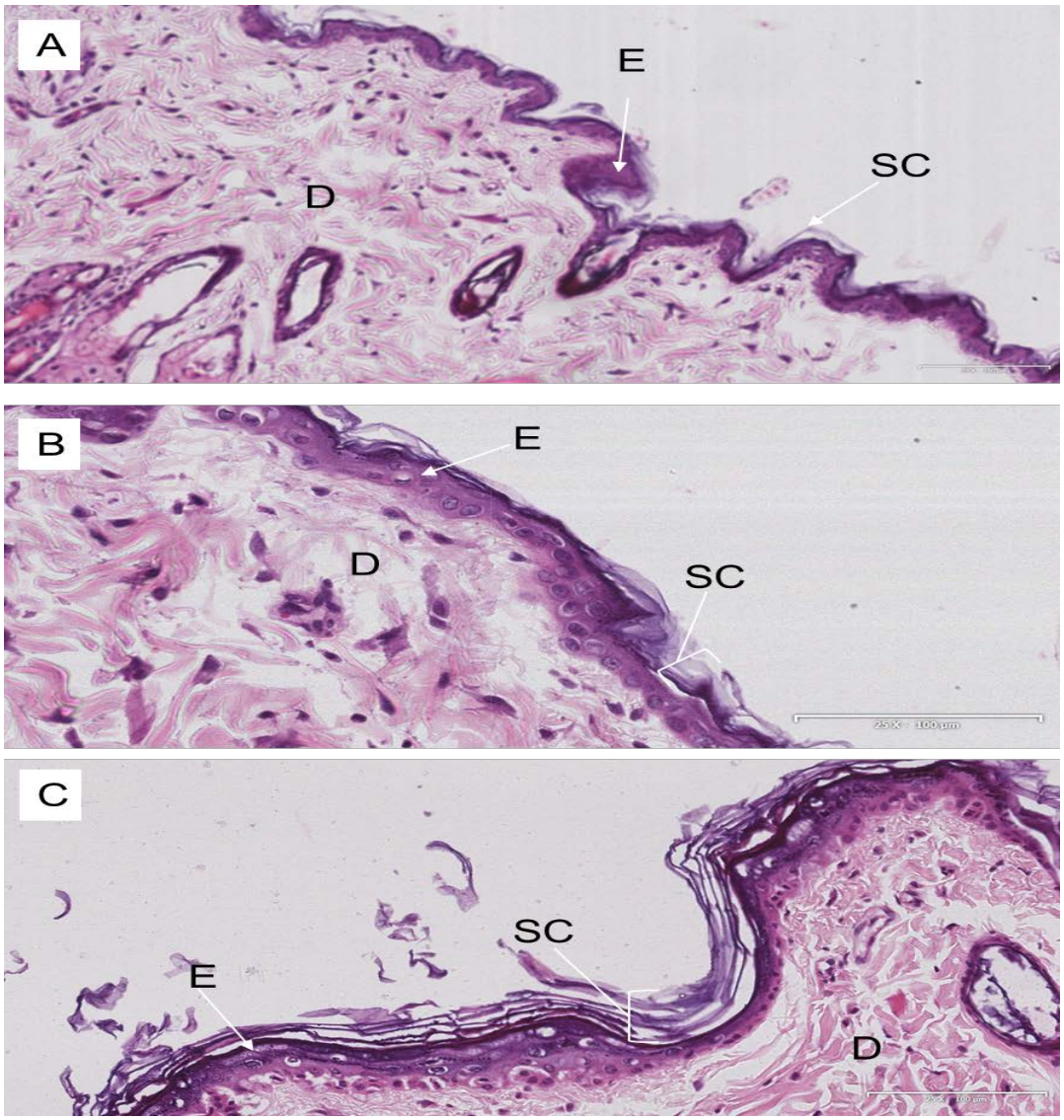

Fig. 4 LM images of H&E stained samples of the control and the treated skin; (A) untreated skin (control), (B) samples exposed to TNF gel, (C) skin exposed to TNEG (D: dermis; E: epidermis; SC: stratum corneum).

The control skin sample (Fig. 4A) displayed normal thin skin morphology which is characteristic of skin from this region [64] and clearly showed complete and undamaged SC. The skin treated with TNF gel (Fig. 4B) showed histomorphological similarities to that of the control skin (Fig. 4A) as the SC layer in the TNF gel treated skin (Fig. 4B) also appeared intact and the underlying layers of the epidermis didn't present any observable signs of morphological changes. The TNEG treated sample (Fig 4C) displayed clearly evident changes in the appearance and the morphology

of the SC layer. The TNEG treatment showed an increase in the thickness of the SC layer which could be a result of the disruption/fluidisation of lipids found in this layer (Fig. 7C). This disruption/fluidisation causes the SC to appear loosely packed [65] and therefore appears thicker than the SC layer of the control skin (Fig. 4A). There were large intercellular spaces in SC layer of the TNEG treated samples (Fig. 4C). The disruption/fluidisation caused by the exposure of the TNEG in the SC layer would lead to a consequent decline in the barrier function presented by normal skin, which would encourage the transport of drugs across the epidermis into the dermal layers of the skin. The histomorphological observations made in the TNEG treated skin correlate directly with the findings of the *ex vivo* permeation study which displayed a significantly higher permeation of TNF from the TNEG (Table 3). While there were significant morphological changes in the SC of the TNEG treated skin (Fig. 4C), the underlying epidermal layers showed no signs of damage or change from the normal morphology of the control image (Fig 4A). The regenerative basal layer of the TNEG treated samples (Fig. 4C) appeared normal and unharmed by the drug formulation. This study indicated that the TNEG exposure would only have temporary effects on the epidermal layer of the skin.

3.8 Stability

The stability of the TNEG was assessed over a 90-day period at room temperature and 4 °C. Student *t*-tests, followed by Mann-Whitney tests revealed that over the 90-day period there were no statistically significant changes in the characteristics (MGD, PDI and ZP) of the NE at both the storage conditions as compared to their respective controls (Table 4). The TNEGs showed no signs of phase separation for the duration of the stability studies.

Table 4. Effect of storage conditions and time on MGD, PDI and ZP of TNEG (n=3).

Time (Days)	**MGD (nm)**		**PDI**		**ZP**	
	4º C	**R.T**	**4º C**	**R.T**	**4º C**	**R.T**
0	136.13 ± 5.21	136.13 ± 5.21	0.182 ± 0.02	0.182 ± 0.02	-20.9 ± 2.08	-20.9 ± 2.08
30	141.60 ± 3.48	148.93 ± 6.01	0.210 ± 0.015	0.221 ± 0.013	-23.53 ± 2.68	-25.7 ± 0.50
60	145.30 ± 2.45	155.03 ± 2.77	0.222 ± 0.022	0.231 ± 0.016	-24.77 ± 1.36	-26.77 ± 0.42
90	146.67 ± 2.61	161.53 ± 2.31	0.241 ± 0.041	0.242 ± 0.014	-24.97 ± 2.44	-27.67 ± 1.66

3.9 Conclusions

Transdermal drug delivery is hampered by the barrier properties of the skin. Innovative strategies are required to optimize the applicability of this route and increase the range of drugs that can be effectively delivered through the skin. In this study, LLA1E which is a recently reported novel transdermal permeation enhancer was successfully employed as an oily phase excipient, in the formulation of TNEGs. The formulated TNEG with a low drug loading of 0.25% w/w displayed high incorporation efficiency and superior *ex vivo* transdermal permeation enhancement of TNF. This is the first study to report the formulation of a TNF NE and NEG system, as well as to successfully deliver therapeutically relevant amounts of TNF via the transdermal route using a nano drug delivery system. The TEER and histological studies revealed that the effects of the TNEG on the skin were temporary. It can therefore be concluded that LLA1E is a promising oily phase excipient in the formulation development of lipid based transdermal nano formulations and the proposed TNEG in this paper has the potential to revolutionize HIV/AIDS treatment.

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Supplementary Material

Nanoemulgel using a bicephalous heteolipid as a novel approach to enhance transdermal permeation of tenofovir

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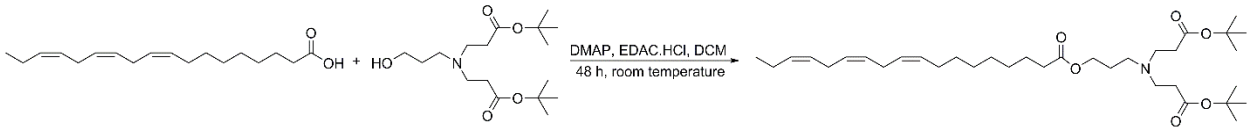
Materials and Method for the synthesis of LLA1E

Materials

Linolenic Acid, N-(3-dimethylaminopropyl) - N-ethylcarbodiimide hydrochloride (EDAC.HCl) and *p*-dimethylaminopyridine (DMAP) was 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 was purchased from Sigma-Aldrich (Steinheim, Germany). All other reagents and solvents used were of analytical grade and were procured from Merck Chemicals.

Method

Synthesis of dendritic ester of linolenic acid (LLA) (Scheme 1)



To a stirred mixture of dendron 1 (1.2 equiv.), synthesized using a previously reported method [1] and DMAP (0.75 equiv.) in DCM was added EDAC.HCl (1.5 equiv.) followed by LLA (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) [2].

References

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CHAPTER 6
CONCLUSION

CHAPTER 6
CONCLUSION

6.1 General conclusions

Due to its increasing prevalence, HIV and AIDS is one of the most serious public health diseases worldwide. Although TNF is considered the most successful ARV to treat and prevent HIV and AIDS, its current oral administration has numerous limitations which negatively impact on the effective treatment/management of the disease. The current HIV and AIDS crisis coupled with limitations of oral TNF therapy therefore drive the need for identifying innovative drug delivery approaches. Drug delivery scientists have thus explored the use of novel drug delivery systems and alternate routes of drug administration for TNF. Although TDD offers an attractive alternative for the systemic delivery of drugs, this route has not been explored for the delivery of TNF. Despite the advantages of TDD, the barrier properties of the skin limit the applicability of this route. The use of FAs as transdermal CPEs has been advocated to efficiently decrease the barrier properties of the skin. FA esters have shown greater promise as transdermal permeation enhancers than their respective parent material. Therefore, the modification of existing FA CPEs to produce novel FA derivatives that display superior performance is highly desirable. Another strategy that has emerged as an effective means of promoting TDD is the use of biocompatible nanomaterials. NEGs have unique characteristics that promote enhanced drug permeation via the transdermal route and have therefore been used for the TDD of various classes of drugs. However, to date there is no report on the formulation of NEG for transdermal delivery of TNF.

The broad aim of this study was to explore the potential of lipid-based strategies for enhancing transdermal permeation of TNF. The specific research aims of this study were therefore: (1) to synthesize and characterize novel biocompatible dendritic ester derivatives of UFAs and explore their potential as promising permeation enhancers for the transdermal delivery of TNF. (2) To evaluate the novel application of UFA esters of cholesterol as promising transdermal permeation enhancers using TNF as a model drug. (3) To synthesize and characterize novel biocompatible mono, di and tri-ester derivatives of FAs and explore their potential as promising transdermal permeation enhancers using TNF as a model drug. (4) To explore the potential of novel linolenic

acid based heterolipid, LLA1E (a novel transdermal permeation enhancer), as an oily phase in the development of a nanoemulgel for the transdermal drug delivery of TNF.

In order to achieve aim 1, the objectives were to:

i) Synthesize novel ester dendritic derivatives of palmitoleic (PA), linoleic (LA), linolenic (LLA) and arachidonic acid (AA) and characterize the derivatives using FTIR, NMR (^{1}H and ^{13}C) and HRMS techniques.

ii) Determine the lipophilicity and pH responsiveness as well as the *in vitro* biosafety of the novel ester derivatives.

iii) Explore the *in vitro* potential of the novel UFA ester derivatives as transdermal permeation enhancers for TNF and assess the effects of the enhancer treatment on the skin using transepithelial electrical resistance as well as light and transmission electron microscopy.

To achieve aim 2, the objectives were to:

i) Synthesize cholesterol ester derivatives of oleic (OA), linoleic (LA), linolenic (LLA), Lauric (LuA), Palmitic (PA) and stearic acid (SA) and characterize the derivatives using FTIR, NMR (^{1}H and ^{13}C) techniques as well as determining their lipophilicity.

ii) Explore the *in vitro* potential of the cholesterol ester derivatives as transdermal permeation enhancers for TNF and assess the effects of the enhancer treatment on the skin using transepithelial electrical resistance and light microscopy.

To achieve aim 3, the objectives were to:

i) Synthesize novel mono, di and tri-ester derivatives of FAs bearing β-alanine *t*-butyl ester head group using oleic (OA), linoleic (LA), linolenic (LLA) and stearic acid (SA) and characterize the derivatives using FTIR, NMR (^{1}H and ^{13}C) and HRMS techniques.

ii) Determine the lipophilicity and the *in vitro* biosafety of the novel ester derivatives.

iii) Explore the *in vitro* potential of the novel ester derivatives as transdermal permeation enhancers for TNF and assess the effects of the enhancer treatment on the skin using transepithelial electrical resistance and light microscopy.

To achieve aim 4, the objectives were to:

i) To determine the optimal ratio of surfactant and co-surfactant (S_{mix}) to oil (LLA1E) by constructing pseudo-ternary phase diagrams.

ii) Evaluate the TNF NE and TNF NEG in terms of mean globule diameter, polydispersity index, zeta potential, viscosity, morphology and stability.

iii) Explore the *ex vivo* potential of the novel TNF nanoemulgel to enhance the transdermal delivery of TNF and assess the effects of the nano formulation on the skin using transepithelial electrical resistance and light microscopy.

The main conclusions generated from the research data are summarised below:

1. Aim 1:
    - Novel ester dendritic derivatives of palmitoleic (PA), linoleic (LA), linolenic (LLA) and arachidonic acid (AA) were successfully synthesized and their physical characteristics were confirmed using FTIR, NMR (^{1}H and ^{13}C) and HRMS techniques.
    - All the derivatives synthesized in this study had a higher lipophilicity as compared to their respective parent UFA. This is a desirable property as the greater the lipophilic nature of an enhancer, the greater the permeability across the skin. The MTT assay results showed that none of the synthesized UFA ester dendritic derivatives were toxic against the HeLa cell line. The percentage cell viability between 85 to 95% for all derivatives across the concentration ranges studied with no dose dependent trends observed. These findings therefore suggest that the use of the synthesized UFA ester dendritic derivatives in biological studies would be safe. Further, all the synthesized UFA ester dendritic derivatives consist of tertiary nitrogen in their structure and their potential to show pH dependency were proven experimentally. This pH dependent property may attract attention, especially in pH based drug targeting. It therefore contributes to widening the pool of novel biocompatible excipients for drug delivery applications in addition to their use as transdermal chemical permeation enhancers.
    - All synthesized UFA ester dendritic 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 TEER evaluations. Histomorphological evaluations suggested that TNF used the transcellular and intercellular route of transport across the epidermis. There was no significant loss in the integrity of 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.

2. Aim 2:
   - Cholesterol ester derivatives of oleic (OA), linoleic (LA), linolenic (LLA), Lauric (LuA), Palmitic (PA) and stearic acid (SA) were successfully synthesized and their physical characteristics were confirmed using FTIR, NMR (^{1}H and ^{13}C) techniques. All the cholesterol ester derivatives synthesized in this study had a higher lipophilicity as compared to their respective parent FA. The greater the lipophilic nature of the derivatives suggests their ability to encourage greater permeability across the skin.
   - All cholesterol UFA esters at 1% w/w were found to be more effective enhancers when compared to their respective parent FAs and saturated FA counterparts. Cholesteryl linolenate (Chol-LLA) showed the most superior performance (ER = 3.71). Attributes such as increased lipophilicity, optimal carbon chain length and the optimum number of double bonds favor Chol-LLA as the superior permeation enhancer. Additionally, the concentration dependent study showed that the greatest ER for Chol-LLA (5.93) was achieved at a concentration of 2% w/w. The histomorphological and TEER evaluations supported the results of the permeability studies. The histomorphological studies revealed that the disruption and fluidization of lipids in the SC was responsible for the permeability enhancement of TNF by Chol-LLA **(**2% w/w). These findings showed no significant loss in the integrity of the epidermis, with drug and enhancer treatment having temporary effects on the barrier property of the epidermis. Cholesterol UFA esters can therefore be considered as new CPEs for exploitation in topical formulations for various classes of drugs.

3. Aim 3:
    - Novel mono, di and tri-ester derivatives of FAs bearing β-alanine *t*-butyl ester head group using oleic (OA), linoleic (LA), linolenic (LLA) and stearic acid (SA) were successfully synthesized and their physical characteristics were confirmed using FTIR, NMR (^{1}H and ^{13}C) and HRMS techniques.
    - All the derivatives synthesized in this study had a higher lipophilicity as compared to their respective parent FA and the lipophilicity increased as the number of FA chains increased. The MTT assay findings displayed non-toxicity for all of the synthesized derivatives against MCF-7, Hep G2 and A549 cell lines. The percentage cell viability was between 76.55 to 92.17 % for all derivatives across the concentration ranges studied with no dose dependent trends observed.
    - The results showed that all the novel ester derivatives were able to enhance the permeability of TNF at a concentration of 1% w/w; however, there was no direct correlation between FA chain number and the permeation enhancement efficacy of the derivatives. The results revealed that all the FA esters displayed greater permeation enhancement activity as compared to their parent FAs. These findings confirm the superiority of these novel ester derivatives as compared to their respective parent material. The UFAs and their respective derivatives in this study showed a greater increase in ER when compared to the SFA (SA) and its derivatives. A concentration affect study on the optimal enhancer from each series revealed that MOAPE at 1% w/w from the mono ester derivative series achieved the highest permeation enhancement of TNF with an ER of 5.87. MOAPE displayed superior permeation enhancement at all concentrations when compared to OA its parent lipid. Further histomorphological and TEER investigations displayed that 1% w/w MOAPE caused no adverse effects to the skin and the skin's integrity, and that the effects of the enhancer treatment were temporary. These findings show that the reported novel FA esters showing promise as effective permeation enhancers and the novel mono ester derivative of OA (MOAPE) adds to the pool of CPEs available to formulation scientists and can be safely incorporated into TDD systems for several classes of drugs.

4. Aim 4:
    - The results showed that the 1:1 (Km = 1) Smix displayed a larger region of nanoemulsion existence as compared to the 2:1 (Km = 2) Smix. This demonstrated that Solutol HS 15®/PEG 400 at 1:1 ratio was able to nanoemulsify LLA1E to a greater extent and was therefore selected for use in the NE formulation.
    - TNEs had a MGD of 129.06 ± 3.35 nm, PDI of 0.192 ± 0.038 and ZP of 20.9 ± 2.02 mV with an incorporation efficiency of 91.94 ± 0.84%. There was no significant change is these properties after incorporation of TNEs into hydrogel as MGD, PDI and ZP of TNEGs were found to be 136.13 ± 5.21 nm, 0.182 ± 0.020 and -20.9 ± 2.08 mV respectively. The viscosity of the TNE and the TNEG was calculated at 1.24 ± 0.01 and 147.67 ± 32.64 respectively. This shows that there was a 119-fold increase in the viscosity when TNE was converted to TNEG. The viscosity of the TNEG achieved in this study is suitable for a hydrogel thickened NEs intended for transdermal application. The surface morphology of the TNE displayed the spherical shaped NE droplets and short-term stability investigations of the TNEGs performed at 4 °C and room temperature for 3 months confirmed its stability.
    - *Ex vivo* permeation studies showed that the TNEG significantly enhanced the TNF permeation by 39.65-fold, with a cumulative amount of 1866.54 ± 108.62 µg.cm $^{-2}$. Histological and TEER assessments showed no permanent effects on the skin by TNEG. Therefore, this novel TNEG nanosystem has the potential of further translation into clinical trials for optimal treatment alternatives for HIV/AIDs patients.

The findings of this study therefore confirmed the potential of newly developed lipid-based approaches to overcome the barrier properties of the SC and deliver therapeutically relevant doses of TNF across the skin.

6.2 Significance of the findings in the study

All four TNF transdermal permeation enhancement approaches successfully developed in this study are novel and have displayed evidence of overcoming the natural barrier properties of the skin. The advantages of these novel strategies have the potential to allow a larger variety of drugs to be delivered via this route. The novel approaches of this study have successfully

demonstrated that therapeutically relevant doses of TNF can be delivered transdermally and this route therefore functions as an attractive alternative to overcome the limitations of current oral TNF administration. The significance of this study is highlighted below:

New pharmaceutical products:

The developed ester dendritic derivatives of PA, LA, LLA and AA and the mono, di and tri-ester of OA, LA, LLA and SA which bear β-alanine *t*-butyl ester head group as well as the TNF loaded NEG are new pharmaceutical products that can promote and improve innovate drug delivery approaches in industrial pharmaceutical R&D.

Improved patient therapy and disease treatment:

All the developed permeation enhancement approaches have successfully decreased the barrier properties of the skin and therefore significantly enhanced the transdermal delivery of TNF. This should improve patient therapy by minimizing doses, lowering side effects and increasing patient compliance. These approaches should therefore improve patient amenability to this treatment regimen.

Creation of new knowledge to the scientific community:

The research findings of this study have contributed to the dissemination of new scientific knowledge within the domains of pharmaceutical sciences and biomaterials chemistry.

- In the novel dendritic ester derivatives study, new knowledge from the development of superior biocompatible novel CPEs from parent FAs was generated. Additionally, the applicability of TNF for transdermal delivery was established, and its possible route of transport across the skin was elucidated from light and transmission electron microscopy.
- In the UFAs esters of cholesterol study, new knowledge was generated from the identification and combination of existing materials which possess advantageous inherent features which contributed favourably to their novel applicability in TDD approaches.
- In the mono, di and tri-ester FA derivatives study, new knowledge was generated from the design and synthesis of novel biosafe derivatives that targeted the lipid domains of the SC and successfully decreased the barrier properties of the SC.

- In the TNF NEG study, new knowledge was generated from the translation of a previously identified novel CPE into a nano TDD dosage form which was essential in realising its downstream pharmaceutical applications.

Stimulation of new research:

The findings of the various studies and the successful development of numerous superior novel CPEs and TNEG can stimulate innovative approaches in various research areas including the following:

- All the synthesized dendritic ester derivatives consist of tertiary nitrogen in their structure and their potential to show pH dependency has been proven experimentally. This pH dependent property may attract attention, especially in pH based drug targeting. Therefore, in addition to their use as transdermal CPEs, they also contribute to widening the pool of novel biocompatible excipients for drug delivery applications. Due to their pH titratable nature they are potential excipients in the development of pH responsive drug delivery systems for antimicrobial or cancer therapeutic applications.
- The proven efficacy of the UFAs esters of cholesterol as transdermal CPEs in this study demonstrate that these ester hybrids of cholesterol and UFAs can also be considered as multifunctional lipids (as CPEs and membrane stabilizers) in the future formulation development of transdermal liposomes for the treatment of multiple disease conditions.
- Additionally, the safety profile and lipidic nature of the novel FA dendritic ester derivatives and the mono, di and tri-esters of FAs can be further exploited for their potential applications in lipid based colloidal drug delivery systems such as nanoemulsions, self-microemulsifying drug delivery systems and nanostructured lipid carriers for several classes of drugs.
- The formulated TNEG successfully demonstrated the translation of a previously identified CPE into an efficient nano drug delivery system. These positive findings would encourage further research utilising other such reported materials in the design and implementation of effective dosage forms for various biomedical applications.

6.3 Recommendations for future studies

The various lipid based approaches in this study were successful in decreasing the barrier properties of the skin and significantly enhancing the transdermal permeation of TNF, however additional studies are necessary to improve/optimize the formulation of these novel lipids to ensure eventual regulatory approval for patient use.

The following studies are therefore suggested:

- From the dendritic ester derivatives study, apart from LLA1E which was identified as the optimal enhancer in that series, the remaining UFA dendritic ester derivatives i.e LA1E, PA1E and AA1E also showed permeation enhancement potential and were able to considerably increase the amount of TNF delivered across the skin. These biosafe UFA derivatives can therefore be further exploited for their applications in lipid based colloidal drug delivery systems such as nanoemulsions, self-microemulsifying drug delivery systems, nanostructured lipid carriers etc.
- The UFAs esters of cholesterol study confirmed that all of the cholesterol esters serve as transdermal permeation enhancers. In combination with the reported property of cholesterol being an efficient liposome membrane stabilizer, these UFA esters of cholesterol can now be considered as multifunctional lipids (as CPEs and membrane stabilizers) in the future formulation development of transdermal liposomes.
- All of the derivatives in the mono, di and tri-ester derivatives of FAs study showed the potential to enhance the transdermal permeation of TNF. The next phase would be to formulate lipid based colloidal drug delivery systems such as nanoemulsions, self-microemulsifying drug delivery systems, nanostructured lipid carriers etc. using these novel biosafe derivatives.
- The successfully developed NEG system for transdermal TNF delivery can be explored for its *in vivo* transdermal drug delivery potential and to generate the bioavailability and the pharmacokinetic data that will be useful to make further modifications to the formulations.
- Long-term stability studies should be performed on the TNEG system according to ICH guidelines to confirm its stability and shelf life.

- All the novel approaches to enhance the transdermal delivery of TNF were successful using the synthesized lipid derivatives, therefore molecular modelling studies should be performed to understand the interaction between these lipid derivatives and the components of the SC/ skin to establish a the exact mechanism of permeation enhancement.
- A scale up method could be developed for the NEG and TNEG which would facilitate their translation for pharmaceutical industry.

6.4 Conclusion

The findings of this study demonstrate the potential of the various lipid based strategies to overcome the natural barrier properties of the SC and deliver therapeutically relevant doses of TNF via the skin. In addition to this study having contributed significantly to the approaches that reversibly decrease the skin's barrier properties, it has also proven the applicability of TDD as an attractive alternate route for administration of TNF. The significant efficacy of FA ester derivatives in this study has further confirmed the superiority for this class of CPEs. The combination of materials chemistry, nanobiotechnology and formulation science serves an effective platform for developing advancements that address the limitations associated with TDD and oral TNF administration. This multidisciplinary approach has the potential to stimulate innovative strides towards the improvement of HIV and AIDS therapeutics thereby enhancing disease management.

APPENDIX

A1. Proof of manuscript submission to European Journal of Pharmaceutical Sciences

Elsevier Editorial System(tm) for European Journal of Pharmaceutical Sciences
Manuscript Draft

Manuscript Number: EJPS-D-16-01525

Title: Novel mono, di and tri- fatty acid esters bearing secondary amino acid ester head group as transdermal permeation enhancers

Article Type: Research Paper

Keywords: fatty acids; esterification; synthesis; chemical permeation enhancers; tenofovir; transdermal

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Manuscript Region of Origin: SOUTH AFRICA

Abstract: The use of chemical permeation enhancers (CPEs) has widened the pool of drugs that can be delivered via the transdermal route. This study explored the synthesis and characterization of novel mono, di and tri-fatty acid (FA) esters bearing β-alanine t-butyl ester head group as transdermal permeation enhancers using tenofovir (TNF) as a model drug. The synthesized compounds were non-toxic to mammalian cells confirming their safety for pharmaceutical applications. All the synthesized derivatives displayed better transdermal permeation enhancement capabilities as compared to their respective individual FAs. The results showed that there was no correlation between number of aliphatic carbon chains and enhancement ratio (ER). The mono oleate derivative (MOAPE) displayed the greatest ER for TNF (5.87) at 1% w/w. Histological investigations of the rat skin treated with MOAPE revealed fluidization of the stratum corneum. Histological and transepithelial electrical resistance (TEER) studies corroborated with the findings of the in vitro permeation experiments and revealed that there was no significant change to the viable epidermis of the skin after 1% MOAPE exposure. The TEER findings also suggested that the permeation enhancement effects of MOAPE were not permanent and the results indicated a return towards original skin integrity after removal of the enhancer formulation.

B1. International conference poster presentation

Novel Bicephalous Lipids for Transdermal Permeation Enhancement of Tenofovir.

S. Rambharose, R. S. Kalhapure, K. G. Akamanchi and T. Govender

Introduction

Transdermal drug delivery has the potential to improve bioavailability of various drugs, decrease dosages required and reduce drug side effects. However the major shortcoming of drug delivery across the skin is its natural barrier property. 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 stratum corneum. This research 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.

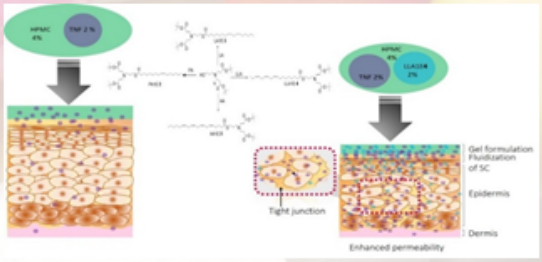


Fig. 1 Illustrates enhanced transdermal drug permeability through the application of novel fatty acid derivatives and drugs within a polymer formulation

Methods

The lipid derivatives were structurally characterised using NMR (^{1}H and ^{13}C) and HRMS techniques. Following the successful synthesis and characterisation of all the FA derivatives (PA1E 2, LA1E 3, LLA1E 4, and AA1E 5), an *in vitro* cell culture system using the MTT assay was employed to determine the biological safety of these derivatives. In vitro permeation studies were conducted at 37± 1 °C using modified vertical Franz type diffusion cells. Transepithelial electrical resistance (TEER) measurements was performed using a Millicell ERS meter. Histological evaluations were performed on freshly harvested excised skin using LM and TEM techniques. Statistical analysis was performed using GraphPad Prism®

Results

The structures of the derivatives were confirmed by FTIR, NMR (^{1}H and ^{13}C) and HRMS. *In vitro* cytotoxicity studies revealed their biocompatibility. Amongst the UFAs, only PA and LLA exhibited transdermal enhancer potential [enhancement ratio (ER) of 1.35 and 2.9 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).

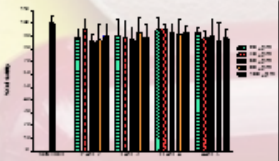


Fig. 2 Cytotoxicity assay displaying percentage cell viability after exposure to various concentrations of the UFA derivatives on HeLa cells.

	Treatment	Amount permeated (µg/cm²)	Jss (flux) (µg/cm²/h)	Permeability (P x 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

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).

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. The increases in the permeability parameters due to the addition of LLA were statically significant (p = 0.0243). 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.

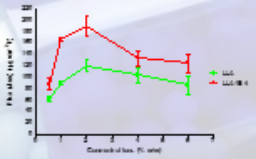


Fig. 3 Concentration effects of LLA and LLA1E 4 on the flux of TNF (n = 6).

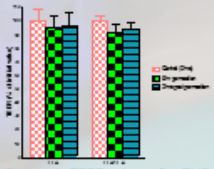


Fig. 4 % Change in TEER values after TNF permeation with either LLA or LLA1E 4 at a concentration of 2% w/w (n = 6).

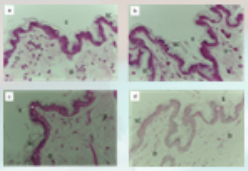


Fig. 5 Photomicrographs of the control and the treated skin selections for light microscopy (LM) stained with H&E; (40) (a) control/untreated, (b) treated with TNF gel, (c) treated with 2% w/w LLA TNF gel, (d) treated with 2% w/w LLA1E 4 TNF gel. (D: dermis; E: epidermis; SC: stratum corneum).

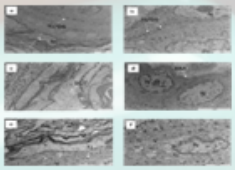


Fig. 6 Electron micrographs of the control and the treated skin selections for transmission electron microscopy (TEM): (a/b) control/untreated (8000), (c) treated with 2% w/w LLA TNF gel (8000), (d) treated with 2% w/w LLA TNF gel (10 000), (e) treated with 2% w/w LLA1E 4 TNF gel (8000), (f) treated with 2% w/w LLA1E 4 TNF gel (12 000). (SC: stratum corneum; TJ/DS: tight Junctions/ desmosomes; N: nucleus; BM: basement membrane; V: vacuoles).

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. 4). 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.

Conclusion

This study has 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.

References

Rambharose S, Kalhapure RS, Akamanchi KG, Govender T. Journal of Materials Chemistry B. 2015;3(32):6662-75.

Corresponding email: Rambharoses@ukzn.ac.za Kalhapure@ukzn.ac.za ; Govenderth@ukzn.ac.za

C1. Local conference poster presentation

1.

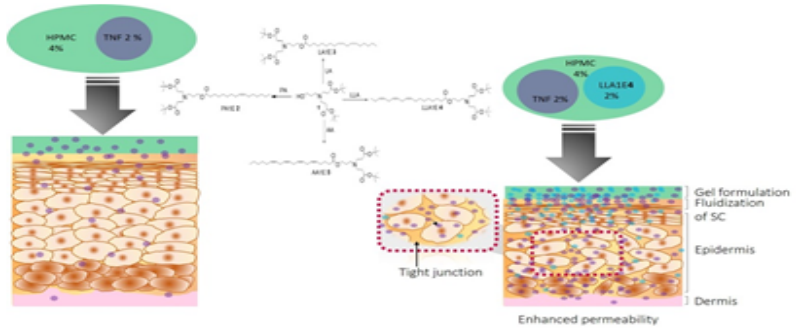
UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

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

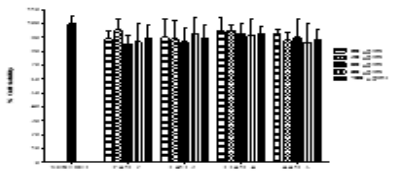
S. Rambharose, R. S. Kalhapure, K. G. Akamanchi and T. Govender

Introduction

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.

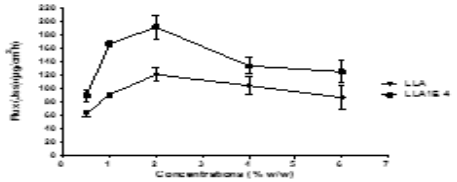


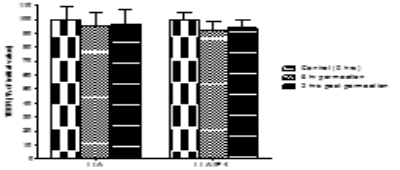
The structures of the derivatives were confirmed by FTIR, NMR (1H and 13C) and HRMS. The in vitro cytotoxicity study revealed their biocompatiblity. Amongst the UFAs, only PA and LLA exhibited transdermal enhancer potential [enhancement ratio (ER) of 1.35 and 2.9 respectively]. All synthesized derivatives 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).

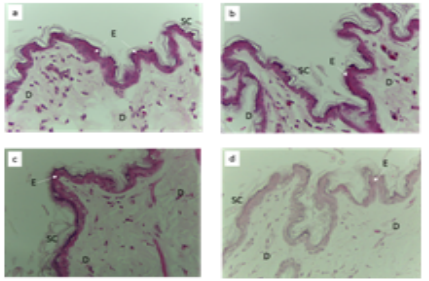


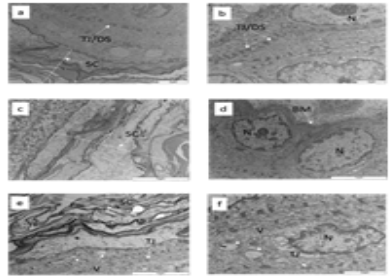
	Treatment	Amount permeated ($\mu g/cm^2$)	Jss(flux) ($\mu g/cm^2/h$)	Permeability ($Px\ 10^2$)	ER	P value
ol	TNF	226.85 ± 33.89	31.48 ± 6.21	0.157 ± 0.03	1	
xxx 1	LA	196.18 ± 22.46	29.398 ± 2.53	0.146 ± 0.01	0.93	0.1658
xxx 2	LA1E 3	514.79 ± 26.89	82.308 ± 4.33	0.411 ± 0.02	2.61	0.2039
xxx 3	LLA	360.72 ± 20.01	*91.316 ± 3.244	0.456 ± 0.16	2.9	0.0243
xxx 4	LLA1E 4	1014.57 ± 24.90	*167.3 ± 3.49	0.836 ±0.17	5.31	0.0066
xxx 5	AA	189.67 ± 19.30	29.82 ± 3.78	0.149 ± 0.18	0.94	0.3262
xxx 6	AA1E 5	510.18 ± 15.19	83.466 ± 1.58	0.417 ± 0.008	2.65	0.0832
xxx 7	PA	269.99 ± 5.16	42.588 ± 0.69	0.212 ± 0.004	1.35	0.6860
xxx 8	PA1E 2	707.84 ± 37.36	*110.3 ± 10.73	0.551 ± 0.05	3.5	0.0039

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.









This study has 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.

Rambharose, Sanjeev, Rahul S. Kalhapure, Krishnacharya G. Akamanchi, and Thirumala Govender. "Novel dendritic derivatives of unsaturated fatty acids as promising transdermal permeation enhancers for tenofovir." Journal of Materials Chemistry B 3, no. 32 (2015): 6662-6675.

UKZN INSPIRING GREATNESS

2.

Dual use of a Novel Dendritic Heterolipid: A Transdermal Permeation Enhancer and an Oil Phase in Nanoemulsion of Tenofovir

UNIVERSITY OF KWAZULU-NATAL INYUVESI YAKWAZULU-NATALI

S. Rambharose, R. S. Kalhapure and T. Govender

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.

Advantages of TDD

- Bypass first pass effect
- Controlled release
- Avoidance of peaks and troughs in plasma
- No GIT distress
- Self-administration
- Non-invasive and improved patient comfort and compliance
- TDD has the potential to improve bioavailability of various drugs, decrease dosages required and reduce drug side effects

Limitations in TDD

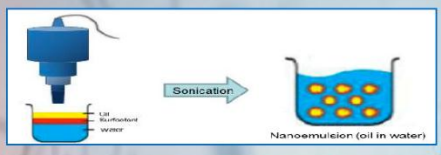
Natural barrier property: This barrier property of the skin is mainly due to the thin outer layer of the epidermis, stratum corneum (SC).

There is a need to develop novel materials to enhance transdermal permeation

Solution

Novel transdermal permeation enhancement technologies include: iontophoresis, electroporation, ultrasound, microneedles, chemical permeation enhancers and transdermal Nano carriers.

The design and application of lipid based nanoparticles provide an ideal platform to develop and advance TDD technologies.



- HIV/Aids infection is a global health concern. Treatment involves the use of antiretroviral drugs such as Tenofovir (TNF).
- TNF is characterised as a nucleotide analog and is considered one of the most successful drug to treat HIV infection

Limitations associated with the Oral dosing of TNF

- Administered as an ester prodrug
- GIT metabolism, poor intestinal permeability, associated side effects
- Low bioavailability= increased dosage

Types of nanoemulsions

- Oil in water
- Water in oil
- Bi-continuous Nano emulsions

In all Nanoemulsion formulations, the interface is stabilized by an appropriate combination of surfactants and/or co-surfactants.

There is a need to develop novel lipids as the oil-phase component

Methods

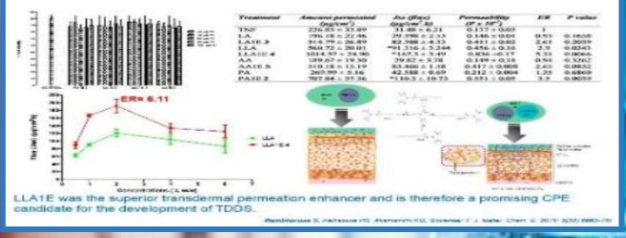
Purpose

Develop a nanoemulsion using a multifunctional excipient where the oily phase is composed of a lipid transdermal permeation enhancer

Objectives

1. Investigate the use of a lipid transdermal permeation enhancer (LLA1E) as the oily phase
2. Characterise the formulation in terms of size, PDI and zeta potential
3. Optimise the developed formulation (drug loading and stability)

Previous report on UFAs



Materials

- Oil phase: 2% (*w/w*) LLA1E
- Surfactants : Solutol HS 15 and PEG 400

Preparation of nanoemulsion

- Construction of ternary phase diagram (*Chemix*) for optimal ratio of surfactant: lipid, Optimising drug loading

Evaluation of nanoemulsion

- Size, PDI, Zeta potential- Malvern ZetaSizer Nano ZS90, Morphology-TEM and Stability (4°C and room temperature for 60 days)

Results and discussion

- Optimal formulation **1:1 SMIX** with **1:2 ratio of Lipid: SMIX**
- Optimised nanoemulsion: 0.25% TNF, 2% LLA1E, 4% SMIX in 10ml water - sonicated at 30% amplitude for 10 mins

Formulation	MGD (nm)	PDI	Z.P
Blank	116.5 ± 0.47	0.158 ± 0.009	- 7.49 ± 1.59
0.25% TNF	120.0 ± 1.90	0.190 ± 0.001	21.03 ± 3.66

Table 1: The affect of drug loading on the parameters of the optimised nanoemulsion. (n=3)

$$\% IE = \frac{W2-W1}{W2} X\ 100$$

% IE = 91.93 %

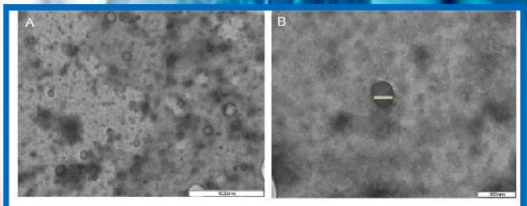
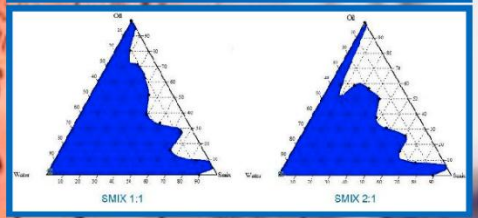


Figure 1: TEM images of the optimised TNF loaded nanoemulsion. A – population of lipid particles ; B – isolated particle measuring 131.35 nm



Time (Days)	MGD (nm)		PDI		Z.P	
	4 °C	R.T	4 °C	R.T	4 °C	R.T
0	126.6 ± 1.90	126.6 ± 1.90	0.190 ± 0.001	0.190 ± 0.001	21.63 ± 3.66	21.63 ± 3.66
7	159.7 ± 1.22	149.8 ± 1.55	0.218 ± 0.006	0.220 ± 0.003	23.79 ± 0.42	26.3 ± 1.05
30	168.86 ± 2.10	231.03 ± 4.27	0.247 ± 0.004	0.416 ± 0.001	24.73 ± 3.17	26.16 ± 2.25
60	186.63 ± 13.03	253.13 ± 17.53	0.278 ± 0.03	0.438 ± 0.06	28.4 ± 2.62	24.86 ± 3.59

Table 2: Effect of storage conditions and time on MGD, PDI and Z.P of TNF NE (n=3)

Conclusion

- LLA1E can serve as an effective oil phase
- A metastable TNF nanoemulsion was successfully formulated
- The NE is expected to perform dual capabilities of transdermal permeation enhancement strategies, Viz., nanosystem and CPE

Significance: Novel nanosystem for improved and sustained delivery of TNF which can be translated to clinics in future, for optimal treatment of HIV/AIDs patients

Ongoing studies and future work

Preparation of hydrogel:

- Determination of MGD, PDI and Z.P, Perform in vitro transdermal permeation studies
- Determine stability of the hydrogel, Perform rheological studies on the hydrogel
- *In vitro* Trans Epithelial Electrical Resistance (TEER) and histo-morphological studies on skin samples after transdermal permeation.

References

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D1. Animal ethics approval

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

12 February 2014

Reference: 054/14/Animal

Prof. T Govender
Pharmaceutical Sciences
School of Health Sciences
University of KwaZulu-Natal
WESTVILLE Campus

Dear Prof. Govender

Ethical Approval of Research Projects on Animals

I have pleasure in informing you that the Animal Research Ethics Committee has granted ethical approval for **2014** on the following project:

"Development and evaluation of a nano-integrated transdermal (NIT) patch for enhancing antiretroviral therapy."

Yours sincerely

Professor Theresa HT Coetzer
Chairperson: Animal Research Ethics Committee

Cc Registrar – Mr C Baloyi
Research Office – Dr N Singh
HOS – Prof. S Essack
BRU – Dr S Singh

Animal Ethics Committee
Professor Theresa HT Coetzer (Chair)
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