Novel amphiphilic lipid augments the co-delivery of erlotinib and IL36 siRNA into the skin for psoriasis treatment

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Running Title: Transdermal delivery of tyrosine kinase inhibitor and IL36 siRNA for psoriasis

Abstract

In this study, we demonstrate for the first time the concurrent transdermal delivery of erlotinib and IL36 α siRNA as a potential dual therapy for psoriasis. The objectives were to develop and evaluate lipid nanocarriers (CYnLIP) using a novel pyrrolidinium lipid to disrupt the skin barrier for enhanced transdermal delivery. CYnLIP (132.00 $\pm$ 6.23nm) had encapsulation efficiency of 49.04 $\pm$ 2.54% for erlotinib. DSC confirmed encapsulation of erlotinib within CYnLIP. Atomic Force Microscopy demonstrated notable topographical changes in the stratum corneum of skin permeated with CYnLIP that were absent in skin hydrated with water. Peak force distance curves also exhibited a more permeable membrane for CYnLIP-incubated skin than hydrated skin. Permeation studies showed enhanced ($p<0.01$) skin retention of erlotinib by CYnLIP (40.76-fold) than solution and more pronounced fluorescence at deeper layers of the skin for fluorescein-labeled siRNA-CYnLIP than solution. The enhanced co-transdermal delivery of erlotinib and IL36 α siRNA by CYnLIP efficaciously treated psoriatic-like plaques in C57BL/6 mice (PASI score of 1) compared to imiquimod-only treatment (PASI score of 4). IHC and western blotting revealed reduction in epidermal hyperplasia (Ki67) and in the dermal infiltration of inflammatory cytokines (IL36 α , pSTAT3, TNF α , NF κ B, IL23 and IL17) for erlotinib/IL36 α siRNA-CYnLIP ($p<0.05$) comparable to Tacrolimus but markedly less than imiquimod-only treatment.

Keywords:

Skin delivery

siRNA

Psoriasis

Gene delivery

Lipid nanocarriers

Transdermal

1. Introduction

Psoriasis is a chronic autoimmune skin disorder that presents as erythematous lesions with epidermal hyperplasia (acanthosis) and extensive inflammation [1]. Further, the corneocytes (dead keratinocytes) of the stratum corneum tend to retain their nuclei (parakeratosis) due to uncharacteristic terminal differentiation of the keratinocytes and this manifests as silvery scaling of the lesions formed. There is also dermal infiltration of inflammatory cells such as dendritic and T cells, which results in the activation of cytokines including IL23, IL17, IL22, TNF α and NF κ B that promote the chronic inflammation further [2].

There is burgeoning research evidence that strongly suggests the crucial roles of members of the IL1 family of cytokines in the pathogenesis of psoriasis. Particularly, the IL36 cytokine family (IL36 α (IL1F6), IL36 β (IL1F8) and IL36 γ (IL1F9)) has been reported to augment the expressions of TH17 cytokines such as IL17A in a feedback mechanism that results in the activation of IL6, IL23 and TNF α [3, 4]. Noteworthy, diverse studies have shown the induction of IL36 cytokines in both human psoriatic skin and psoriatic-like mouse models. Of importance in this study were the novel IL36 α and its receptor, IL36R, which have both been demonstrated extensively to induce the expressions of MAP-kinase and NF κ B that cause the chronic inflammation associated with psoriasis [5]. Studies have illustrated that the functional deletion of the antagonist of IL36R resulted in the formation of severe psoriasis in humans [6]. Additionally, the deletion of *IL36R* gene in C57BL/6 mice protected them from manifesting psoriatic-like inflammation such as the classical erythema, scaling and epidermal thickening that are usually observed [7]. However, no study has explored the avenue of transdermal delivery of IL36 α siRNA as a therapeutic potential for the interference of the mRNA and inhibition of IL36R activation for the treatment of psoriasis.

Further, studies have illustrated the elevated activity of RAS-MAPK and JAK-STAT signaling in psoriatic skin as a consequence of tyrosine hyperphosphorylation of the epidermal growth factor receptor (EGFR) cytoplasmic domain [8]. This influenced the selection of erlotinib, a classical tyrosine kinase inhibitor (TKI) for the dual treatment of psoriatic-like plaques in C57BL/6 mice with IL36 α siRNA. This is due to the fact that erlotinib has an established role in mitigating MAP-kinase expression via the EGFR signaling pathway. Also, erlotinib has been illustrated substantially to selectively inhibit the phosphorylation of STAT3, a cytokine vital for the promotion of epidermal hyperplasia. We have shown in previous studies the relevance of STAT3 in psoriasis formation and the significant healing of psoriatic-plaques after transdermal treatment with the siRNA to knock down the gene [9].

Importantly, clinical research data has indicated a significant role of erlotinib in the treatment of psoriasis. Researchers have reported that psoriasis-associated inflammation remitted spontaneously in lung cancer patients who were undergoing chemotherapy with erlotinib [10, 11]. Additionally, Onyama et al have reported of a patient presenting with metastatic lung adenocarcinoma who exhibited severe psoriatic skin lesions that decreased significantly after onset of oral administration of erlotinib [12]. However, the patient experienced acneform eruptions and a rash in the seborrheic areas, which are consistent with the adverse reactions of tyrosine kinase inhibitors. Hence, in this study, we explored the transdermal route of drug delivery as a viable alternate for erlotinib administration for the management of psoriasis to limit its adverse effects associated with oral delivery. There are no reports of erlotinib transdermal delivery systems and due to its hydrophobic nature (log p 2.39), an efficient delivery strategy is required for enhanced skin deposition.

The transdermal method of drug delivery is a viable approach for consideration because it is generally non-invasive and it circumvents the hepatic-portal circulation, which makes it an ideal delivery method for drugs that are highly susceptible to first pass metabolism. However, the stratum corneum comprises largely of lipids (ceramides, fatty acids and cholesterol) that form highly impermeable bilayered matrix and is hence impossible to permeate by macromolecules and hydrophilic drugs. Some approaches that have been employed to enhance the skin distribution of therapeutic cargos include the use of chemical permeation enhancers and phospholipids [13-15]. These enhancers have been proposed to disturb the ordered structure of the stratum corneum, extract keratin and lipids from the corneocytes and lipid matrix, respectively and to fluidize the lipid matrix for enhanced skin delivery [16, 17].

Note worthily, nano and micro particles, comprising of biodegradable polymers and lipids, have been utilized effectively to augment the transdermal delivery of diverse therapeutic molecules in a controlled-release fashion [18-24]. We have previously used a novel synthesized cationic amphiphilic lipid that has the capability to temporarily fluidize the lipid matrix to improve the transdermal delivery of dual siRNAs and small molecules for the management of skin inflammatory conditions [9, 25]. This lipid has pyrrolidinium physicochemical characteristics and has demonstrated to be a potent permeation enhancer for the skin delivery of therapeutic agents [26, 27].

In the present study, we demonstrate for the first time the enhanced transdermal delivery of erlotinib and IL36 α siRNA via lipid nanocarriers formulated with the novel pyrrolidinium lipid as a potential therapy for the management of psoriasis. The objectives of the study were 1) to employ the novel pyrrolidinium lipid to formulate cationic lipid nanocarriers (CYnLIP) for the effective encapsulation of erlotinib and conjugation of IL36 α siRNA for enhanced concurrent

transdermal delivery, 2) to assess the efficacy of the erlotinib/IL36 α siRNA-CYnLIP system for the treatment of psoriatic-like plaques in C57BL/6 mice and 3) to investigate the combined effect of their delivery on the TH17/IL23 signaling pathway.

2. Materials and Methods

2.1 Materials

The cationic pyrrolidinium lipid (DOPyCl) was synthesized per [26]. 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC) was purchased from Avanti Lipids (Alabaster, AL). Erlotinib (99% HPLC) was purchased from AK Scientific Inc. (Union City, CA). IL 36 α (IL1F6) siRNA was purchased from OriGene Technologies, Inc. (Rockville, MD, USA). Imiquimod (IMQ) was purchased from VWR International (Suwanee, GA, USA). Topgraf[®] (Tacrolimus) was purchased from GlaxoSmithKline Pharmaceutical Limited (Thane, MH, India). Lipofectamine-2000 transfection reagent was procured from Life Technologies (Grand Island, NY, USA). RIPA buffer was purchased from G-Biosciences (Maryland Heights, MO, USA). BCA Protein Assay Reagent Kit was purchased from Pierce (Rockford, IL, USA). Antibodies against IL36 α and EGFR were purchased from R&D Systems (Minneapolis, MN) and Cell Signaling (Danvers, MA), respectively. pcDNA3-eGFP was purchased from Addgene (Cambridge, MA) from the library of Doug Golenbock's lab. Fluorescein-labeled siRNA, antibodies against TNF α , pSTAT3, Ki67, IL-17, IL-23, β -actin, secondary HRP-conjugated antibody and ABC staining immunohistochemistry (IHC) kit were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Phosphate buffer saline (PBS, pH 7.4), DEPC-treated water, cetyl trimethylammonium bromide (CTAB), HPLC grade water and ethanol were procured from Sigma-Aldrich Co (ST. Louis, MO, USA). All other chemicals used in this research study were of analytical grade.

2.2 Cell lines and cell cultures

Human Embryonic Kidney cells (HEK-293) were purchased from Invitrogen (Grand Island, NY, USA) and A431 squamous skin cancer cells were a kind donation by Dr Santosh Katiyar's lab. Both cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St Louis, MO, USA) and supplemented with 10% fetal bovine serum (FBS) from Invitrogen (Grand Island, NY, USA) and antibiotic-antimycotic mixture comprising penicillin (5000U/ml), streptomycin (0.1 mg/ml) and neomycin (0.2mg/ml) from Sigma-Aldrich (St Louis, MO, USA) at 37°C in the presence of 5% CO₂ and 95% relative humidity. The growth media was replaced every 2 days and the cells were subcultured when 80-90% confluent with 0.25% trypsin-EDTA (Invitrogen; grand Island, NY, USA).

2.3 Animals

C57BL/6 mice (6 weeks old; male; Charles River Laboratories, Wilmington, MA, USA) were used for the development of the imiquimod-induced psoriatic-like plaque model. The mice were housed in cages with bedding and maintained under controlled conditions of 12:12h light: dark cycle, 22 ± 2°C and 50 ± 15% RH. They were fed with Harlan Teklad and provided with water ad libitum. The mice were housed at Florida A&M University in accordance with the Guide for the Care and Use of Laboratory Animals and Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The mice were acclimatized to laboratory conditions for one week before the onset of all experiments. The Animal Care and Use Committee, Florida A&M University, FL, USA approved all the animal protocols used.

2.4 HPLC method

The HPLC method followed was that recommended by Faivre et al [28] with modifications. Briefly, the HPLC system from Waters Corporation (Milford, MA) was used with a Vydac

reverse phase C18 (300 Å pore size silica) analytical column (5 mm, 4.6x250 mm) (Grace Vydac, Columbia, MD). The mobile phase comprised of an isocratic system that contained ammonium acetate buffer (5mM, pH 4.5) and acetonitrile in a ratio of 55 to 45 set at a flow rate of 1 ml/min. The amount of erlotinib was measured at λ_{max} of 331 nm.

2.5 Preparation of cationic lipid nanocarriers (CYnLIP)

CYnLIP was formulated with the synthesized pyrrolidinium lipid (DOPyCl) with DSPC, CTAB and cholesterol in weight (mg) ratio of 0.6:6.7:1.0:1.7 by the thin film hydration method. Briefly, the lipids and erlotinib (5mg/120mg of lipid) were dissolved in chloroform and dried under vacuum using the Buchi Rotavapor R-124 (Buchi, Switzerland) to form a thin and even film. The lipid and drug film was then hydrated with water at 50 °C. The resulting formulation was then sonicated for 2min (Branson probe sonicator) to break down the particles and evenly disperse the particles.

For the preparation of IL36 α siRNA-CYnLIP and erlotinib/IL36 α siRNA-CYnLIP, the formulated CYnLIP and erlotinib-CYnLIP were respectively incubated with the siRNA for 30 mins for complete conjugation. The formulation was stabilized for 24 hrs and employed for all experiments.

2.6 Physicochemical characterization of CYnLIP

CYnLIP was characterized for the mean particle size, polydispersity index (PI) and zeta potential (ζ potential, mV) using Nicomp 380 ZLS (Agilent Technologies, Santa Clara, CA, USA), which uses dynamic light scattering to measure the particle size distribution and Nicomp 380 ZLS analyzer, which measures the particle electrophoretic mobility, respectively. Each measurement was done in triplicate. The free drug was separated from the formulation before use.

The total drug content was estimated by dissolving 100 µl of erlotinib-CYnLIP in 400µl of methanol, which was then centrifuged at 10,000 rpm for 15 min and the supernatant collected and analyzed by HPLC. The entrapment efficiency was determined using the methods proposed by Shah et al, (Shah P et al, Biomaterials, 2012). Briefly, the dispersion was placed within the donor compartment of a vivaspin centrifuge filter membrane of molecular weight cutoff (MWCO) of 100 kDa and centrifuged at 5000 rpm for 15 min. The amount of drug collected within the receiver compartment (RC) was then measured by HPLC. The entrapment efficiency was calculated using the formula:

Entrapment efficiency (%) = [(the total drug content – amount of drug in RC) X 100%]/ the total drug content.

2.7 Differential Scanning Calorimetry (DSC) of CYnLIP

DSC was performed with a DSCQ100 System (TA Instrument, USA) using established methods. About 5 mg of erlotinib, blank CYnLIP and erlotinib-CYnLIP (lyophilized) was respectively weighed into aluminum pan (TA Instrument, USA) and sealed hermatically with lid. The samples were then heated at temperature range of 25 to 200 °C with a heating rate of 5°C/min under nitrogen purge. The data was processed with the Universal Software analysis 2000 (TA Instrument, USA).

2.8 Intermittent Contact Atomic Force Microscopy (IC-AFM) of CYnLIP

IC-AFM analysis of CYnLIP was performed via the tapping mode (Bruker Nanoscope V) using otepsa probe. The topography of the sample was obtained from the up and downwards movements of the probe as it scanned across a given area of the sample. All images used for the evaluation of the sample characteristics were taken with image size of 1 µm. CYnLIP was diluted 500-fold and air-dried on a microscope slide overnight. All measurements were

performed at room temperature (22 °C). The Bruker Analysis software was employed for all image processing. Topographic 3D AFM images were presented with nanoscale (nm).

2.9 Cell viability assay of CYnLIP

The cell viability of blank CYnLIP was estimated in HEK 293 cells using the Alamar Blue assay. Briefly, the cells were seeded in 96 well plates at density of 10^4 cells/well and incubated overnight in 5% CO₂ at 37°C. The cells were treated with approximate cationic lipid concentration range from 1 µM to 30 µM and incubated for 24 hours. The cells were then incubated with 20 µl of 0.5mg/ml of alamar blue reagent for 4 hours at 37°C and the fluorescence was measured with a Tecan[®] 200 PRO spectrophotometer microplate reader at excitation/emission wavelengths of 550/580 nm. The relative cell viability (%) of the treated cells was estimated with the equation,

$$[(\text{Fluorescence of treated cells} / \text{Fluorescence of control cells}) * 100]$$

where control cells were cells that were not treated.

2.10 Gel electrophoresis

To ascertain the efficient conjugation of the siRNA to CYnLIP as well as the integrity of the siRNA, gel electrophoretic assay was performed using 2% agarose gel. Briefly, scrambled siRNA (10 µM) was incubated with varying amounts of CYnLIP in the ratio of ~1: 1, 2.5, 4 and 5 for 15 to 30 min. Scrambled siRNA diluted with DEPC water (in place of CYnLIP) to the required concentration was used as the control. 20 µl of each mixture was loaded into a well of the agarose gel with glycerin and run under a constant voltage of 50V for 1 hr in TAE buffer containing 0.5 µg/ml of ethidium bromide. The same volume of loading dye (Pierce, Rockford, IL, USA) was used to track the migration of the siRNA. The respective location of each siRNA

band after migration was visualized under UV transilluminator (Gel DocTM XR+ System, BioRad, Hercules, CA, USA) at a wavelength of 365 nm.

Likewise, a similar validation process was used for pcDNA3-eGFP to verify its successful conjugation to CYnLIP. pcDNA3-eGFP plasmid was obtained from ampicillin-resistant DH5alpha bacteria that was cultured overnight at 37°C in LB-AMP growth medium. The plasmid was purified using the RNeasy Kit according to the manufacturer's protocol and was quantified using the NanoDrop 1000 (Thermo Scientific, Wilmington, DE). The same procedure noted above for the gel electrophoresis of siRNA was followed for the DNA with some modifications. Briefly, pcDNA-eGFP (283.2 ng/μl) was incubated with CYnLIP of varying amounts in the ratio of ~1: 1, 2.5 and 4 for 15 to 30 min. Each resulting mixture (20μl) was then loaded into a well and run under the same voltage (as noted above) for 1 hr. This was run alongside 1 kb DNA ladder. The analysis was carried out in comparison to pcDNA3-eGFP solution (i.e. pcDNA3-eGFP dissolved with DEPC-treated water).

2.11 *In vitro* cellular internalization and transfection by CYnLIP

The efficiency of CYnLIP to deliver siRNA internally as well as transfect DNA plasmid into cells was determined with fluorescein-labeled siRNA and pcDNA3-eGFP, respectively in A431 squamous skin cancer cells. The cells were plated at density of 50×10^4 cells/well in 24-well plate and incubated overnight in 5% CO₂ at 37°C. The cells were washed twice with DEPC-treated water after the medium was aspirated and replaced with Opti-MEM[®] Reduced Serum Medium (Gibco[®], Life Technologies, Grand Island, NY, USA).

For uptake studies, the cells were incubated with fluorescein-labeled siRNA-CYnLIP (30nM of siRNA) for 2 hr after which, the cells were washed with DEPC-treated water and analyzed for fluorescence using Olympus BX40 microscope equipped with computer-controlled digital

camera (DP71, Olympus Center Valley, PA, USA) in comparison with fluorescein-labeled siRNA solution and fluorescein-labeled lipofectamine-2000.

For transfection studies, the cells were incubated with approximately, 20µg/ml of pcDNA3-eGFP-CYnLIP for 4 h and replaced with fresh Opti-MEM[®] Reduced Serum Medium. The cells were then incubated for 12 hr and analyzed for the expression of green fluorescence protein (GFP) in comparison with solution and lipofectamine-2000, respectively.

2.12 *In vitro* skin permeation studies

2.12.1 Preparation of skin

Dermatomed human skin was purchased from Platinum Training (Henderson, NV) and transported in 10% glycerin in saline solution with a thickness of 0.5 ± 0.1 mm. The skin was stored at -80°C . This method of transport and storage has been validated in our lab extensively to preserve the barrier characteristics of the skin for permeation studies [23, 29]. Prior to use, the dermatomed human skin was thawed and rinsed with distilled water for 15-20 min to remove excess glycerin.

Full thickness rat skin was obtained from the dorsal region of CD[®](SD) hrBi hairless rats after sacrificing by an overdose of halothane anesthesia. The skin was collected and the subcutaneous fat and connective tissue were removed with caution. The skin was then preserved in 10% glycerin in saline solution and stored at -80°C until used in accordance with established methods by our lab. Prior to use, the skin was rinsed with distilled water for 15-20 min to remove excess glycerin.

2.12.2 Permeation studies with CYnLIP

2.12.2.1 Permeation studies with erlotinib-CYnLIP

To determine the extent of skin permeation of erlotinib by CYnLIP, skin permeation studies were carried out using dermatomed human skin. Briefly, the skin was mounted on Franz diffusion cells (Permeagear Inc., Riegelsville, PA, USA) between the donor and receiver compartments with the stratum corneum in direct contact with the donor compartment. The receiver compartment was filled with PBS (pH 7.4), stirred at 300 rpm and maintained at $32 \pm 0.5^{\circ}\text{C}$ using a circulating water bath. The studies were carried out for 24 h under non-occlusive conditions. 150 μl of erlotinib-CYnLIP was applied to the skin in the donor compartment and incubated for 24 hr. Afterwards, the excess formulation was removed from the surface of the skin with a cotton swab, washed with 50% v/v ethanol in water and then blotted dry with lint-free absorbent wipes. The total dosing area of 0.636 cm^2 of skin was collected with a biopsy punch and analyzed by HPLC [30]. Erlotinib suspended in water was used as control. The skin permeation studies were carried out in triplicate ($n=3$) and the average was determined.

2.12.2.2 Permeation studies with fluorescein-labeled siRNA-CYnLIP

To determine the extent of permeation and skin distribution of siRNA by CYnLIP, the permeation studies were performed with full thickness rat skin using the above-mentioned procedure. 150 μl of fluorescein-labeled siRNA-CYnLIP was applied to the skin in the donor compartment and incubated for 6 hr. The skin was then cryosectioned with a cryotome (Shandon, England) and analyzed for green fluorescence, which depicts degree of permeation of siRNA, using fluorescence microscope (Olympus BX40 microscope equipped with computer-controlled digital camera). Fluorescein-labeled siRNA dissolved in DEPC-treated water was used as control.

2.12.2.3 AFM studies of skin permeated with blank CYnLIP

To investigate the interaction of CYnLIP with the skin lipid matrix for enhanced transdermal delivery, permeation studies were performed in full thickness rat with blank CYnLIP. The procedure and receiver compartments were maintained as mentioned above. 150 μ l of blank CYnLIP was applied to the skin in the donor compartment and incubated for 4 hr. Skin permeated with water (hydrated skin) was maintained as a control. Following the permeation studies, the skin was cryosectioned laterally (20 μ m thick) to separate the stratum corneum and this was explored for morphological changes in comparison to the hydrated skin in a ScanAssyst AFM analysis using a scanassyst (silica nitrile) probe. Peak force distance curves were derived and used to establish the effect of the CYnLIP permeation on the disruption of the stratum corneum lipid membrane.

2.13 *In vitro* drug release studies

Further, CYnLIP was incorporated into Hydroxypropyl methylcellulose (HPMC) polymeric matrix to increase its viscosity and consistency as well as skin contact time by decreasing run-off. Varying amounts of the HPMC polymer (0.5, 1.0 and 1.5 %w/v) were dispersed into formulation under constant stirring to form a complete gel and was stabilized at room temperature for 24h prior to use.

In vitro drug release studies were then performed to investigate the amount of drug released from the different concentrations of polymer matrix over 72 h using PBS (pH 7.4). A semi-permeable dialysis membrane of molecular weight cut off (MWCO) of 50,000 Da (Sigma Aldrich Co, MO) was used for the studies. The membrane was mounted between the donor and receiver compartments of the Franz diffusion cells (Permeagear Inc., Riegelsville, PA) and 150 μ l of each erlotinib-CYnLIP HPMC gel was applied evenly on the surface of the membrane in the donor compartment and the studies were carried out as mentioned above for the skin permeation

studies. The experiment was carried out in triplicate. At predetermined time intervals (0.5, 1, 2, 3, 4, 6, 10, 24, 48 and 72 h), 0.3 ml samples were collected from the receiver compartment. To maintain sink conditions, 0.5% of tween 80 was added as a solubilizer because erlotinib is an insoluble drug. Also, each sample volume withdrawn was replaced with fresh buffer solution throughout the sampling period to maintain a volume above that which would provide complete dissolution of the insoluble drug. The collected samples were then analyzed for drug content using the HPLC.

The different erlotinib-CYnLIP HPMC gels were also used for skin permeation studies (24 hr) to determine the effect of polymer concentration on the retention and permeation of the drug by the formulation. The concentration of polymer that resulted in the maximum skin retention of erlotinib by CYnLIP was selected and used for the *in vivo* psoriasis studies.

2.14 Psoriasis studies

2.14.1 *In vivo* imiquimod induced psoriatic-like plaque model

Imiquimod (IMQ)-induced psoriatic-like plaque model in C57BL/6 mice [31] was used to evaluate the anti-psoriatic effect of the optimized formulation. Briefly, IMQ cream (prepared as previously optimized by Shah et al [31] was applied topically to the shaven dorsal regions of C57BL/6 mice (4mg/day/animal) to induce inflammation (n=6). The animals were then treated by applying erlotinib-CYnLIP, IL36 α siRNA-CYnLIP and erlotinib/IL36 α siRNA-CYnLIP, respectively to the inflamed dorsal regions for 5 consecutive days. Tacrolimus (Topgraf[®]) and blank CYnLIP were used as positive and negative controls, respectively. Tacrolimus was used because the plaques formed resembled a typical skin inflammatory condition and this topical medication is classically used in such situations.

2.14.2 PASI Scoring and Trans-epidermal water loss (TEWL) determination of skin

The clinical scoring method for the Psoriasis Area and Severity Index (PASI) was used to score the severity of the inflammation induced on the dorsal regions of the mice daily based on the extent of erythema, scaling and epidermal thickening. The PASI score was assigned daily after initiating treatment on a scale of 0 to 4 where 0- none; 1- slight; 2- moderate; 3- marked; 4- very severe inflammation.

The trans-epidermal water loss (TEWL) was estimated using Delfin VapoMeter (Delfin Technologies Ltd, Finland) as a measure of the extent of skin damage and healing with each day of treatment. The device was placed gently on the dorsal region of the mice each day of treatment and the value corresponding to water loss was recorded.

The combination index (CI) was estimated as proposed by Shah et al [29]. The equation noted below was used-

Combination Index (CI) = [Response of erlotinib/IL36 α siRNA-CYnLIP/Response of erlotinib-CYnLIP] / [Response of erlotinib/IL36 α siRNA-CYnLIP/Response of IL 36 α siRNA-CYnLIP]

CI > 1.3 indicated antagonism, CI = 1.1-1.3 indicated moderate antagonism, CI = 0.9-1.1 indicated additive effect, CI = 0.6-0.8 indicated moderate synergism, CI = 0.4-0.6 indicated synergism and CI = 0.2-0.4 indicated strong synergism.

2.14.3 Histology

H&E staining of the excised skin samples at the end of the 5 day treatment was performed as described by [32]. Briefly, formalin-fixed, paraffin-embedded skin sections were deparaffinized, rehydrated and stained with hematoxylin and counterstained with eosin. The stained skin sections were mounted and viewed with the Olympus BX40 light microscope equipped with a computer-controlled digital camera (DP71, Olympus Center Valley, PA).

Immunohistochemical studies were carried out on paraffin-embedded skin sections using the protocol specified by ImmunoCruzTM mouse ABC staining kit. Briefly, the section slides were washed in xylene and hydrated in varying concentrations of alcohol. The sections were then incubated with primary antibodies against Ki67 overnight at 4°C. Horseradish peroxidase-conjugated secondary antibody was then applied for the detection of the primary antibody. The section slides were subsequently stained with DAB chromogen and counterstained with hematoxylin. Olympus BX40 microscope equipped with a computer-controlled digital camera was used for the visual analysis of the images of the sections.

Brown staining of the skin sections was considered as positive result.

2.14.4 Western blot analysis

Western blot analysis was performed as described earlier by [33] using the excised skin at the end of the 5 day treatment. Briefly, the skin samples were minced in lysis cocktail composed of RIPA lysis buffer, protease inhibitor (PI) and phenylmethylsulfonyl fluoride (PMSF). The concentration of the extracted protein was estimated per the manufacturer's protocol using BCA Protein Assay Reagent Kit. 50 µg of the extracted protein for each sample was loaded into a gel and subjected to SDS-polyacrylamide electrophoresis (SDS-PAGE). The blots were incubated with primary antibodies for EGFR, IL36, TNFα, NFκB, IL23, IL17, pSTAT3 and β-actin and subsequently detected with Horseradish peroxidase-conjugated secondary antibodies using a SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) and BioRad ChemiDocTM XRS+ imaging system (Hercules, CA, USA). Data has been represented as the mean ± SD (n =6).

2.15 Statistical Analysis

The data of the studies presented in this article were expressed as the mean $\pm$ standard deviation for at least three replicates; the multiple groups were compared using a one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison Test and between two groups by student's t test analysis. The mean differences were considered significant in all experiments valued at * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

3. Results

3.1 Physicochemical characterization of CYnLIP

CYnLIP formed a monodispersion with average particle size of 132.00 ± 6.23 nm (P.I.- 0.15 ± 0.003). The particles were largely positively charged with zeta potential of $+39.76 \pm 1.91$ mV (table 1). The entrapment efficiency estimated for erlotinib in CYnLIP was $49.04 \pm 2.54\%$. Intermolecular interaction was the main force in play in the formation of CYnLIP and erlotinib. Erlotinib, being highly insoluble, associates with the hydrophobic tails of the lipid bilayer and is entrapped within the layers for thermodynamic stability. The stability of the nanocarriers was not affected by the conjugation of IL36 siRNA whilst there was minimal change in the particle size.

Physicochemical characterization of optimized erlotinib CYnLIP				
Formulation	Particle size (nm)	Polydispersity	Zeta Potential (mV)	Entrapment efficiency (%)
Erlotinib CYnLIP	132.00 ± 6.23	0.15 ± 0.003	$+39.76 \pm 1.91$	49.04 ± 2.54

Table 1: Physicochemical characterization of the erlotinib CYnLIP

3.2 Differential Scanning Calorimetry (DSC) of CYnLIP

DSC was performed to ascertain if the drug was encapsulated within CYnLIP. The thermograms of erlotinib, lyophilized erlotinib-CYnLIP and blank CYnLIP have been shown in figure 1. The drug showed a sharp endothermic peak at temperature range of 155 to 157 °C. Blank CYnLIP, which comprised of all the lipids with the exception of the drug showed two broad peaks at 100 °C and 130 °C, respectively. However, there was no visible peak for the drug in the thermogram for erlotinib-CYnLIP, indicative that the drug was entrapped within the lipid bilayer. Further, the thermogram for erlotinib-CYnLIP revealed that the endothermic peaks observed earlier for the blank CYnLIP shifted to 90 °C and 140 °C, respectively with the latter peak becoming less prominent.

Figure 1

Figure 1: Differential scanning calorimetric analysis showing complete solubilization of erlotinib in CYnLIP.

3.3 Intermittent Contact Atomic Force Microscopy (IC-AFM) of CYnLIP

The analysis confirmed the monodispersion, size and homogeneous distribution of CYnLIP that was comparable to the results obtained from the dynamic light scattering particle size determination (figure 2a). The images also demonstrated that the nanocarriers formed uniform spherical morphology with size range of 80 to 100 nm.

3.4 Cell viability assay of blank CYnLIP

Cell viability assay was performed to determine the tolerance of normal human cells to CYnLIP. Blank CYnLIP demonstrated to be relatively non-toxic to HEK 293 cells after 24 hr incubation (figure 2b). There was approximately 80% cell viability at the maximum concentration of ~30 μM of total cationic lipid.

Figure 2

Figure 2: A) Atomic force microscopy (AFM) of CYnLIP by soft tapping in air. B) Cell viability of blank CYnLIP in embryonic kidney cells (HEK 293) after 24 hr incubation.

Figure 3

Figure 3: A) Gel electrophoresis assay of conjugated siRNA to CYnLIP- i) loading dye, ii) free siRNA, iii) to vi) siRNA (10 μ M) incubated with varying amounts of CYnLIP in the ratio of ~1: 1, 2.5, 4 and 5, respectively. B) Cellular internalization of fluorescein-labeled siRNA-CYnLIP in A431 squamous skin cancer cells.

3.5 Gel electrophoresis

The results revealed that CYnLIP successfully conjugated to both the siRNA and DNA plasmid and inhibited their migration under an applied electrical field (figure 3a and 4a). Their corresponding bands were observed to be still present within the wells they were loaded in after the gel electrophoresis run. This illustrates the effective electrostatic complexation of the nucleic acids to the positively charged nanocarriers, which resulted in the complete neutralization of

their negative charges and hence the inhibition of their electrophoretic mobility. Further, each of the different volumes of CYnLIP containing varying concentrations of cationic lipid bound efficiently to the nucleic acid and prevented their migration. In contrast, both free siRNA and DNA plasmid migrated freely under the electric potential applied because their negative charges were free and not complexed.

Figure 4

Figure 4: A) Gel electrophoresis of conjugated pcDNA3-eGFP to CYnLIP- i) free pcDNA3-eGFP, ii) to iv) pcDNA3-eGFP: total cationic lipid- i) free DNA plasmid, ii), iii) & iv) pcDNA-eGFP (283.2 ng/ μ l) incubated with CYnLIP of varying amounts in the ratio of $\sim$ 1: 1, 2.5 and 4, respectively and v) DNA molecular weight ladder (1kb). B) Transfection of pcDNA3-eGFP-CYnLIP into A431 squamous skin cancer cells.

3.6 Cellular internalization of fluorescein-labeled siRNA-CYnLIP and transfection of pcDNA3-eGFP CYnLIP

Cellular uptake studies carried out with fluorescein-labeled siRNA ascertained the efficiency of CYnLIP in transporting the macromolecule across the cell membrane (figure 3b). There was pronounced green fluorescence observed in A431 cells treated with fluorescein-labeled siRNA-CYnLIP after the 2 hr incubation that was comparable to the fluorescence observed for fluorescein-labeled siRNA delivered by lipofectamine-2000 but markedly greater than the free siRNA.

For transfection study, pcDNA3-eGFP was effectively delivered into A431 cells by CYnLIP, which resulted in the successful transcription of the mRNA and translation of the green fluorescence protein (GFP) (figure 4b). There was more pronounced green fluorescence detected within the cells, which corresponded to the amount of GFP translated after transfection with pcDNA3-eGFP-CYnLIP. This was comparable to the green fluorescence observed for pcDNA3-eGFP-lipofectamine-2000 but significantly greater than the free DNA plasmid.

3.7 Permeation studies with CYnLIP

The results showed that CYnLIP effectively transported erlotinib across the stratum corneum and deposited the drug within the deeper layers of the skin. CYnLIP caused significantly ($p < 0.001$) enhanced retention of erlotinib within the skin layers (10.35 ± 0.50 μg per gram of skin) that was 40.76-fold more than solution (figure 5a). Further, the amount of erlotinib that permeated into the receiver compartment for CYnLIP (6.07 ± 0.25 ng/cm^2) was significantly ($p < 0.001$) greater than solution (no drug detected) (figure 5b) with calculated flux of 0.25 $\text{ng}/\text{cm}^2\text{hr}$.

For permeation studies of fluorescein-labeled siRNA-CYnLIP in full thickness rat skin, there was pronounced green fluorescence distributed within the deeper layers of the skin (figure 5c).

However, for the free siRNA, fluorescence was observed only within the stratum corneum, clearly illustrating the relevance and effectiveness of CYnLIP in surmounting the skin barrier to deliver the siRNA.

Figure 5

Figure 5: A) Skin retention of erlotinib by CYnLIP in comparison to solution in dermatomed human skin after 24 hr permeation studies. B) Permeated amount of erlotinib into receiver compartment after 24 hr. C) Skin retention of fluorescein-labeled siRNA-CYnLIP in full thickness rat skin after 6 hr permeation studies. Data has been represented as the mean $\pm$ SD (n =3). ***p<0.001

The AFM data reiterated the above results by revealing that the stratum corneum of the rat skin was topographically altered after 4 hr permeation studies with blank CYnLIP, which was distinctly different from the hydrated skin (permeated with water for 4 hr) (figure 6a and 6b). Further, CYnLIP caused significant changes in the lipid membrane, which were evident in the peak force distance curves derived (figure 6c and 6d). The disruption of the lipid membrane may therefore account for the observed enhanced permeability of the skin incubated with CYnLIP and for the resulting delivery of the cargos into the deeper layers of the skin.

Figure 6

Figure 6: IC-AFM analysis of stratum corneum (20 μm thick) after 4 hr permeation studies with A) water and B) blank CYnLIP in full thickness rat skin. There were prominent topographical differences observed between CYnLIP treated skin and untreated skin. C) and D) represent the differences observed between CYnLIP treated skin and untreated skin.

peak force distance curves for blank skin (permeated with water) and skin permeated with blank CYnLIP, respectively.

3.8 Drug release studies

The results revealed that the amount of HPMC incorporated did not influence the rate and extent of release of erlotinib from CYnLIP (figure 7a). There was a quick and sustained drug release for all three gels over the duration of the release studies. However, the amount of polymer affected the amount of erlotinib that was retained within the skin layers as well as the amount that permeated into the receiver compartment (figure 7b and 7c). There was increase in drug deposition with increase in amount of HPMC polymer with 1.5% gel demonstrating the maximum skin retention and permeation of erlotinib. The estimated amount of erlotinib retained within the skin layers for 0.5%, 1% and 1.5% HPMC gels were 2.28 ± 0.001 , 2.56 ± 0.001 and 10.08 ± 0.005 μg per gram of skin, respectively and the permeated amounts were 0.014 ± 0.005 , 0.015 ± 0.009 and 0.021 ± 0.015 $\mu\text{g}/\text{cm}^2$.

Figure 7

Figure 7: A) Drug release, B) Skin retention and C) Drug permeation of 0.5, 1.0 and 1.5% erlotinib CYNLIP-HPMC gel, respectively. Data has been represented as the mean $\pm$ SD (n =3).

***p<0.01

3.9 PASI Scoring of psoriatic-plaques and Trans-epidermal water loss (TEWL) determination

The PASI score represented the severity of scaling, erythema, epidermal thickness and extension of rete-ridges formed from IMQ application. Reduction in the score is indicative of the degree of healing associated with treatment. The score was 4 for all the groups prior to treatment. However, the PASI score was significantly reduced for erlotinib/IL36 α siRNA-CYNLIP treated

mice (score of 1) by the 5th day of treatment (figure 8a), which was comparable to Tacrolimus (Topgraf®). The PASI score for IMQ-only treatment (negative control) remained 4 by the end of 5th day whilst the scores for erlotinib-CYnLIP and IL36 α siRNA-CYnLIP treated groups reduced to 2. The PASI scores have been represented in Table 2.

Psoriatic Area and Severity Index (PASI) Score						
Day	0	1	2	3	4	5
No IMQ, no drug	0	0	0	0	0	0
IMQ only	4	4	4	4	4	4
IL36 siRNA CYnLIP	4	4	3	3	3	2
Erlotinib CYnLIP	4	4	4	2	2	2
IL36 siRNA/erlotinib CYnLIP	4	4	3	3	2	1
Blank CYnLIP	4	4	4	4	4	3
Topgraf®	4	4	3	2	2	1

Table 2: PASI scoring of the psoriatic dorsal regions of C57BL/6 mice after initiation of treatment.

The combination index calculated at the end of the 5th day of treatment was 1.0 for erlotinib/IL36 α siRNA-CYnLIP, suggestive of an additive therapeutic effect for erlotinib and IL36 α siRNA.

Similarly, the recorded TEWL was reduced ($p < 0.05$) for erlotinib/IL36 α siRNA-CYnLIP by ~1.5 and 2-fold less than blank CYnLIP and negative control (only imiquimod), respectively (figure 8b).

Figure 8: A) Pictures of dorsal regions of C57BL/6 mice showing psoriasis and the effect of treatment with erlotinib/IL 36 siRNA-CYnLIP. B) Measurement of the trans-epidermal water loss (TEWL) of the various treatment groups. The value recorded for erlotinib/IL 36 siRNA-CYnLIP was comparable to Tacrolimus (Topgraf). Data has been represented as the mean $\pm$ SD (n =6).

3.10 Histology

H&E staining revealed extensive epidermal thickening (acantosis), rete ridge extensions and thickening of the stratum corneum (hyperkeratosis) for the negative control (IMQ-only treatment). These were prominently decreased for erlotinib/IL36 α siRNA-CYnLIP, which illustrated similar morphology as the positive control (no IMQ, no treatment) and Topgraf (figure 9) but less than either erlotinib-CYnLIP or IL36 α siRNA-CYnLIP.

IHC staining showed markedly reduced brown staining for Ki67 (keratinocyte proliferation marker) and EGFR for erlotinib/IL36 α siRNA-CYnLIP signifying reduced inflammation, proliferation of keratinocytes and epidermal thickening (figure 9).

Figure 9: Histological analysis of skin after psoriasis treatment with erlotinib/IL36 siRNA-CYnLIP in comparison to Topgraf and the individual therapies. Brown stain is indicative of positive results for Ki67.

3.11 Western blot analysis

Western blot confirmed the decreased expressions of EGFR and IL36 α for erlotinib-CYnLIP and IL36 α siRNA-CYnLIP treated mice, respectively; thus revealing the successful inhibition of the EGFR signaling pathway and knock down of the *IL36 α* gene (figure 10a and 10b). However, there was more significant ($p < 0.01$) reduction of both proteins for erlotinib/IL36 α siRNA-CYnLIP treated mice compared to the individual treatments (erlotinib-CYnLIP and IL36 α siRNA-CYnLIP). Further, the results illustrated reduced skin inflammation (NF κ B), keratinocyte proliferation stimulation (pSTAT3) and dermal cytokine infiltration (TNF α , IL23 and IL17) for erlotinib/IL36 α siRNA-CYnLIP, which was significantly ($p < 0.01$) less than Topgraf, blank CYnLIP and imiquimod-only treatment.

Figure 10a

Figure 10b

Figure 10: Western blot analysis of skin after psoriasis treatment showing A) protein bands and B) quantified protein expressions for inflammatory markers. There was significant reduction of inflammation for erlotinib/IL 36 siRNA-CYnLIP treated group compared to imiquimod only and blank CYnLIP treatments. Data has been represented as the mean $\pm$ SD (n =6). **p<0.01

4. Discussion

The results of the present study strongly suggest the potential therapeutic benefits of delivering erlotinib and IL36α siRNA concurrently, for the first time, across the skin for the treatment of psoriasis. Transdermal drug delivery has gained a lot of attention because it has proven to be ideal for the transport of different molecules for various therapeutic effects, both locally and

systemically [34-36]. Lipid nanocarriers have been extensively demonstrated to efficiently surmount the skin barrier by creating hydration channels within the stratum corneum and by disrupting the lipid matrix for enhanced transdermal drug delivery [37, 38]. Several researchers have established the biocompatibility of the lipids used and the vital protection of diverse therapeutic cargos by delivery systems such as nanoemulsions, liposomes and solid lipid nanoparticles against degradation for improved percutaneous delivery and the management of numerous skin disease conditions [39, 40].

Importantly, lipid nanocarriers have been established as ideal carriers for the transdermal transport of both hydrophilic and macromolecular drugs. Marepally et al have illustrated significant healing of psoriatic-plaques in mice after treatment with dual siRNA delivered by fusogenic nanoparticles formulated with a novel pyrrolidinium lipid [9, 26]. In the study, the authors proposed that the pyrrolidinium lipid, which possesses a cationic head group and oleyl fatty acid chains enhanced the percutaneous delivery of dual siRNAs by disrupting the ordered lipid lamellae domain of the stratum corneum. This hypothesis was supported in the present study by the atomic force microscopic analysis, which revealed that in incubating rat skin with the blank formulation developed with the novel pyrrolidinium lipid, there were distinct topographical changes observed that were absent in skin that was incubated with water only (figure 6a and 6b). Also, there was increased skin surface roughness and disruption of the lipid membrane. Noteworthy, this result corresponded accurately with the enhanced permeation and distribution of erlotinib ($p < 0.001$) and fluorescein-labeled siRNA into the deeper layers of the skin by the lipid nanocarriers. Both erlotinib and fluorescein-labeled siRNA suspended in aqueous medium failed to permeate the skin and this revealed their impermeable and negligible diffusive nature across the stratum corneum owing to their physicochemical characteristics.

These delivery limitations were however surpassed by the lipid nanocarriers; this observation may be attributed to their permeation enhancing potential achieved by the interference of the ordered skin barrier structure as well as the creation of hydrophilic pores within the lipid matrix. Notwithstanding, the nanocarriers proved to be relatively non-toxic and hence, possessed reduced probability to cause skin damage. This was illustrated in the tolerance of HEK 293 cells to the nanocarriers. The cells showed approximately 80% cell viability after 24-hour incubation with the blank formulation, which was representative of the duration of application of the formulation during the in vivo studies. Moreover, other widely used permeation enhancers such as oleic acid follow similar mechanism to enhance permeation and they are well tolerated.

Additionally, the incorporation of the lipid nanocarriers into HPMC matrix gel facilitated the formation of a hydration film on the skin surface that may have further contributed to the formation of hydrophilic channels within the stratum corneum and hence, enhanced the skin permeation of the therapeutic cargos [31, 41]. Boakye et al have shown in a study that oleic acid surface-modified nanostructured lipid carriers incorporated into HPMC gel significantly potentiated the topical delivery of a highly lipophilic antioxidant for skin cancer chemoprevention compared to the aqueous dispersion [32]. This was apparent in the results, which indicated increased skin deposition of erlotinib with increased polymer concentration up to 1.5%.

The nanocarriers formed a monodispersion that effectively encapsulated erlotinib as demonstrated in the AFM and DSC studies. The DSC analysis revealed the disappearance of the endothermic peak for erlotinib in CYNLIP, signifying its entrapment within the lipid bilayers of the nanocarriers. However, the low entrapment efficiency of erlotinib within the nanocarriers may be due to its poor aqueous solubility. Also, the nanocarriers were positively charged largely

due to the cationic pyrrolidinium lipid (DOPyCl); hence they efficiently interacted with the negatively charged siRNA via electrostatic attractions for the formation of a stable system. This was apparent in the gel retardation assay that showed that the siRNA was bound and protected by the nanocarriers such that they did not migrate under the application of electric current. Notably, the lipid nanocarriers by proficiently conjugating to the fluorescein-labeled siRNA and the pcDNA3-eGFP, enhanced their cellular transport; further illustrating the delivery superiority of the nanocarrier system. Nucleic acids are generally problematic to transfer subcellularly due to both their large sizes and negative charges, which are opposed by the similarly charged cellular membranes [42, 43]. This principle was demonstrated in both the uptake and transfection studies, which illustrated negligible expression of green fluorescence in skin cancer cells that were incubated with the free fluorescein-labeled siRNA and pcDNA3-eGFP plasmid, respectively. However, there was augmented expression of the fluorescence observed when the cells were incubated with the siRNA and plasmid that had previously been conjugated to the lipid nanocarriers. This is critically indicative of the vital role of lipid nanocarriers in the transport of macromolecules across the cell membrane for therapeutic functions [44, 45].

The delivery proficiency of the lipid nanocarriers additionally translated into effective therapeutic outcome with considerable healing of the psoriatic-like plaques observed following treatment with erlotinib/IL36 α siRNA-CYnLIP, which was comparable to the positive control (no imiquimod, no treatment). The recorded PASI score and TEWL value for erlotinib/IL36 α siRNA-CYnLIP were not significantly different from Topgraf (Tacrolimus); however, the molecular studies of the treated skin tissue revealed otherwise. There was more successful healing for the dual therapy than Topgraf and was evident in the significantly ($p < 0.05$) greater inhibition of the infiltration of inflammatory cytokines into the dermis for the former.

Importantly, the histology of skin treated with erlotinib/IL36 α siRNA-CYnLIP after psoriasis induction was more comparable to the positive control than Topgraf; there was more pronounced decrease in epidermal thickening and extension of rete ridges with minimized hyperkeratosis (thickening of stratum corneum).

Further, the separate cargos were limited in achieving maximum healing but in combination, they demonstrated additive therapeutic outcome with increased recovery, hence supporting scientific findings that suggest that combination therapies generally attain greater curative results than monotherapies [46, 47]. However, both erlotinib and IL36 siRNA independently reduced the observed inflammation considerably more than the imiquimod-only and blank CYnLIP treated groups, further illustrating the inadequate healing of the psoriatic plaques by the body in the absence of remedy [25].

Both erlotinib and IL36 α siRNA proved to be efficacious in reducing the psoriasis-associated inflammation by markedly decreasing the infiltration of classical dermal cytokines such as IL17, IL23, TNF α and NF κ B [48-50]. However, IL 36 α knock down demonstrated to be more relevant for the downregulation of TNF α , IL17 and IL23 inflammatory cytokines than erlotinib, therefore supporting the diverse research data that reveal the critical role of the cytokine and its receptor, IL36R in the induction of psoriasis [3]. Our results are in agreement with reported studies that have illustrated that in knockout mice with *IL23* and *IL17* genes deleted, the induction of IL36 α overexpression via imiquimod application resulted in residual inflammation that was absent in knockout mice with the deletion of *IL36 α* gene. The authors suggested the initiation of psoriasis by IL36 α mainly via the IL23/IL17 signaling axis but also the probability of lesion formation mediated by IL36 α through other minor avenues as well [7]. In the present study, the inhibition of IL36 α expression potentially restored the inflamed dorsal skin of the mice to normalcy and

reduced epidermal thickening notably compared to imiquimod-only treatment. Erlotinib also played a significant role in mediating psoriasis management by pronouncedly reducing the expressions of EGFR, pSTAT3 and IL17 compared to imiquimod-only and blank CYnLIP treatment with corresponding decrease in epidermal thickening that was equivalent to IL36 α siRNA.

Finally, there was reduced expression of phosphorylated STAT3 for the dual treatment that was more enhanced than the separate treatments and Topgraf with consequently decreased expression of Ki67, a keratinocyte hyperproliferation protein. These results are therefore in agreement with the studies of Marepally et al, who demonstrated considerable reduction of acanthosis and Ki67 expression after the skin delivery of STAT3 siRNA in psoriasis management [9].

5. Conclusion

The novel pyrrolidinium lipid formed monodispersed nanocarriers that served as efficient carriers for both erlotinib and IL36 α siRNA. The lipid nanocarriers enhanced the skin deposition of erlotinib ($p < 0.001$) and the siRNA. Further, the improved synchronized transdermal delivery of erlotinib and IL36 α siRNA by CYnLIP efficaciously treated the psoriatic-like plaques induced in C57BL/6 mice and reduced the infiltration of dermal cytokines that are responsible for the chronic inflammation associated with psoriasis. Overall, the dual delivery of erlotinib and IL36 α siRNA demonstrated great therapeutic value for psoriasis treatment in an additive therapeutic manner that was more beneficial than the individual agents and the marketed formulation of Tacrolimus (Topgraf[®]).

Acknowledgements

The authors would want to extend their gratitude to Dr Hernan Flores-Rozas of the Department of Pharmacology and Toxicology, Florida A & M University; to Dr Gautam Behl of the

Department of Pharmaceutics, Florida A & M University and to Dr Eric Lochner of the Department of Physics, Florida State University, Tallahassee, FL for their enormous guidance and contribution to this project.

This project was supported by the National Institutes of Health's Minority Biomedical Research Support (MBRS)-SC1 program [Grant#SC1 GM092779-01]; the National Institute on Minority Health and Health Disparities (NIMHD) P20 program (Grant #1P20 MD006738-03) and the Department of Defense (DOD) Breast Cancer Program [Grant # W81XWH-11-1-0211].

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Graphical Abstract