Probing the effects of fish oil on the delivery and inflammation-inducing potential of imiquimod

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ABSTRACT

Imiquimod is a chemotherapeutic agent for many skin-associated diseases, but it has also been associated with inflammatory side effects. The aim of this study was to prevent the inflammatory effect of commercial imiquimod (Aldara®) by controlled release of imiquimod through a hydrogel/oleogel colloidal mixture (CA bigel) containing fish oil as an anti-inflammatory agent. Imiquimod permeability from Aldara® cream and bigel through mice skin was evaluated, and the drug content residing in the skin *via* the tape stripping technique was quantified. The fish oil fatty acid content in skin along with its lipophilic environment was also determined. An inflammation study was conducted using animal models, and Aldara® cream was found to potentially cause psoriasis-like inflammation, which could be owing to prolonged application and excessive drug permeation. Controlled release of imiquimod along with fish oil through CA bigel may have caused reduced imiquimod inflammation. NMR studies and computerized molecular modeling were also conducted to observe whether the fish oil and imiquimod formed a complex that was responsible for improving imiquimod transport and reducing its side effects. NMR spectra showed dose-dependent chemical shifts and molecular modeling revealed π–σ interaction between EPA and imiquimod, which could help reduce imiquimod inflammation.

1. Introduction

In dermatology, topical drugs are intended to treat diseased areas of the skin such that the drug distribution is restricted to the affected tissue alone. The permeation of topical drugs through the skin leading to systemic circulation is generally not desired, and may, in some instances, lead to systemic side effects. Imiquimod is an immune response modifier that is currently used to treat skin diseases such as actinic keratosis, basal cell carcinoma, neoplasia, and perianal warts in adults (Graells et al., 2014; Hadley et al., 2006; Jin et al., 2009; Tarbet et al., 2011; Teicher et al., 1997), but it is also known for its inflammatory-like side effects, such as severe skin inflammation (Cantisani et al., 2012; Rosenblatt and de Campos, 2012). Fish oil is a unique candidate for reducing inflammatory side effects. It possesses omega-3 fatty acids (especially eicosapentaenoic acid and docosahexaenoic acid), which are associated with many beneficial pharmacological activities in various diseases, such as cardiovascular disease (Darren and Bruce, 2004), breast cancer (Neil et al., 2013), and pancreatic cancer (Liam et al., 2014), and they are also considered anti-inflammatory agents (Carmelo et al., 2005; Zulfakar et al., 2012).

Furthermore, the fish oil omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have also been shown to enhance the topical permeation of drugs (Zulfakar et al., 2010) and have been utilized as a drug delivery vehicle (Huri et al., 2013; Rehman et al., 2014). Following topical application to the skin, polyunsaturated fatty acids are believed to become incorporated within keratinocytes (Christopher and Charles, 2007; Nugteren et al., 1995), and the delivery of these fatty acids together with imiquimod may help improve skin permeation as well as reduce the inflammatory side effects of the drug.

In an earlier paper, we formulated a bigel, a hydrogel/oleogel mixture, composed of fish oil and a polymer (Khurram et al., 2014) that highlighted the controlled delivery of imiquimod. The present study is divided into two sections. First, the permeability of the commercial product of 5% imiquimod (Aldara®) and the formulated fish oil–carbopol bigel containing 5% imiquimod were compared using a mouse skin model. Then, animal models (Swiss albino mice) were used to determine the extent of the

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inflammatory side effects of imiquimod and the reduction of those side effects by controlled drug delivery using carbopol–fish oil bigels. Histopathological studies were also conducted to confirm the possible side effects of imiquimod topical administration.

Last, we investigated the possibility of an interaction between fish oil and imiquimod using proton NMR, and the type of interaction and its potential influence were predicted through molecular modeling. Molecular models were constructed to predict the possible geometrical parameters of EPA- or DHA-imiquimod complexes and their binding energies to assess the possibility of the pull effect phenomenon (Christopher et al., 2007; Kadir et al., 1987).

2. Materials and methodology

2.1. Materials

Blackmores Fish Oil (42% EPA and 21% DHA) was purchased from Blackmores (Warriewood, Australia), and benzalkonium chloride and carbopol 940 were purchased from ACROS (New Jersey, USA). Aldara® cream was purchased from a local pharmacy, triethanolamine was obtained from Friendemann Schmidt (Parkwood, Australia), and beeswax was obtained from R & M Chemicals (Selangor, Malaysia). Cetrimide, butylated hydroxyanisole, and imiquimod powder were purchased from Sigma–Aldrich (St. Louis, USA). Deuterated chloroform, hexane, and sodium methoxide were purchased from Merck (Frankfurt, Germany), and Swiss albino mice were obtained from an animal house at the National University of Malaysia.

2.2. Preparation of bigel

Bigels were prepared as previously described (containing 5% imiquimod in each formulation) (Khurram et al., 2014) by first individually preparing carbopol hydrogel and fish oil oleogel, and then through the physical mixing of both gels, a homogenous gel was formed (bigel). Each bigel was prepared by mixing 50:50 ratio of hydrogel and oleogel (Table 1) using mechanical stirring (800 rpm) for 15 min at room temperature. All the formulations were stored at $5 \pm 2\,^{\circ}C$. CA bigel consisted of 5% imiquimod dispersed in oleogel phase, whereas CB bigel was the blank bigel composed of the same ratio of hydrogel and oleogel as that of CA bigel.

2.3. Preparation of the skin model

The Swiss albino mice were anesthetized and killed by cervical dislocation, and the fur on the dorsal side was removed with a clipper (Aesculap, Melsungen, Germany), while taking precaution to avoid any skin abrasion. The dorsal portion of the skin was excised from the body, and subcutaneous tissues were surgically removed. Skin was washed with PBS, carefully wiped with a cotton swab dipped in isopropyl alcohol to remove adhering fats, and mounted on a Franz cell (SC facing the donor compartment) for *ex vivo* analysis. The receptor compartment of the Franz diffusion cell (PermeaGear Inc., USA) had a volume of 3.5 mL and a contact area of 0.95 cm^2. It was filled with cetrimide solution that acted as a receptor medium, and skin temperature was maintained at $32 \pm 1\,^{\circ}C$ with water bath and stirred at 300 rpm. The donor compartment was equilibrated with the receiver compartment for 1 h to facilitate skin hydration. After equilibration, sample (50 mg) was applied to the skin, and the permeation at different time intervals was analyzed for 48 h. Statistical significance of *ex vivo* permeation study between Aldara® and CA-bigel was analyzed using paired sample *t*-tests with SPSS 20 software (IBM Cooperation, USA).

Table 1
Ratio of hydrogel and oleogel in bigel formulations.

Bigel	Hydrogel	Oleogel	Imiquimod (%)
CA	50	50	5
CB	50	50	–

Furthermore, the drug permeation data were fitted to different mathematical models (Korsmeyer–Peppas, Higuchi, Hixson–Crowell, zero order, and first order models) to analyze the release mechanism. All equations were fitted to the whole release curves, except for Peppas equation fitted only up to 60% of drug release. The correlation coefficients were determined from regression plots and this mathematical modeling of release kinetics would highlight the drug transport mechanism involved in controlled release. The equations of different kinetic release used in this study are given below.

$$\text{Zero order equation} : Q = Q_0 - K_0 t \quad (1)$$

$$\text{First order equation} : \text{Log}Q = \text{Log}Q_0 - K_1 t \quad (2)$$

$$\text{Higuchi equation} : Q = K_2 t^{1/2} \quad (3)$$

$$\text{Hixson} - \text{Crowell equation} : Q0^{1/3} - Qt^{1/3} = Kst \quad (4)$$

$$\text{Korsmeyer} - \text{Peppas equation} : \frac{Q}{Q_0} = Kt^n \quad (5)$$

In the equations, K_0–K_2 are release constants, Q/Q_0 represents the fraction of drug release at time *t*, and *n* is diffusion constant that indicates the general release mechanism. The "*n*" value from the release exponent of Korsmeyer–Peppas model could be used to characterize the release mechanisms.

2.4. *Depth profiling* via *the tape stripping technique*

Depth profiling of imiquimod was used to estimate the amount of drug retained inside the Franz cell-treated skin layers. After 48 h, the skin mounted on Franz diffusion cells was carefully removed, and the remaining portion of the formulation adhering to the skin was scraped off with a spatula. To determine the amount of drug retained in stratum corneum (SC) and the epidermis and dermis (EPD), a tape stripping technique was employed to separate the stratum corneum (SC) from the viable epidermis and dermis (Coderch et al., 1996; Lademann et al., 2009; Rohrbach et al., 1977). Ten strips were taken from the skin by applying adhesive tape and removing it with a pair of forceps. The first 2 strips were discarded, and the subsequent strips were placed into a jar to which extraction solvent was added. Imiquimod was extracted from skin samples using a 7:3 (v/v) methanol:acetate buffer (pH 4.0) (De Paula et al., 2008; Zahid et al., 2013) solution by mechanically stirring overnight in a water bath (BW-20G, Lab Companion, USA), followed by ultracentrifugation (Beckman Coulter, California, USA) for 20 min (12,000 rpm at 10 °C) and sonication for 2 h at room temperature. Fatty acids were also extracted from the skin samples using hexane, and later they were converted into fatty acid methyl esters (FAMEs). Imiquimod and fatty acids were analyzed using liquid chromatography and gas chromatography, respectively.

2.4.1. Skin content of imiquimod

The chromatographic system consisted of an RP-HPLC (Shimadzu LC-20AT with an SIL-20A autosampler and an SPD-M20A DAD detector, Shimadzu Corp., Kyoto, Japan) and Altima C8 column (4.6 × 150 mm; 5 μm). The mobile phase for imiquimod analysis consisted of acetonitrile, water, and phosphoric acid (250:750:10) adjusted to a pH of 2.7, delivered at a flow rate of 1.5 mL/min with an injection volume of 20 μL. The imiquimod permeated through the skin membrane into the receptor phase of Franz cells. Drug was extracted from skin layers through the tape stripping technique and was monitored at a wavelength of 254 nm and calculated against a calibration curve.

2.4.2. Skin content of fatty acids

Fatty acid analysis was conducted using a gas chromatography (GC) 2010 instrument (Shimadzu Corp., Kyoto, Japan) with a flame ionization detector (FID). The injector temperature was maintained at 250 °C, and the detector temperature was maintained at 275 °C. The temperature of the column was maintained at 180 °C for 2 min, and then it gradually increased to 240 °C at a rate of 4 °C/min. Nitrogen was used as a carrier gas with a flow rate of 60 cm/s. Fatty acid methyl esters (FAMEs) were prepared by adding 0.5 mL 0.5 M sodium methoxide solution (vortexed for 1 min) (Khurram et al., 2014), and the amount of EPA and DHA that penetrated into the skin membrane was determined.

2.5. ATR-FTIR

To investigate the influence of fish oil on skin layers, the Fourier Transformation Infrared (FTIR) spectroscopic technique by attenuated total reflection (ATR) method was performed. Different layers of skin (separated through the tape stripping technique) were examined from 4000 to 550 cm^{-1}. The influence of fish oil fatty acids on the structure of the skin membrane was studied, and its influence on imiquimod inflammatory side effects was predicted. Franz cell-treated blank skin samples (without any formulation applied) were used as a control for the comparison.

2.6. Imiquimod skin inflammation analysis

The skin inflammation study was conducted on female Swiss albino mice. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Universiti Kebangsaan Malaysia. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Universiti Kebangsaan Malaysia (approval number: FF/2014/HANIF/24-SEPT.16/612-OCT.-2014-FEB.-2015). The mice were evenly divided into 4 groups with each group consisting of 6 animals. Group A was the control group with no treatment, Group B was treated with commercial 5% imiquimod cream (Aldara®), Group C was treated with 5% imiquimod carbopol–fish oil bigel (CA) and Group D was treated with blank bigel formulation (CB). Group B, C, and D were treated with 62.5 mg (3.25 mg of active ingredient) of formulation each day for 14 days, and every 2 days they were monitored for transdermal epidermal water loss (TEWL) and the presence of erythema. After 14 days, skin samples were taken, and histopathological studies were also conducted to analyze the presence of any inflammatory effects of imiquimod on the skin.

2.7. Nuclear magnetic resonance

To investigate any possibility of interaction between fish oil and imiquimod, proton NMR studies were conducted. Saturated solutions of imiquimod in fish oil, 75:25, 50:50, and 25:75 mixtures of fish oil and imiquimod, and pure fish oil and imiquimod were prepared. To achieve a constant amount of imiquimod, equivalent volumes of saturated solutions in $CDCl_3$ were transferred into NMR tubes. ^1H NMR spectra were acquired on a Bruker Avance DPX600 spectrometer operating at 600 MHz and at 25 °C. The chemical shifts were determined relative to the imiquimod and fish oil control.

2.8. Molecular modeling

Computerized molecular modeling of imiquimod and fatty acids (EPA and DHA) were performed to predict the type of interaction between the fish oil and imiquimod and the influence

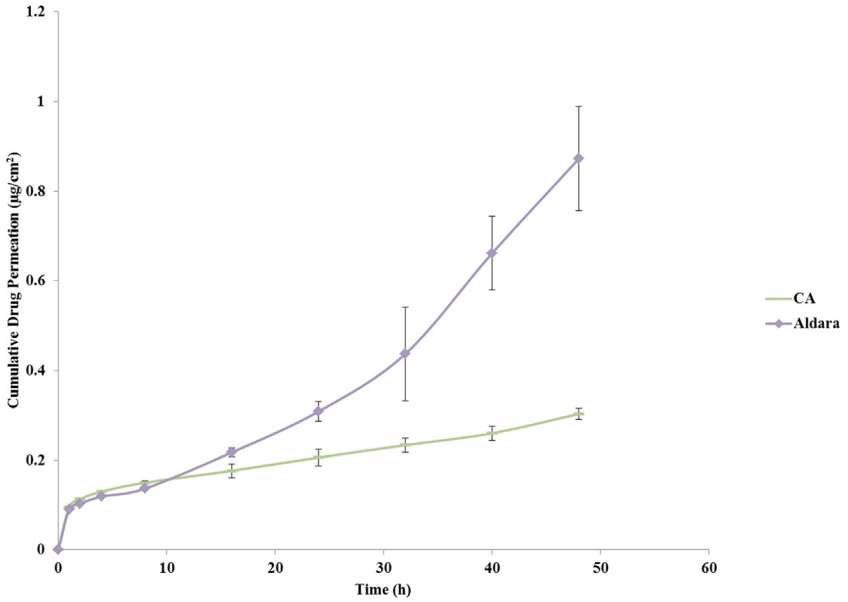

Fig. 1. Cumulative permeation of imiquimod through Aldara® cream and CA bigel formulation for the period of 48 h using excised mice skin. Aldara® cream exhibited high permeation across the skin membrane compared to the CA bigel formulation ($p < 0.05$).

this interaction could have on imiquimod activity. All of the 2D and 3D structures of the individual imiquimod molecules and the EPA and DHA fatty acid compounds were built with ChemBioOffice® 2008 (PerkinElmer, Inc., Waltham, MA, USA). Minimization using the CHARM energy protocol section in Discovery Studio® 3.1 (Accelrys, Inc., San Diego, CA, USA) has been established in our lab (Rullah et al., 2014, 2015). The calculations were performed at 1000 steps of steepest descent with an RMS gradient tolerance of 3, followed by conjugate gradient minimization with the maximum number of cycles set to 2000 for the minimization and 0.1 kcal/(mol × Å) of minimization for the RMS Gradient. Docking studies were performed with the CDOCKER protocol section in Discovery Studio® 3.1. Complexes between imiquimod and EPA or DHA were constructed by bringing together optimized structures and re-optimizing them using the same method. The conformational energy landscape of these complexes was explored by randomly altering the mutual orientation of drug and fatty acid as well as the dihedral angles of any rotatable bonds. This was followed by full energy minimization. During the docking process, the fatty acids as macromolecules were held rigid while imiquimod was allowed to flex during the refinement. Imiquimod was heated to 700 K in 2000 steps, and the cooling temperature was set to 300 K in 5000 steps. Grid extension was set automatically based on individual fatty acids. Finally, 10 conformation imiquimod and EPA or DHA poses were ranked according to their CDOCKER interaction energy, and the predicted binding interactions were analyzed.

3. Results and discussion

3.1. Imiquimod permeation analysis

Imiquimod permeation through skin was studied on excised mice skin using the commercial product 5% imiquimod (Aldara®) and bigel formulation (CA). Since Aldara® is in a cream form, it unsurprisingly showed higher drug permeation (0.87 ± 0.12 μg/cm^2) compared to the CA bigel formulation (0.30 ± 0.01 μg/cm^2). The CA bigel formulation exhibited more of a controlled release pattern (as shown in Fig. 1), whereas Aldara® cream does not show any controlled release and led to a massive permeation of the imiquimod inside the receptor phase. The study was conducted for 48 h to investigate the outcome of prolonged imiquimod formulation use, but the permeation comparison up to 8 h, which is the normal time after which the applied formulation might be washed off or removed from the skin surface, between both the Aldara® cream and CA bigel was quite similar (0.14 ± 0.03 μg/cm^2 and 0.15 ± 0.01 μg/cm^2, respectively). The permeation from Aldara® and CA-bigel was also found to be statistically significant ($p < 0.05$). The higher skin permeation displayed by imiquimod from Aldara® cream could possibly contributes to the imiquimod-related inflammatory side effects. On the other hand, whether the lesser amounts of imiquimod permeating from the bigel formulation results in reduced clinical efficacy is yet to be determined. Further work on induced tumor in animal model is currently ongoing, with preliminary data suggestive of comparable tumor inhibition to the commercial cream (unpublished data).

The kinetic mechanism from the drug permeation from all the formulations plotted and calculated the coefficients of the drug release (R^2) and exponent "n" for Kormeyer-model are presented in Table 2. The ideal kinetic models for drug release and permeation were estimated using the different mathematical equation plots: cumulative amount of drug permeated *vs.* time (zero-order model), log cumulative percentage of drug remaining *vs.* time (first-order model), cumulative percentage drug permeated *vs.* square root of time (Higuchi model), cube root of drug percentage remaining in matrix *vs.* time (Hixson–Crowell model) and log cumulative percentage drug permeated *vs.* log time (Korsmeyer–Peppas model). From the regression equations it was observed that the Aldara® follow a first-order release pattern and CA bigel followed the zero-order equation. Higuchi equation was also a good fit for the CA bigel permeation. To confirm the diffusion controlled mechanism the data was fitted into the Korsmeyer's model and CA bigel showed a good linearity (0.99) and the diffusional coefficient "n" value of the was 0.23 indicating that the diffusion is indeed the dominant mechanism of drug permeation and is classified as quasi-Fickian diffusion (partial diffusion). Whereas, Aldara cream had a good linearity fit with Hixson–Crowell model and it could be assumed that the drug release and permeation through the skin is controlled by the drug particles dissolution and not by the diffusion which occurs in bigels due to polymer matrix.

3.2. Depth profiling

3.2.1. Skin content of imiquimod

Imiquimod entrapped in SC and EPD was extracted out from the skin samples by a previously described method and was analyzed by HPLC. The content of imiquimod inside the skin was found to be higher for Aldara® cream than for CA bigel (1.57 ± 0.52 mg/cm^2 and 0.93 ± 0.05 mg/cm^2, respectively) as shown in Fig. 2A. Similarly, in the epidermis and EPD layers, the amount of imiquimod following Aldara® cream treatment was also higher than CA bigel (0.85 ± 0.34 mg/cm^2 and 0.30 ± 0.04 mg/cm^2). These results correlate with the permeation analysis, but the higher entrapment of imiquimod in SC from Aldara® could also result in inflammatory action. If the skin is damaged, as in the case of skin carcinoma, the SC will no longer be there as a permeation barrier and all the entrapped imiquimod might penetrate into the body and cause severe, adverse reactions.

3.2.2. Skin content of fatty acids

The fish oil fatty acids EPA and DHA are known for their anti-inflammatory activities. CA bigel contains these fatty acids, and if they also penetrate into the SC and epidermis of the skin, it might help reduce some of the inflammatory side effects of imiquimod. To measure the content of EPA and DHA in SC and EPD following CA bigel treatment, depth profiling *via* the tape stripping technique was performed. The extracted samples were analyzed by gas chromatography. The amount of EPA present in the SC was greater than the amount present inside the EPD (5.56 ± 0.32 mg/cm^2 and 2.36 ± 0.02 mg/cm^2, respectively), as shown in Fig. 2B. The same trend was observed for DHA content in SC and EPD as well (4.35 ± 1.04 mg/cm^2 and 2.10 ± 0.05 mg/cm^2, respectively). As

Table 2
Correlation values (R^2) of drug permeation kinetic equations and "n" value of diffusion constant from Korsemeyer–Peppas model indicating the release mechanism.

Formulation	Zero order	First order	Higuchi	Hixson–Crowell	Korsemeyer–Peppas	
	R^2	R^2	R^2	R^2	R^2	n
Aldara	0.94	0.99	0.84	0.99	0.91	0.43
CA	0.99	0.94	0.98	0.95	0.99	0.23

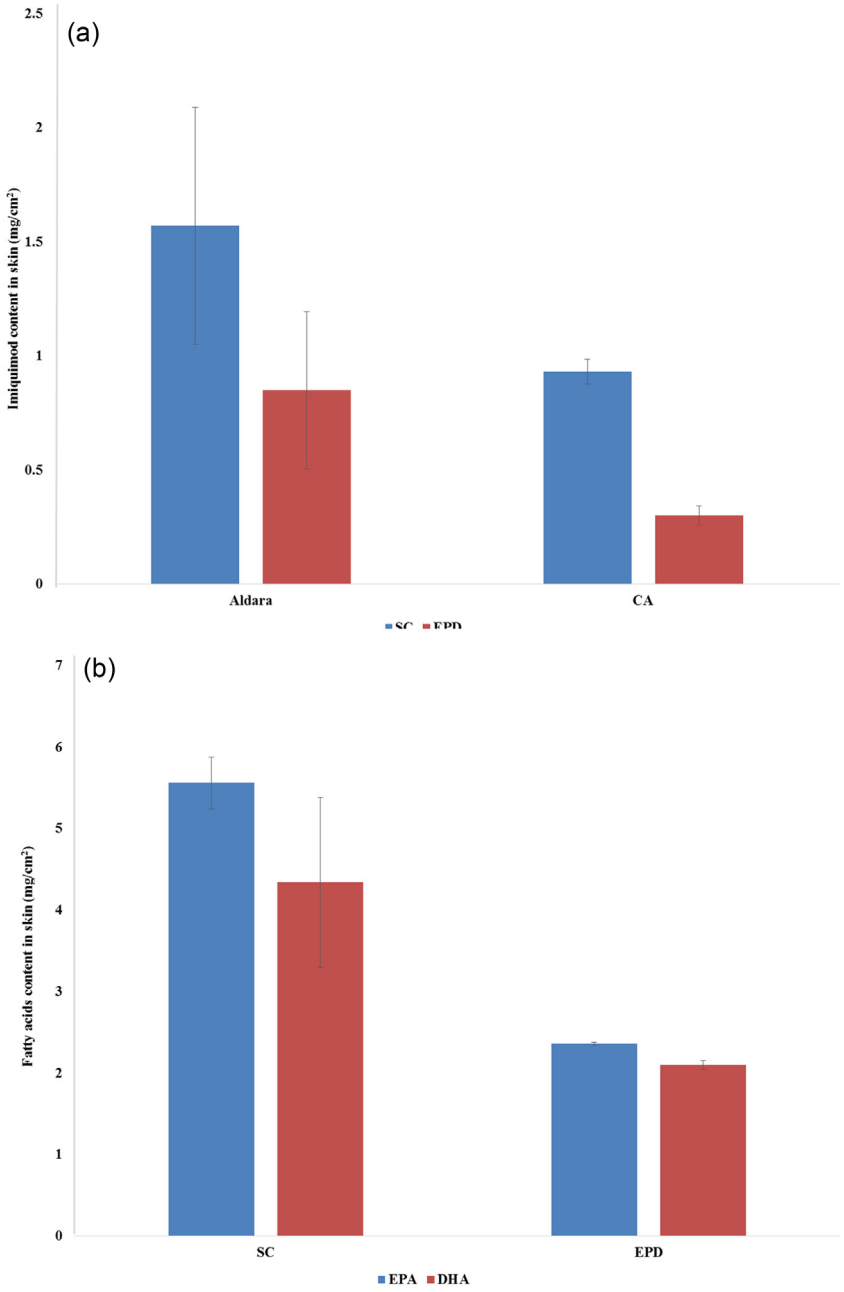

Fig. 2. (A) Content of imiquimod for Aldara® cream and CA bigel in stratum corneum (SC), epidermis and dermis of the skin (EPD). High presence of imiquimod was found in SC and EPD regions of the skin for both Aldara® cream and CA bigel formulation. (B) Primary fish oil fatty acids (EPA and DHA) are appreciably present in both stratum corneum (SC) and epidermis and dermis of the skin (EPD) in the CA-bigel treatment group, which may explain the inflammatory side effects-reducing potential of the formulation.

observed earlier in the imiquimod skin content analysis, the amount of imiquimod following CA bigel treatment was also higher in SC than the epidermis. Through GC analysis, it can be predicted that the fatty acids (EPA and DHA) present in the skin might help increase the lipophilic environment of the skin. The presence of these omega-3 anti-inflammatory fatty acids along with imiquimod inside the skin may help reduce the imiquimod-related inflammatory side effects.

3.3. ATR-FTIR

To confirm that the CA bigel formulation increased the lipophilic environment of the skin, skin samples were treated for 48 h with Franz diffusion cells and were investigated through ATR-FTIR, and the tape stripping technique was employed to separate the stratum corneum and epidermis layers. The FTIR spectrum of untreated blank SC (control) showed various peaks (Fig. 3). The absorption bands in the wave numbers between the 3000 and 2700 cm^{-1} bond were due to C—H stretching of the alkyl groups present in both proteins and lipids. The bands at 2955 cm^{-1}, 2920 cm^{-1}, 2870 cm^{-1}, and 2850 cm^{-1} were due to —CH_2 and —CH_3 vibrations of the long chain hydrocarbons of lipids. These narrow bands were attributed to the long alkyl chains of fatty acids, ceramides, and cholesterol, which are the major components of the SC lipids. The two strong bands (1650 cm^{-1} and 1550 cm^{-1}) were due to the amide I and amide II stretching vibrations of SC proteins. The amide I and amide II bands arise from C=O stretching vibration and C—N bending vibration, respectively. The amide I band

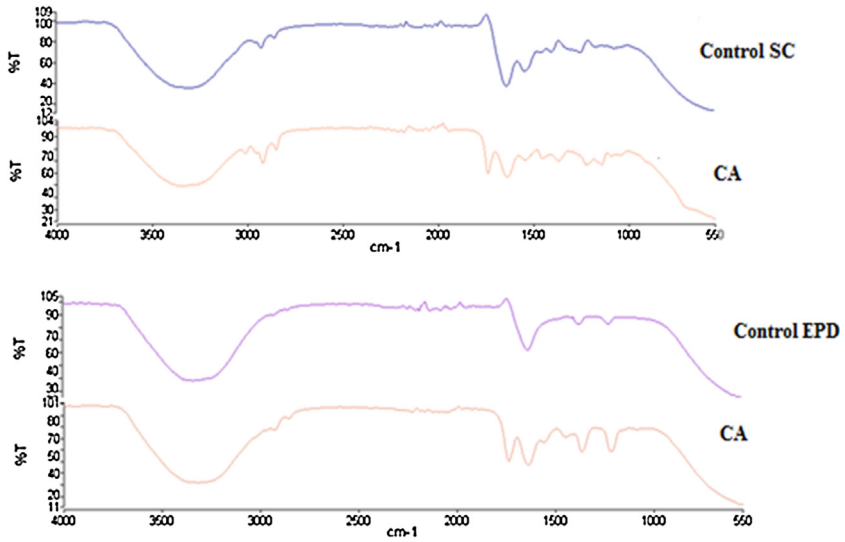

Fig. 3. ATR-FTIR spectrum of untreated and bigel treated skin skin samples exhibiting the increased in lipophlic nature of the skin through fish oil fatty acids from CA bigel.

consists of component bands, representing various secondary structures of keratin (Faiyaz et al., 2008). When skin was treated with bigel formulation (CA), increased signal intensity was observed (around 2700 cm^{-1}–3000 cm^{-1}), along with the presence of a new peak around 1740 cm^{-1}, representing a C=O ester stretch from fish oil triglycerols. The increase in the intensity of peaks is due to the presence of fish oil inside the formulations, which increased the lipophilic nature of the skin, thus resulting in higher peak intensity. Fish oil displayed higher intensity at 2700 cm^{-1}–3000 cm^{-1} (in the SC and EPD layers), which represents the CH_2=CH_2 bonds of fatty acids present inside fish oil. From the ATR-FTIR analysis, an increase in the lipophilic environment can be confirmed, which could be beneficial in reducing skin inflammation. These results also correlate with the GC data, and suggest that increased concentrations of EPA and DHA could be responsible for the lipophilic environment inside the skin. The increase in the lipophilic environment may help reduce inflammation across the skin membrane.

3.4. Imiquimod skin inflammation analysis

To investigate the inflammatory side effects of prolonged imiquimod use, a skin inflammation study was conducted using female Swiss albino mice for 14 days, and parameters like TEWL and erythema were observed to detect any presence of inflammatory symptoms. During the study, marked increases in TEWL and erythema were observed in mice of Group B, which were being treated with Aldara® cream. By the end of the study, the TEWL of Group B animals was found to be 22.36 ± 4.79 and the erythema value was 201.78 ± 40.41, which are quite high compared to the other groups (as shown in Fig. 4A and B). Group C, which was being treated with CA bigel, also showed higher values of TEWL and erythema (5.42 ± 0.62 and 100.06 ± 13.81, respectively), but they were quite low compared to Group B animals. Group D was administered blank bigel (CB) formulation and showed similar results to the untreated control Group A. From the TEWL and erythema results, it was evident that the prolonged application of imiquimod on skin may result in skin inflammation, as was observed in both Group B and Group C, but Group B showed very severe inflammation whereas Group C's inflammation was very mild. The discrepancy between the nature of the inflammation between the two groups may be due to two possibilities: (1) the controlled release of imiquimod through CA bigel and (2) the increased lipophilic environment of the skin owing to the anti-inflammatory fish oil fatty acids (EPA and DHA), as discussed earlier.

The physical appearance of Group B mice (treated with Aldara® cream) skin also showed signs of hair loss, the appearance of silvery white scales, and erythematous papules (Fig. 5), which resulted in marked increases in erythema values. Decreased skin hydration due to TEWL, silvery scales with no hair growth, and erythematous papules are the classical symptoms of psoriasis-like inflammation. Furthermore, to investigate this inflammation, histopathological studies of the skin samples were conducted. Marked increases in epidermal layer thickness were observed in Group B, which increased to 60.9–75.1 μm, whereas in the untreated control Group A, the epidermal layer thickness was merely 14.3–26.9 μm (Fig. 6). Even in Group C and Group D, the epidermal layer thickness was found to be between 18.6 μm and 26.6 μm. This epidermal hyperplasia (thickening of the skin) in Group B is also termed acanthosis and is an indicator of psoriasis. Furthermore, Group B histology also exhibited elongated rete-like ridges and the presence of neutrophils in the epidermis (micro-abscesses). There was also evidence of hyperkeratosis, and no such indications were observed in Group C histology. Thus, it could be concluded that prolonged Aldara® cream treatment results in psoriasis-like inflammation, whereas CA-bigel, which shows controlled release of imiquimod along with anti-inflammatory fatty acids (EPA and DHA), avoids the severe inflammatory effects of imiquimod.

3.5. NMR

NMR spectra have been shown to be highly sensitive to the local chemical environment, and the technique has been used previously to probe π–π interactions, where such processes are manifested as up or downfield shifts depending on the magnitude of shielding/deshielding modulation (Kelly et al., 2001). Different ratios of binary mixtures of fish oil:imiquimod (75:25, 50:50, and 25:75) were prepared in order to investigate the influence of fish oil on imiquimod activity or permeation, and proton NMR spectra were obtained (Fig. 7). From the examination of the NMR spectra, it would appear that the protons are most susceptible to shifts in the presence of fish oil. As the isobutyl moiety signal overlaps with the fish oil signal, only chemical shifts of the aromatic protons of imiquimod were measured. Fig. 8 shows the effect of fish oil on the

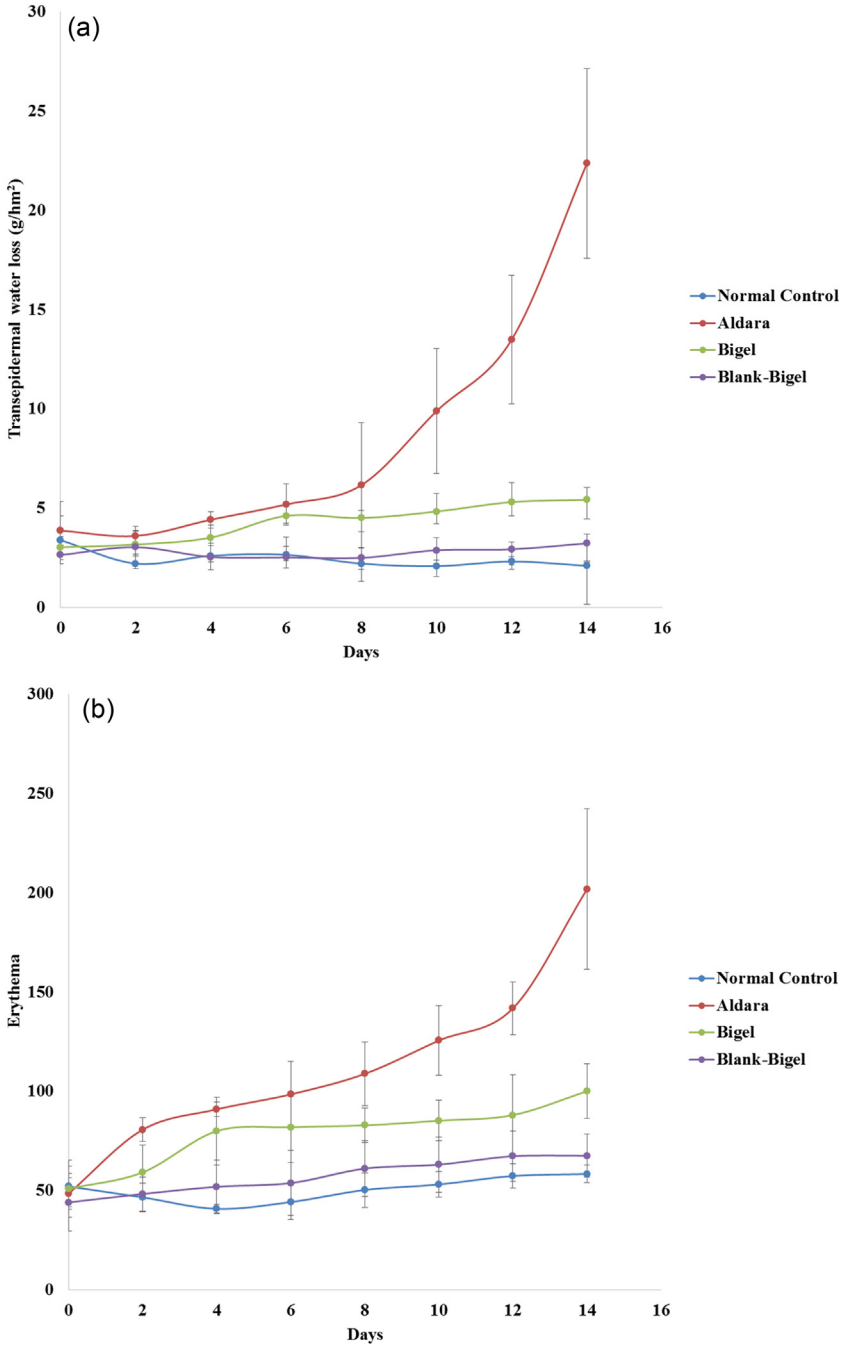

Fig. 4. (A) Transepidermal water loss values obtained from mice for the period of 14 days. Aldara® cream treated mice exhibited highest epidermal water loss as compared to bigel formulations and untreated control mice. (B) Erythema values obtained from mice for the period of 14 days. Aldara® cream treated mice exhibited highest erythema score as compared to bigel formulations and untreated control mice, indicating the presence of an inflammatory reaction.

^{1}H chemical shift of the benzene moiety of imiquimod. It is clear that the addition of fish oil to imiquimod results in dose-dependent downfield chemical shifts in the signals of the aromatic protons in the imiquimod structure.

The chemical shifts of proton Ha, Hb, Hc and Hd decreased with an increase in the mole ratio of fish oil (Fig. 8). This chemical shift pattern can be explained by the fact that the fatty acids in fish oil can influence π–σ interactions, and with the phenomenon of the push and pull effect, the fatty acids inside the fish oil can help increase imiquimod permeation through the skin or reduce it if the fatty acid–drug complex is formed. In turn, this might help reduce the inflammatory side effects. This transdermal delivery of vehicle complexed with drug is termed the pull effect phenomenon. Such facilitated transport of relatively large compounds across skin is the evidence of the pull effect in permeation enhancement (20, 28), but in this case it might also be helpful in anti-inflammatory action. The results show that the chemical shift might be affected by a CH/π interaction between the hydrocarbons and the benzene ring. To further strengthen our hypothesis, molecular docking simulation was also performed.

3.6. Molecular modeling

To elucidate the possible binding interactions of imiquimod with EPA and DHA, a molecular docking study was employed. The Structure Data Files for EPA and DHA were retrieved from

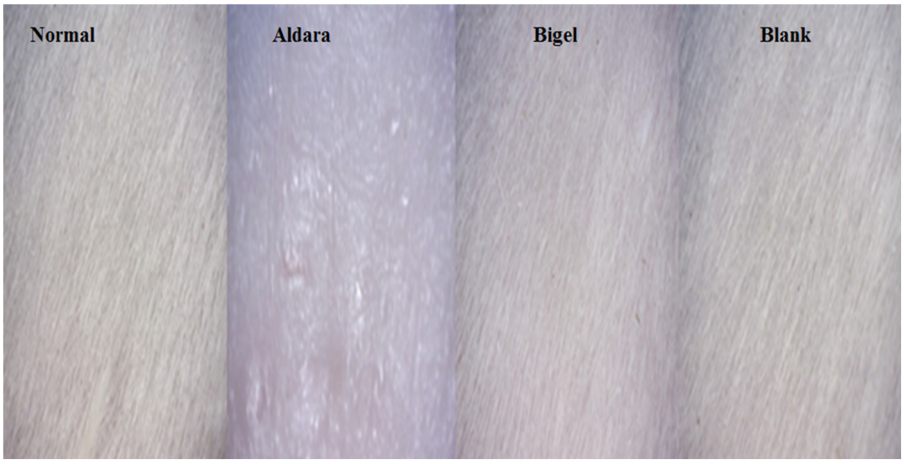

Fig. 5. Aldara® cream treated mice skin exhibited visible signs of psoriasis-like inflammation such as presence of silvery scales and absence of hair growth. Bigels treated skin however, appeared to be similar to the normal mice skin.

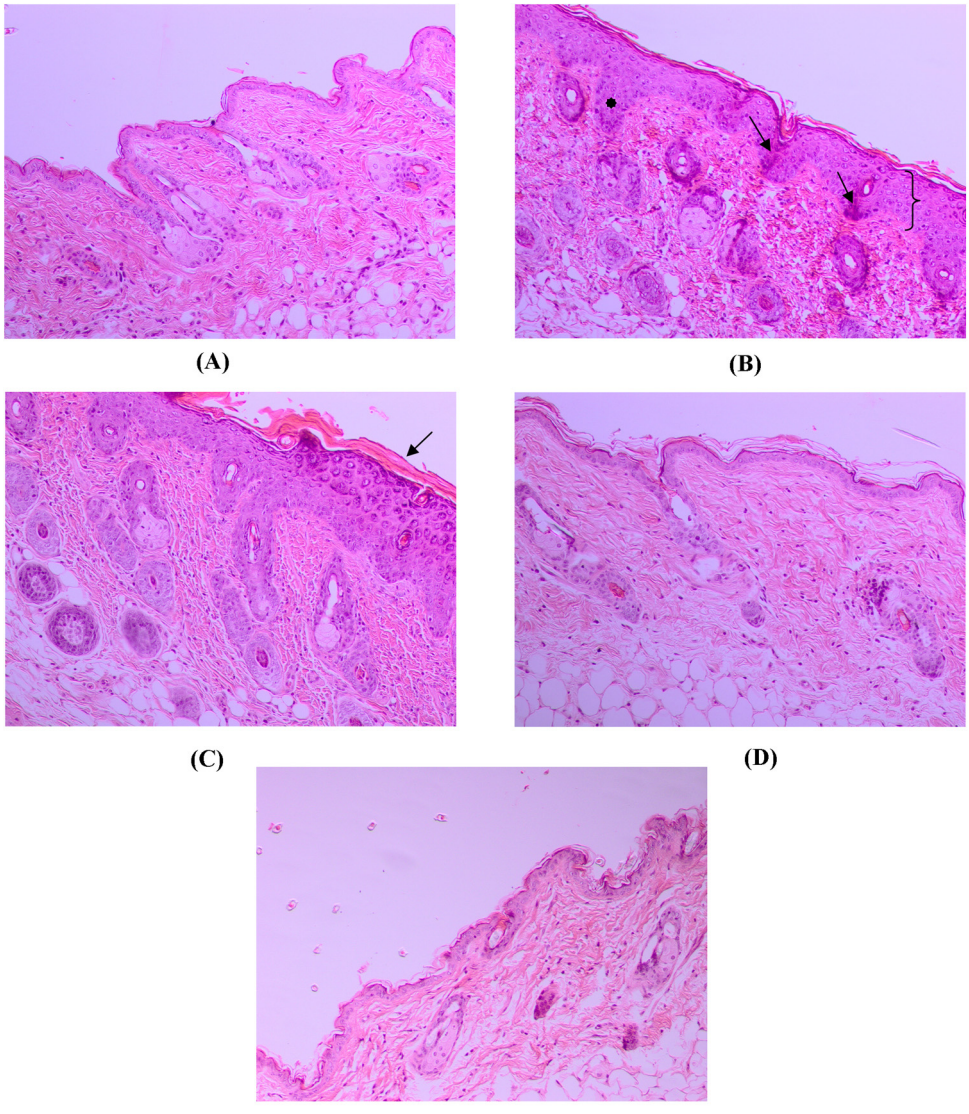

Fig. 6. (A) untreated control normal mice skin histology, (B) Aldara® treated mice showing increased epidermal thickness, micro abscesses and rete-ridges, (C) Aldara® treated mice showing hyperkeratosis, (D) CA bigel treated mice and (E) CB bigel treat skin histology was similar to the normal mice.

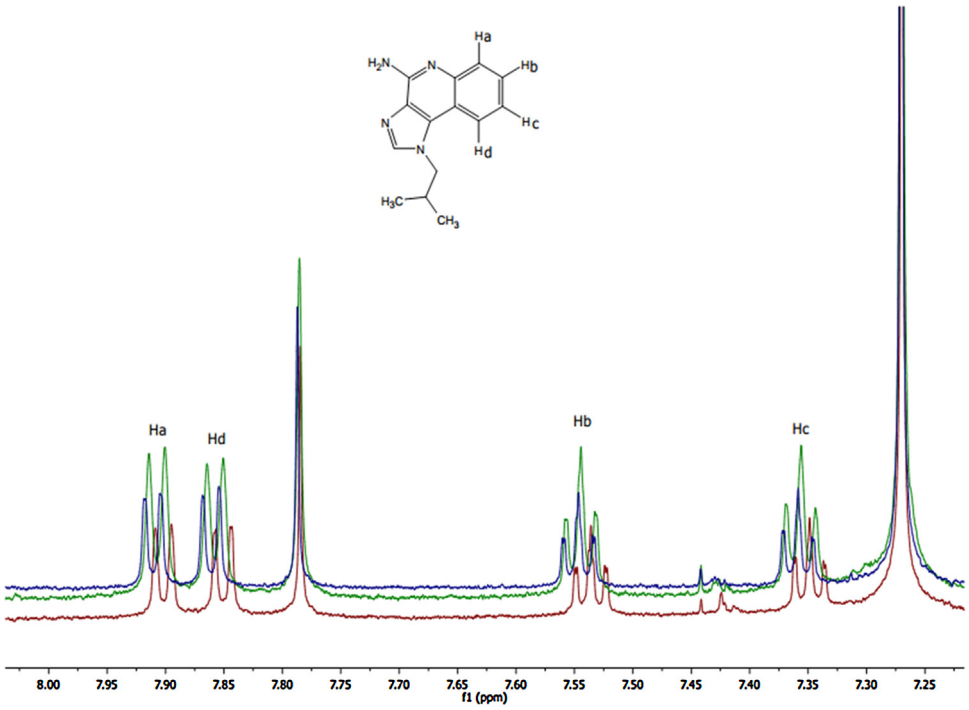

Fig. 7. NMR spectra of the aromatic region of the control imiquimod (A) and the different ratio mixtures of fish oil:imiquimod (purple: 25% fish oil; green: 50% fish oil; red: 75% fish oil). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

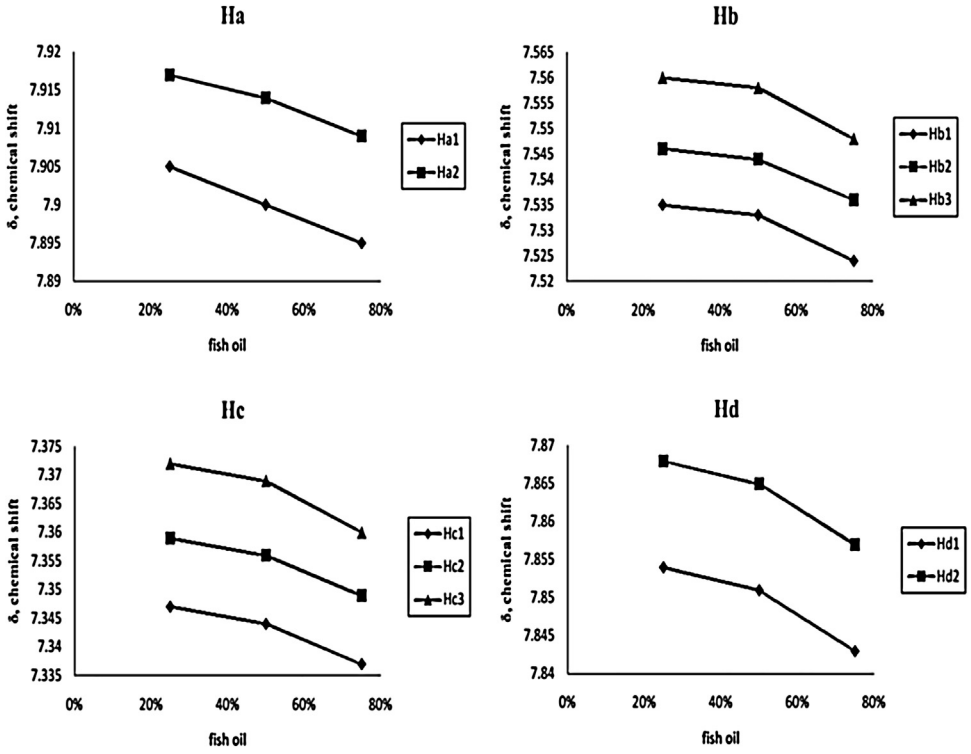

Fig. 8. Relative chemical shift of proton Ha, Hb, Hc and Hd in different ratio mixtures of fish oil:imiquimod, exhibiting the dose dependent downfield proton chemical shifts.

DrugBank (http://www.drugbank.ca). The obtained single crystal-XRD atomic coordinates for EPA and DHA, based on experimental crystals, were extracted from the ligand protein crystal structures of eicosapentanoic acid bound in the cyclooxygenase channel of prostaglandin endoperoxide H synthase-1 (**1IGX**: EPA) and the crystal structure of human brain fatty acid binding protein (**1FDQ**: DHA), which was retrieved from the Brookhaven Protein Data Bank. Molecular models were studied for the possibility of any interactions between molecules, including hydrogen bonding and van der Waals forces. The docking results showed that the aromatic quinoline ring of imiquimod forms a weak π–σ interaction of about 3.6 Å with the proton of EPA (aromatic ring···HC—). There are reasonably close contacts between the aromatic quinoline ring of imiquimod and proton-C_{12} in the EPA chain (Fig. 9), a possible

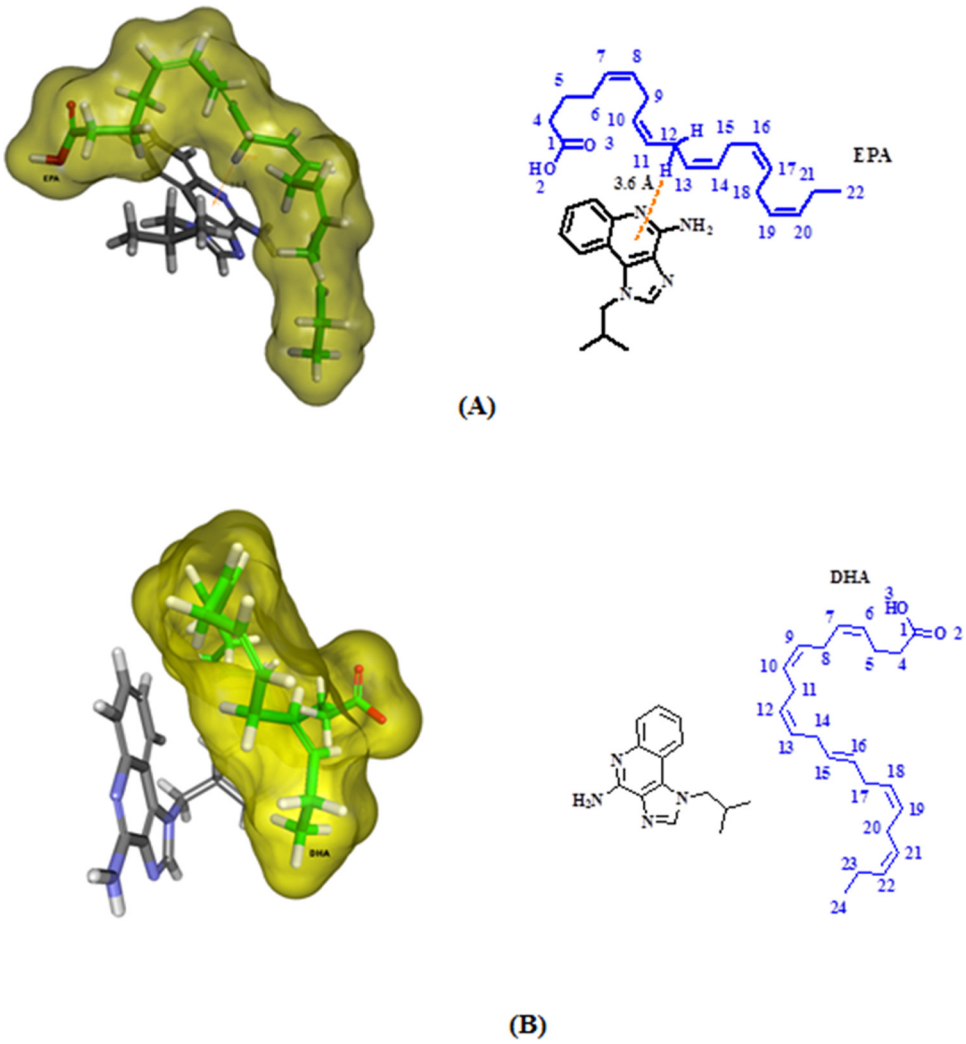

Fig. 9. (a) Molecular model of EPA and imiquimod showing the possible π···σ interaction between the aromatic ring of imiquimod and long of chain of fatty acid. (b) Molecular model of DHA and imiquimod showing lack of any interaction.

source of the increased stability of this complex. Molecular models of imiquimod with EPA also showed a binding energy of 53.14 kJ/mol^{-1}. Imiquimod and DHA did not show any such interaction (Fig. 9); this may be because DHA is not able to fit as easily around the imiquimod molecule owing to competition for space. Therefore, it did not produce any marked effect. EPA has an "open conformation" in the conduction alkene groups (C=C), and this could explain why the aromatic rings of imiquimod form a weak π–σ interaction with the proton of EPA. However, DHA with the "close conformation" and imiquimod show no evidence of any interaction. This π–σ interaction between imiquimod and the EPA molecule model predicts that EPA might influence imiquimod activity.

4. Conclusion

Imiquimod is an efficient chemotherapeutic agent for the treatment of many skin diseases, but it is also associated with inflammatory side effects. Aldara® cream (commercial imiquimod product) results in the massive permeation and high deposition of imiquimod in stratum corneum. This excessive amount of drug could cause inflammation. CA bigel demonstrated the controlled release of imiquimod, which was equal to imiquimod release from Aldara® cream up until 8 h following treatment, and it contained fish oil, which is known for its anti-inflammatory activity. Furthermore, the inflammation study in animal models exhibited psoriasis-like inflammation when animals were applied with Aldara® cream. The histopathological study of the skin confirmed the presence of inflammation caused by Aldara® cream. CA bigel does not cause any severe inflammation, which may be due to the controlled release of drug, the increased lipophilic environment of the stratum corneum, epidermis, and dermis layers, or the help of anti-inflammatory fish oil fatty acids (EPA and DHA). Lesser drug release from bigel formulations compared to Aldara® cream might be considered as a limitation at this point and further investigation on the therapeutic efficacy of the bigel formulation is needed, in view of the reduced imiquimod skin permeation. We also studied the possibility of an interaction between fish oil and imiquimod. Proton NMR data revealed a clear pattern of change in the chemical shifts of aromatic protons in imiquimod, the magnitude of which depends on the concentration of fish oil. Through computational molecular modeling, a chance of complex formation between imiquimod and EPA through a π–σ interaction between the aromatic ring of the drug and a long chain fatty acid was confirmed. Attractive interactions between π–σ systems such as these are possible; arising from electrostatic and dispersion-based forces, they may play an important role in biological systems and molecular recognition. Such interactions may also be beneficial for

synergetic effects, since EPA has a known anti-inflammatory activity, and, along with permeation, it may also help in reducing drug-related inflammatory side effects.

Conflicts of interest

Authors have no conflict of interest to report.

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References

Cantisani, C., Lazic, T., Richetta, A.G., Clerico, R., Mattozzi, C., Calvieri, S., 2012. Imiquimod 5% cream use in dermatology, side effects and recent patents. Recent Pat. Inflamm. Allergy Drug Discov. 6, 65–69.

Carmelo, P., Salvatore, T., Luisa, R., Natale, A.S., Francesco, B., 2005. Invitro percutaneous absorptiion studies and invivo evaluation of antiinflammatory activity of essential fatty acids (EFA) from fish oil extracts. Int. J. Pharm. 299, 41–48.

Christopher, P.T., Charles, M.H., 2007. Probing the skin permeation of eicosapentaenoic acid and ketoprofen 2. Comparative depth profiling and metabolism of eicospentaenoic acid. Eur. J. Pharm. Biopharm. 67, 156–165.

Christopher, P.T., Jamie, P., Thomas, T., Charles, M.H., 2007. Probing the skin permeation of fish oil/EPA and ketoprofen: 1. NMR spectroscopy and molecular modelling. Int. J. Pharm. 338, 207–212.

Coderch, L., Oliva, M., Pons, M., Maza, A.D., Manich, A.M., Parra, J.L., 1996. Percutaneous penetration of liposomes using the tape stripping technique. Int. J. Pharm. 139, 197–203.

Darren, J.H., Bruce, J.H., 2004. Omega-3 fatty acids from fish oils and cardiovascular disease. Mol. Cell. Biochem. 263, 217–225.

De Paula, D., Martins, C.A., Bentley, M.V., 2008. Development and validation of HPLC method for imiquimod determination in skin penetration studies. Biomed. Chromatogr. 22, 1416–1423.

Faiyaz, S., Sanjula, B., Alka, A., Javed, A., Sheikh, S., 2008. Skin permeation mechanism and bioavailability enhancement of celecoxib from transdermally applied nanoemulsion. J. Nanobiotechnol. 6 doi:http://dx.doi.org/10.1186/1477-3155-1186-1188.

Graells, J., Ojeda, R.M., Garcia-Cruz, A., 2014. Effect of imiquimod as compared with surgery on the cancerization field in basal cell carcinoma. Actas Dermosifiliogr. 105, 53–59.

Hadley, G., Derry, S., Moore, R.A., 2006. Imiquimod for actinic keratosis: systematic review and meta-analysis. J. Invest. Dermatol. 126, 1251–1255.

Huri, M.F.D., Shiow-Fern, N.G., Zulfakar, M.H., 2013. Fish oil-based oleogels: physicochemicals characterisation and in vitro release of betamethasone dipropionate. Int. J. Pharm. Pharm. Sci. 5, 458–467.

Lademann, J., Jacobi, U., Surber, C., Weigmann, H.J., Fluhr, J.W., 2009. The tape stripping procedure–evaluation of some critical parameters. Eur. J. Pharm. Biopharm. 72, 317–323.

Jin, J.Y., Jung, Y.J., Choi, S.S., Hwang, J., Chung, E., 2009. Autophagy mediated anti-tumoral of imiquimod in CaCo-2 cells. Biochem. Biophy. Res. Commun. 386, 455–458.

Kadir, R., Stempler, D., Liron, Z., Cohen, S., 1987. Delivery of theophylline into excised human skin from alkanoic acid solutions: a push–pull mechanism. J. Pharm. Sci. 76, 774–779.

Kelly, J.X., Winter, R., Riscoe, M., Peyton, D.H., 2001. A spectroscopic investigation of the binding interactions between 4,5-dihydroxyanthobne and heme. J. Inorg. Biochem. 86, 617–625.

Khurram, R., Mohd Amin, M.C., Zulfakar, M.H., 2014. Development and physical characterization of polymer–fish oil bigel (hydrogel/oleogel) system as a transdermal drug delivery vehicle. J. Oleo. Sci. 63, 961–970.

Liam, D.C., Siong-Seng, L., Ashok, R.V., 2014. Chromosome instability and carcinogenesis: insights from murine models of human pancreatic cancer associated with BRCA2 inactivation. Mol. Oncol. 8, 161–168.

Neil, M.I., Clifford, A.H., Ayca, G., 2013. Omega-3 fatty acids for prevention of breast cancer: an update and the state of science. Curr. Breast Cancer Rep. 5, 247–254.

Nugteren, D.H., Christ-Hazelhof, E., Van der Beek, A., Houtsmuller, U.M.T., 1995. Metabolism of linoleic acid and other essential fatty acids in the epidermis of the rat. Biochim. Biophys. Acta 834, 429–436.

Rehman, K., Tan, C.M., Zulfakar, M.H., 2014. Development and in-vitro characterization of fish oil oleogels containing benzoyl peroxide and salicylic acid as keratolytic agents. Drug Res. 64, 159–165.

Rohrbach, R., Iversen, O.H., Riede, U.N., Sandritter, W., 1977. Effects of cellophane tape stripping of mouse skin on epidermal growth regulators (chalones). Beitr. Pathol. 160, 175–186.

Rosenblatt, A., de Campos, G.H.G., 2012. Local and systemic adverse effects of imiquimod therapy for external anogenital warts in men: report of three cases. Int. J. STD AIDS 23, 909–910.

Rullah, K., Mohd Aluwi, M.F.F., Yamin, B.M., Abdul Bahari, M.N., Wei, L.S., Ahmad, S., Abas, F., Ismail, N.H., Jantan, I., Wai, L.K., 2014. Inhibition of prostaglandin E_2 production by synthetic minor prenylated chalcones and flavonoids: synthesis biological activity, crystal structure, and in silico evaluation. Bioorg. Med. Chem. Lett. 24, 3826–3834.

Rullah, K., Mohd Aluwi, M.F.F., Yamin, B.M., Baharuddin, M.S., Ismail, N.H., Teruna, H. Y., Bukhari, S.N.A., Jantan, I., Jalil, J., Husain, K., Wai, L.K., 2015. Molecular characterization, biological activity, and in silico study of 2-(3,4-dimethoxyphenyl)-3-(4-fluorophenyl)-6-methoxy-4*H*-chromen-4-one as a novel selective COX-2 inhibitor. J. Mol. Struct. 1081, 51–61.

Tarbet, E.B., Larson, D., Anderson, B.J., Bailey, K.W., Wong, M.H., Smee, D.F., 2011. Evaluation of imiquimod for topical treatment of vaccinia virus cutaneous infections in immunosuppressed hairless mice. Antiviral Res. 90, 126–133.

Teicher, B.A., Ara, G., Buxton, D., Leonard, J., Schaub, R.G., 1997. Optimal scheduling of interleukin-12 and chemotherapy in the murine MB-49 bladder carcinoma and B-16 melanoma. Clin. Cancer Res. 3, 1661–1667.

Zahid, H., Haliza, K., Mohd, C.I.M.A., Endang, K., Fhataheya, B., Sahudin, S., 2013. Self-assembled polymeric nanoparticles for percutaneous co-delivery of hydrocortisone/hydroxytyrosol: an ex vivo and in vivo study using an NC/Nga mouse model. Int. J. Pharm. 444, 109–119.

Zulfakar, M.H., Abdelouahab, N., Heard, C.M., 2010. Enhanced topical delivery and ex vivo anti-inflammatory activity from a betamethasone dipropionate formulation containing fish oil. Inflamm. Res. 59, 23–30.

Zulfakar, M.H., Ong, C.M.Y., Heard, C.M., 2012. The effects of betamethasone dipropionate and fish oil on HaCaT proliferation and apoptosis. Int. J. Pharm. 434, 399–405.