



Dictamnine delivered by PLGA nanocarriers ameliorated inflammation in an oxazolone-induced dermatitis mouse model

Chin-Yu Lin^{a,b,*}, Yun-Ting Hsieh^c, Long Yi Chan^a, Ting-Ya Yang^c, Tomoji Maeda^{b,d},
Tsong-Min Chang^e, Huey-Chun Huang^{c,*}

^a Institute of New Drug Development, College of Medicine, China Medical University, Taichung 40402, Taiwan

^b Tsuzuki Institute for Traditional Medicine, College of Pharmacy, China Medical University, Taichung, 40402, Taiwan

^c Department of Medical Laboratory Science and Biotechnology, College of Medicine, China Medical University, Taichung 40402, Taiwan

^d Department of Pharmaceutical Sciences, Nihon Pharmaceutical University, Kitaadachi-gun, Saitama 362-0806, Japan

^e Department of Applied Cosmetology and Master Program of Cosmetic Sciences, Hungkuang University, Taichung 43302, Taiwan

ARTICLE INFO

Keywords:

Atopic dermatitis
Dictamnine
Oxazolone
Polymeric nanocarrier
Drug delivery
Drug nanoformulation

ABSTRACT

Dictamnine is an active pharmaceutical ingredient in *Dictamnus dasycarpus*, a Chinese herbal medicine widely used for the treatment of skin inflammations such as atopic dermatitis (AD). Oxazolone has been demonstrated to induce significant skin inflammation and produce inflammatory cytokine expression identical to that of AD. An *in vitro* HaCaT inflammation model treated with dictamnine, which efficiently scavenged the reactive oxygen species (ROS) and mitochondrial ROS (mROS), and it reduced interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α) expression, NLRP3 inflammasome activation, and NF- κ B expression. To explore the anti-inflammatory mechanism of dictamnine and enhance sustained drug release and penetration into epidermal structures in a dermatitis mouse model, we prepared PLGA-nanocarrier-encapsulated dictamnine (Dic-PLGA-NC) in a specifically designed bioreactor, namely an ultrasound composite streams-impinging mixer (U-SiM). Mouse dermatitis model was treated with Dic-PLGA-NC medication, spleens were collected to evaluate body weight ratio, and skin was retrieved for histological examination and two-photon microscopy. The data demonstrate that Dic-PLGA-NC efficiently penetrated the dermal layer, making it superior to naked dictamnine; moreover, it ameliorated the dermatitis symptoms and inflammatory cytokine expression *in vivo*. Dic-PLGA-NC produced using the U-SiM bioreactor could be used in new manufacturing processes for drugs to treat AD.

Department of Medical Laboratory Science and Biotechnology, College of Medicine, China Medical University, No.91 Hsueh-Shih Road, Taichung 40,402, Taiwan.

1. Introduction

Skin is the largest organ in the human body, and it is the first-line barrier that protects against pathogens and chemical dangers. Skin is divided into the hypodermis, dermis, and epidermis. The epidermis is the most superficial layer and comprises the basal layer, stratum spinosum, stratum granulosum, stratum lucidum, and stratum corneum [1]. Keratinocytes are the most abundant cell type and account for 90% of cells in the epidermis; these cells are responsible for the production of keratin, which forms the major physical barrier that protects against pathogens and prevents internal dehydration [2]. Atopic dermatitis

(AD) is a prevalent skin disease in children from birth to the age of 2 years; it is also termed allergic dermatitis or atopic eczema [3], and it affects up to 20% of children and 1%–3% of adults worldwide [4,5]. Adult-onset AD has been defined only recently. It is characterized by pronounced clinical heterogeneity, and the disease factors remain unclear. Most critical symptoms of AD occur in the epidermis; these symptoms include itching, rough skin, swelling, thickening, debris, and scarring, all of which severely impede patients' quality of life [6] and the families of affected children through sleep, finances, and relationships. Moreover, in addition to physical symptoms, AD has been documented to impact patients in terms of behavioral problems, mood disorders, and medical burdens [7].

Symptoms presenting in the epidermis such as pruritus damage the skin's protective function, resulting in subsequent infection risk. Current prevention and treatment strategies focus on restoring epidermal barrier

* Corresponding authors at: Institute of New Drug Development, College of Medicine, China Medical University, No.91 Hsueh-Shih Road, Taichung 40402, Taiwan.
E-mail addresses: geant@mail.cmu.edu.tw (C.-Y. Lin), lchuang@mail.cmu.edu.tw (H.-C. Huang).

<https://doi.org/10.1016/j.jconrel.2020.10.007>

Received 5 June 2020; Received in revised form 10 September 2020; Accepted 3 October 2020

Available online 5 October 2020

0168-3659/© 2020 Elsevier B.V. All rights reserved.

functions through emollients. Topical corticosteroids or nonspecific immunosuppressive drugs are considered the first-line therapy for acute or severe AD. However, targeted disease-modifying drugs have not yet been developed [8]. Current strategies involve either broad or targeted immunosuppression, for example, through NK cell-boosting interleukin-15 (IL-15) superagonist infusion, which has demonstrated remarkable improvement in an AD mouse model [9]. However, such medical strategies are complex.

Dictamnine is the main component of *Dictamnus dasycarpus* Turcz.—a traditional Chinese medicine that has been widely used to treat skin inflammation for hundreds of years. The exact anti-inflammatory mechanism of dictamnine has not yet been clearly described. Traditionally, the root bark of *D. dasycarpus* is used for the treatment of inflammatory skin diseases such as AD, pruritus, and urticaria in China, Japan, and Korea [10]. In a study, *D. dasycarpus* extract alleviated the oxidative stress and inflammatory responses elicited by oxazolone on skin [11]. Dictamnine is the main active pharmaceutical ingredient (API) extracted from the root bark of *D. dasycarpus*, and it has been shown to have antifungal [12], antiangiogenic, and anticancer properties [13]. Dictamnine has been demonstrated to inhibit epithelial–mesenchymal transition (EMT) in tumors by downregulating HIF-1 α and Slug expression [14]. Notably, the antibacterial and antiyeast functions of dictamnine may be attributed to its photoinduced toxicity, which may be further developed as a powerful photosensitizer [15].

Skin barrier dysfunction in AD leads to pathogen invasion and subsequent immunological responses. Molecular exploration revealed the overexpression of thymic stromal lymphopoietin (TSLP), IL-6, IL-1 β , IL-4, and TNF- α , as well as elevated serum IgE levels and activated Th2 immunocyte-dominant inflammatory responses in patients with AD [6,16–18]. Furthermore, the apparent elevation and infiltration of specific immunocytes, such as mast cells, T lymphocytes, dendritic cells, eosinophils, and basophils are related to AD severity [19–21]. Therefore, to create an optimal, affordable, and representative model for investigating the efficacy of AD treatment, allergen- and hapten-induced models, which have similar pathological features to human AD-like symptoms, are useful for evaluating both pathogenic mechanisms and potential therapeutic agents [22]. Multiple challenges of a hapten, oxazolone, to normal murine skin induce chronic Th2-like hypersensitivity with multiple features identical to those of human AD. Because of its reproducibility, predictability, and affordability, this model is used to evaluate both pathogenic mechanisms and potential therapies for AD [18].

Nanoformulation has emerged as a new strategy for specifically delivering unstable APIs or imaging agent; nanoencapsulation has been demonstrated to prolong API efficacy and showed improved efficacy in solid tumor [23,24]. Polymeric nanoparticles are prepared from biocompatible and biodegradable polymers with sizes ranging from 10 to 1000 nm. The API is dissolved, entrapped, encapsulated, or attached in the polymer nanoparticle carrier. The penetration and transport of nanoformulated APIs through the skin depends on the chemical composition of the carriers and the encapsulation mechanism, both of which influence drug release and physical–chemical properties. Polymeric nanoparticles can modify drug activities, delay and control drug release, and increase drug adhesion or duration of permanence on the skin. Moreover, nanoparticles can be useful as lipophilic drug reservoirs for delivery in the stratum corneum; this has become an important strategy for controlling their permeation into the skin.

The drug efficacy and mechanism of dictamnine for AD treatment remain unclear. Therefore, to investigate the anti-inflammatory mechanism of dictamnine and to enhance sustained medication *in vivo*, we prepared PLGA-nanocarrier-encapsulated dictamnine (Dic-PLGA-NC) by using an ultrasound composite streams-impinging mixer (U-SiM) bioreactor and applied it to a mouse model with AD-like symptoms induced by oxazolone. The mechanism of dictamnine for the modulation of cellular oxidative stress to alleviate inflammatory responses was examined in HaCaT cells—a keratinocyte model. The drug release

profile and penetration of nanoformulated dictamnine were designed and examined using cumulative *in vitro* measurement and two-photon microscopic observation *in vivo*.

2. Materials and methods

2.1. Cell culture and viability detection

HaCaT cell models were used for *in vitro* experiments; detailed methods are disclosed in the supplementary information (SI).

2.2. Protein quantitation and western blot

Detailed information is provided in the SI.

2.3. Cytosolic and mitochondrial reactive oxygen species detection

Detailed information is provided in the SI.

2.4. Immunocytochemical staining and ELISA

Detailed information is provided in the SI.

2.5. Preparation of nanoformulated dictamnine by using the U-SiM bioreactor

Dic-PLGA-NC was prepared using antisolvent flash precipitation; the internal configurations of the U-SiM bioreactor were referenced from a previous report [25]; the bioreactor was modified and fabricated in our university workshops based on literature data and self-tested parameters. The U-SiM bioreactor reservoir was installed using an ultrasonic device providing superimposed turbulent flow and modified Pyrex glass providing high tension stress resistance and low temperature deformation. Stream tubes with internal diameters of 0.260 and 0.603 mm were manufactured using Teflon (Fig. 1A). Dictamnine and PLGA (85/15 L-lactide/glycolide copolymer, Corbion Inc., Cat. PURASORB PLG 8523) were dissolved in N-methyl-2-pyrrolidone (Sigma-Aldrich, Cat. 443,778) and dichloromethane (Sigma-Aldrich, Cat. 270,997), respectively, and then composed to form the drug-containing organic solution (1:3, w/w, 1.2 mg/mL). Poly-vinylpyrrolidone (Mw. 10,000; Sigma-Aldrich, Cat. PVP10; 0.8 mg/mL) was used to prepare the dispersion solution and stabilize the aqueous solution in the dilution reservoir. A 50-mL dispersion solution and a 5-mL drug-containing organic solution were simultaneously injected into the reactor tank, which was prefilled with a 50-mL dispersion solution, at speeds of 30 and 3 mL/min, respectively, by using a syringe pump (Pump System Inc., Cat. NE-1000). Subsequently, the organic solvent and nonencapsulated free dictamnine from the U-SiM reservoir were removed from the suspension by using a dialysis bag with a molecular weight cutoff of 7 kDa in Milli-Q water for 48 h in a dark environment.

2.6. Physical–chemical properties, high-performance liquid chromatography, and cumulative drug release

The particle size distribution of Dic-PLGA-NC manufactured using the U-SiM bioreactor was examined using dynamic light scattering (DLS; Malvern, Cat. ZS90). Measurements were performed in triplicate for each sample. To examine the drug encapsulation efficiency, the Dic-PLGA-NC suspension was placed in the inner tube of an ultrafiltration tube (Amicon Ultra-4 Centrifugal Filter Unit; Merck-Millipore, Cat. C7719) with a molecular weight cutoff of 10 kDa and centrifuged at 4000 $\times$ g for 20 min. The filtrate containing nonencapsulated dictamnine in the outer tube and the unfiltered suspension in the inner tube were then collected for high-performance liquid chromatography (HPLC) as previously described [26].

The morphology of Dic-PLGA-NC was examined using scanning

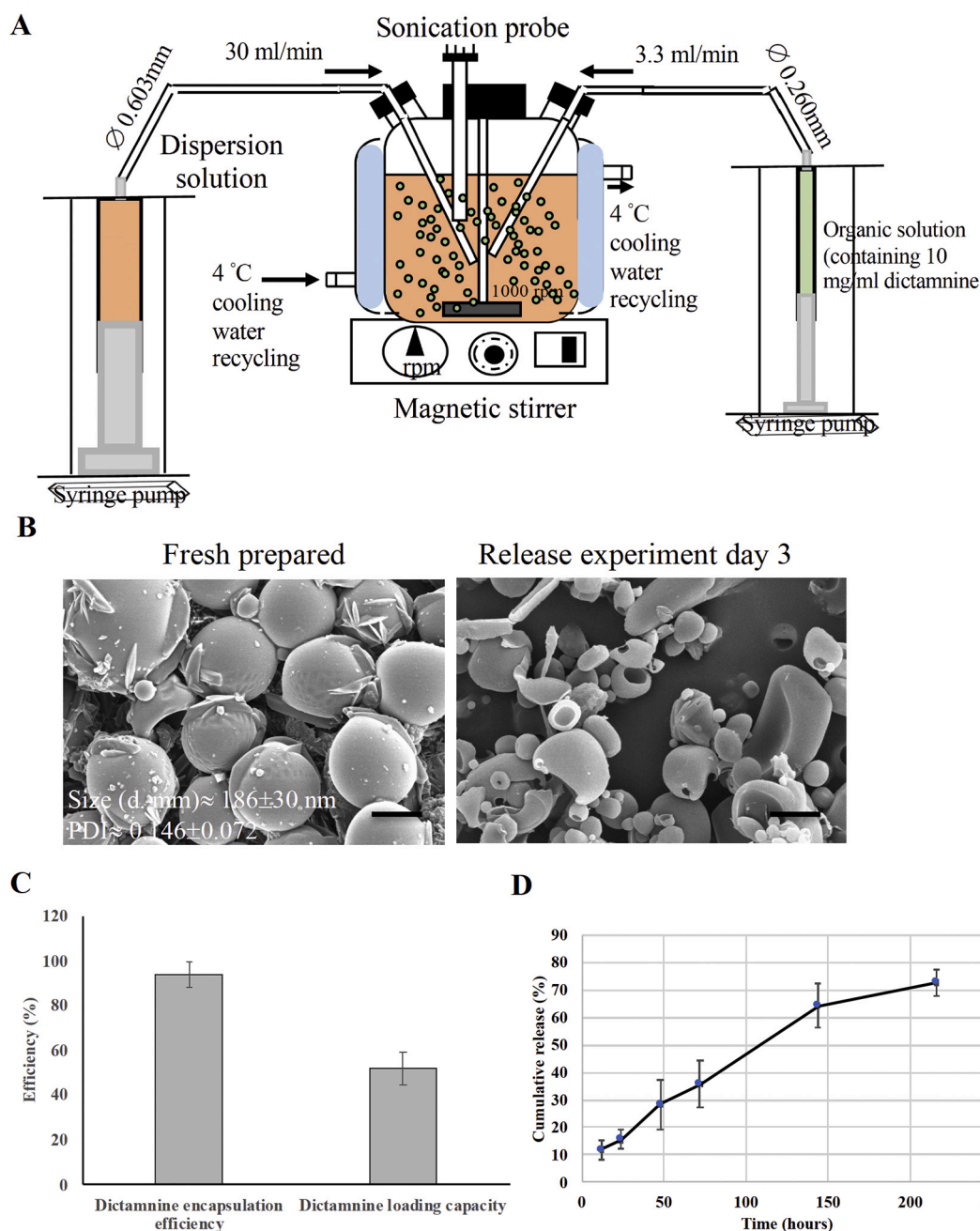


Fig. 1. Manufacture process and physicochemical properties of Dic-PLGA-NC. (A) Schematic of the U-SiM bioreactor used to produce Dic-PLGA-NC. (B) DLS measurement of Dic-PLGA-NC. Representative images show SEM observations of freshly prepared Dic-PLGA-NC and Dic-PLGA-NC collected from the release experiment on day 3. (C) Dictamnine encapsulation efficiency and loading capacity of Dic-PLGA-NC. (D) Cumulative release profile of Dic-PLGA-NC in PBS. Scale bar, 100 nm. Data represented as means $\pm$ SDs ($N > 4$).

electron microscopy (SEM; Hitachi, TM-1000). Before SEM observation, lyophilized Dic-PLGA-NC samples were spread over a double-sided conductive tape that was adhered to a specimen stub. The tape was then coated with gold using a sputter coater to provide the samples with electrical conductivity.

The *in vitro* drug release profile of Dic-PLGA-NC was measured by dispersing particles in phosphate-buffered saline (PBS; pH: 7.4) for 216 h in a dark environment at 30 °C to mimic superficial skin conditions. The immersed PBS was collected for HPLC measurement in accordance with a method modified from a previous study [27]. This demonstrates the physiological release of free dictamnine from Dic-PLGA-NC. Therefore, the drug residue in the nanocarrier suspension was considered to be unreleased dictamnine. Drug-releasing experiments were performed in

triplicate.

The encapsulation efficiency and drug release profile of Dic-PLGA-NC were measured using an HPLC system (Hitachi, Cat. Chromaster 5110) equipped with an ultraviolet detector. Analyses were performed at room temperature by using an Agilent Zorbax SB-C18 column (250 mm $\times$ 4.6 mm, 5 μ m). The mobile phase was acetonitrile: 0.1% H_3PO_4 solution (6:4, v/v) at a flow rate of 1 mL/min. The detector's wavelength was set to 430 nm. The injection volume was 50 μ L for all samples. Following equations were used to calculate encapsulation efficiency (EE %), drug loading capacity, and drug release profiles.

$$\text{Encapsulation (\%)} = \frac{\text{Unfiltered drug} - \text{free drug in filtrate}}{\text{Unfiltered drug}} \times 100\%$$

$$\text{Drug loading capacity (\%)} = \frac{\text{Drug}}{\text{Drug} + \text{polymeric agent}} \times EE \times 100\%$$

$$\text{Released drug (\%)} = \frac{\text{Drug}_{(\text{total})} - \text{Drug}_{(\text{residue})}}{\text{Drug}_{(\text{total})}} \times 100\%$$

2.7. Animal model and drug administration

To establish an AD model in mice, topical oxazolone was repeatedly applied over an extended period to induce allergic contact dermatitis in hairless male Institute of Cancer Research (ICR) mice aged 17 weeks (BioLASCO). The mice were subdivided into groups according to the results of a stratified randomization method. Animals were anesthetized using a 4-mg/kg intramuscular injection of Zoletil 50 (Virbac, Cat. 06516), after which their back hair was shaved, and they were sensitized once with 5% (w/v) oxazolone dissolved in a solvent prepared with acetone and olive oil in 3:1 (v/v). After 3 days, the mice were challenged

with 1% oxazolone once every 2 days for a total of six challenges. For the experimental group, naked or nanoformulated dictamnine formulations were applied to the backs daily for 14 days (Fig. 2A). The dictamnine dose used in all experimental animal groups is 2 µg/mL, applied once daily, 200 µL per administration in carrier solution composed with acetone and olive oil in 3:1 (v/v). The control group animals were treated with ethanol at the same intervals. All mice were sacrificed and sampled on day 31. All animal experiments were approved by the Committee for the Use and Care of Animals of China Medical University.

2.8. Immunohistochemical staining, skin recovery evaluation, spleen and body weight evaluation, and quantitative reverse transcription polymerase chain reaction of tissue-extracted RNA

The standard protocol for immunohistochemical (IHC) staining and primers for quantitative reverse transcription polymerase chain reaction (qRT-PCR) are provided in the SI.

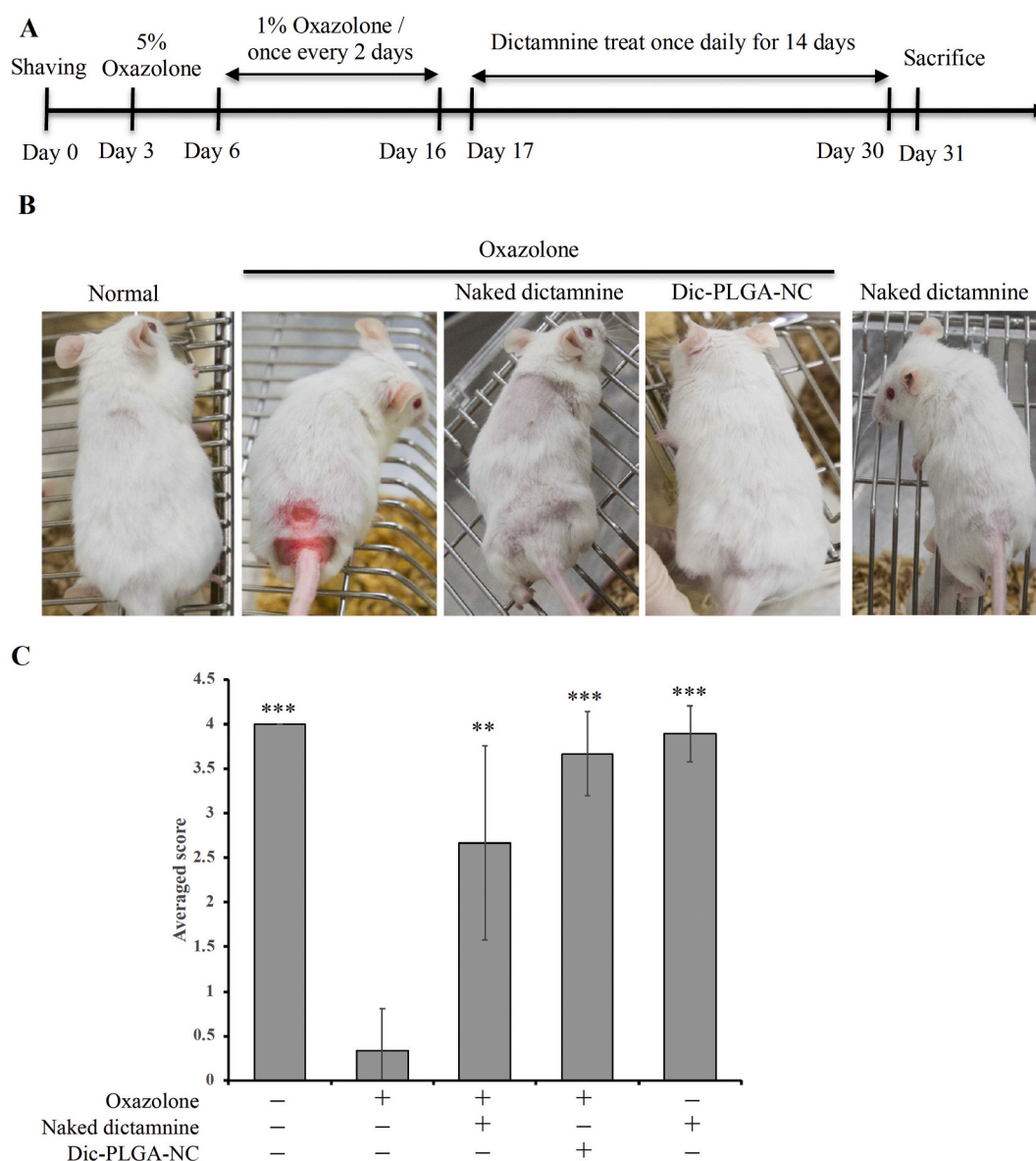


Fig. 2. Anti-inflammatory effect of dictamnine in mice treated with oxazolone. (A) Schematic of the experimental design for the skin damage model and subsequent dictamnine administration. (B) Representative gross appearance of AD animal models with naked dictamnine or Dic-PLGA-NC topical treatment on day 31. (C) Oxazolone-induced extensive skin lesions recovered only after treatment with dictamnine or Dic-PLGA-NC. Bars represent recovery evaluation scores for the mouse skin. Data represented as means $\pm$ SDs of three animals ($N = 3$); $^{**}p < .01$, $^{***}p < .001$ compared with the oxazolone-only group.

2.9. Intravital two-photon microscopy

The standard protocol for two-photon microscopy is provided in the SI.

2.10. Statistical analysis

Data are presented as means $\pm$ SDs at least $N \geq 3$, and statistical comparisons were performed using Student's *t*-test or a two-way analysis of variance; $p < .05$ was considered significant. All calculations were performed using SAS licensed to China Medical University.

3. Results

3.1. Cytotoxicity in HaCaT cell model treated with oxazolone and dictamnine

Oxazolone is commonly used to induce allergic dermatitis in animal models, and it causes Th1- or Th2-dominated inflammatory responses similar to those observed in pathological examinations of AD in humans

[28] (detailed molecular formula is illustrated as Fig. S1A for oxazolone, Fig. S1B for dictamnine, and major components of *Dictamnus dasycarpus* in HPLC analysis, Fig. S1C). Oxazolone has been demonstrated to induce reactive oxygen species (ROS) and mitochondrial ROS (mROS) expression in HaCaT cells and to elevate the expression of AD-related proteins, such as NLRP3 and p65 [11]. Therefore, we established an inflammatory model by using HaCaT cells induced with oxazolone. An MTT assay demonstrated an overall cell survival rate of more than 90% for oxazolone doses of 10–200 μ M. The inflammatory model was established using 100- μ M oxazolone in HaCaT cells (Fig. S2A).

The survival rates of HaCaT cells treated with dictamnine were examined 24 and 48 h after administration. The data showed a survival rate of >90% at doses of 0.2 and 2 μ g/mL; these dictamnine dosages were adopted for subsequent experiments (Fig. S2B).

3.2. Alleviation of intracellular oxidative stress induced by oxazolone in HaCaT cells

Oxidative stress is a result of the imbalance between antioxidative substances and ROS, and it is related to many inflammatory diseases,

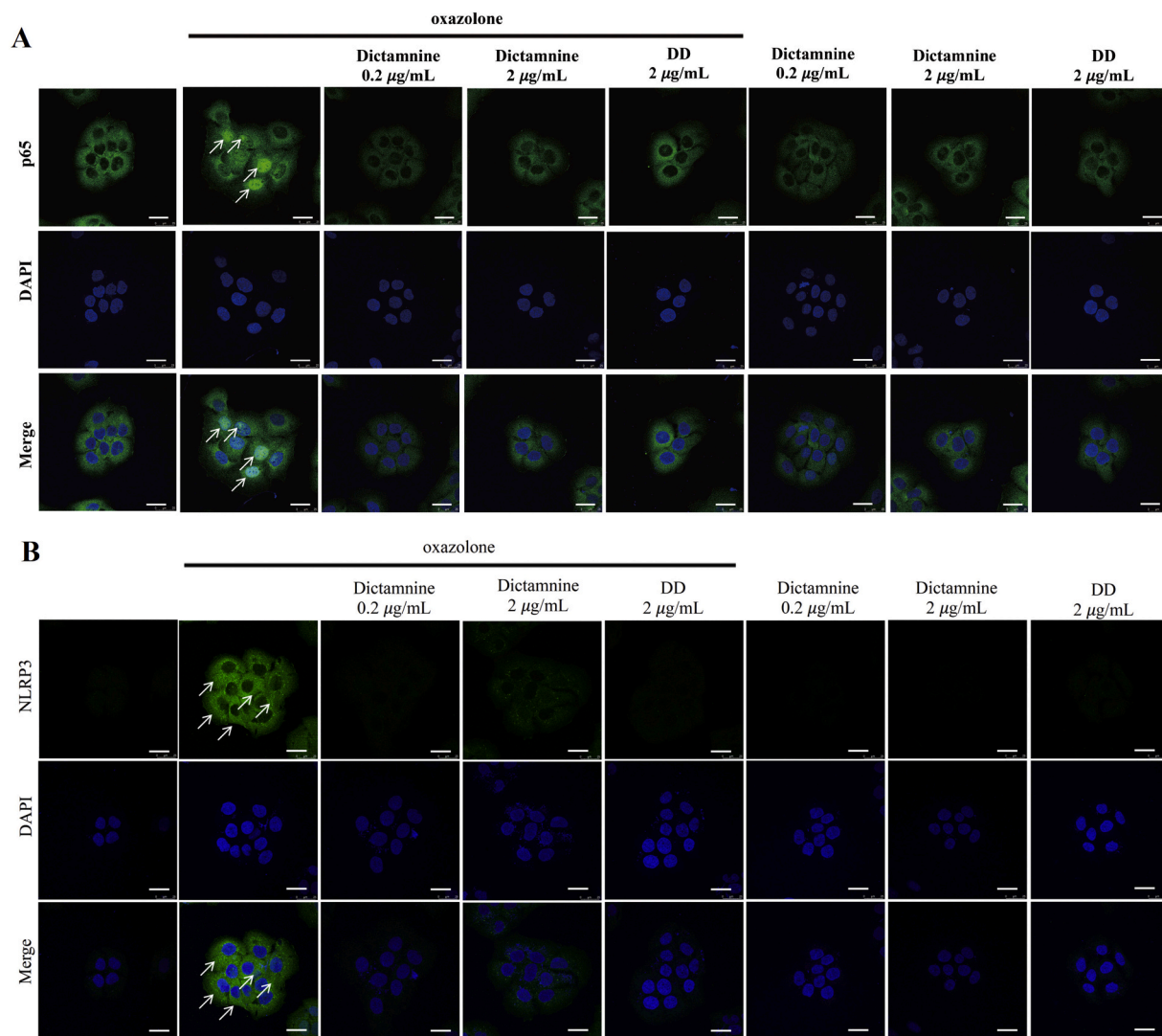


Fig. 3. Dictamnine ameliorated oxazolone-induced p65 nuclear translocation in HaCaT cells. (A) Representative images show cellular localization of endogenous p65 was observed through confocal microscopy in control and oxazolone-stimulated HaCaT cells with or without dictamnine treatment. DD, *Dictamnus dasycarpus*. White arrow indicates nuclear translocation of p65. Scale bar, 25 μ m. (B) Representative images show cellular localization of NLRP3 in HaCaT cells left untreated or stimulated as indicated and analyzed through confocal microscopy. Cellular nuclei were counterstained with DAPI (blue). DD, *Dictamnus dasycarpus*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

such as AD [29]. To examine whether the oxazolone-induced inflammatory scenario in the HaCaT cell model can be alleviated through dictamnine administration, we used DCFH-DA (green fluorescence) and MitoSOX™ (red fluorescence) to track ROS and mROS, respectively. *D. dasycarpus* served as a positive reference in this antioxidative measurement. Data revealed that ROS and mROS expression in HaCaT cells treated with oxazolone were reduced by dose-dependent dictamnine administration, and green and red fluorescence appeared diminished with a dictamnine dose of 2 µg/mL (Fig. S3A). The fluorescence intensity was further quantified using ImageJ, which revealed 52- and 80-fold decreases in ROS expression and 16- and 15-fold decreases in mROS expression for the 0.2 and 2 µg/mL doses, respectively (Fig. S3B).

To examine the activated dimer of NF-κB, which comprises the P65 and P50 subunits translocated to the nucleus, in an oxazolone treatment scenario with dictamnine administration, we examined HaCaT cells treated with oxazolone followed by dictamnine by using a confocal microscope with P65 antibodies labeled using green fluorescence. Data revealed apparent nuclear translocation of P65 during oxazolone treatment (arrows); however, extinguished green fluorescence was observed after dictamnine administration (Fig. 3A).

HaCaT cells treated with oxazolone were examined for NLRP3 aggregation through IHC staining with green fluorescence. Apparent NLRP3 aggregation occurred (arrows, Fig. 3B). However, NLRP3 aggregation was reduced after dictamnine administration, and green fluorescence was extinguished with both high and low doses (Fig. 3B).

3.3. Altered inflammatory cytokine expression in an oxazolone-induced inflammatory cell model

The MAPK pathway comprises multiple subunits, which are regulated and activated by ROS formation [30]. This pathway involves the phosphorylation of ERK, JNK, and p38 protein kinases, which subsequently activate NF-κB and trigger inflammatory diseases [31,32]. To assess whether dictamnine administration modifies the MAPK pathway changes induced by oxazolone treatment, we examined the phosphorylated JNK, p38, and ERK expression levels through western blotting. Data revealed that pJNK and pp38 expressions were elevated after

oxazolone administration but reduced after dictamnine treatment at doses of 0.2 and 2 µg/mL (Fig. 4A).

Western blotting was used to examine the elevated-ROS-mediated phosphorylation of IκB and dissociation with NF-κB, which stimulated the overexpression of P65 in the NF-κB subunit. Data revealed that the expressions of pIκB and P65 were elevated in HaCaT cells treated with oxazolone and reduced after dictamnine administration. The inhibition efficacies of pIκB and P65 were higher at a dictamnine dose of 2 µg/mL than at 0.2 µg/mL (Fig. 4B). Moreover, the mROS-stimulated activation of NLRP3 inflammasome and subsequent mediation of the turnover of procaspase-1 to caspase-1 were examined. Both high and low doses of dictamnine reduced the level of caspase-1 caused by oxazolone treatment (Fig. 4C).

To characterize the release of TNF-α and IL-1β, which are the downstream cytokines produced by cytosolic ROS and mROS overexpression, respectively, HaCaT cells were treated with oxazolone for 4 h and supplemented with dictamnine for another 24 h, and the resulting medium was collected for ELISA analysis. Dictamnine administration significantly reduced both the TNF-α and IL-1β secretions induced by oxazolone-mediated ROS and mROS overexpression (Table 1).

3.4. Preparation, biophysicochemical properties, and drug release profile of Dic-PLGA-NC

To extend the drug release and degradation time for skin administration in the animal model, we applied a nanosized encapsulation system for dictamnine delivery *in vivo* by using the FDA-approved medical polymer PLGA. The dictamnine dose of 2 µg/mL (Fig. 4B) was selected for nanosized encapsulation because of the superior inhibition efficacy toward pIκB and P65 at this dose (Fig. 4B). As illustrated in Fig. 1A, dictamnine was mixed with PLGA (85:15), dissolved in an organic solvent phase, and then injected into the specially designed U-SiM bioreactor. The organic phase stream was continuously impinged with another water-based dispersion solution stream in a controlled ultrasound- and stir-assisted mixing operation to prepare Dic-PLGA-NC. After filtration, washing, and purification, laser scattering measurements revealed that Dic-PLGA-NC particles had an average size of

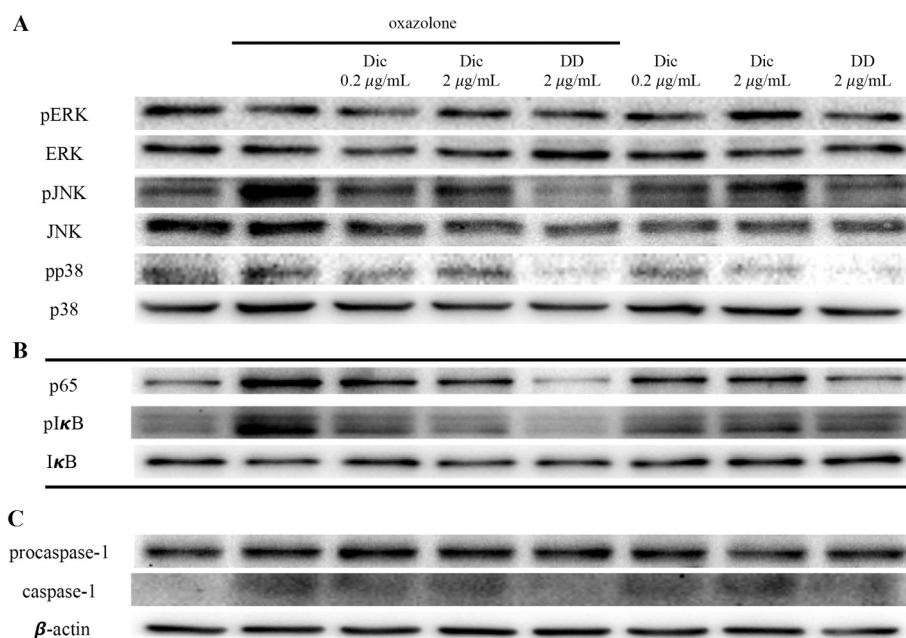


Fig. 4. Dictamnine reduced MAPK and p65 activation and procaspase-1 cleavage in oxazolone-induced inflammation in HaCaT cells. Representative immunoblotting images for JNK (and total), p38 (and total), ERK (and total), p65, IκB (and total), procaspase-1, caspase-1, and β-actin on lysates from HaCaT cells. HaCaT cells were pretreated with 100 µM oxazolone for 4 h before incubation with dictamnine for 24 h at the indicated concentrations.

Table 1

The concentration of IL-1 β and TNF- α in oxazolone-induced HaCaT cells culture supernatants decreased significantly when dictamnine was added. Data are expressed as the means $\pm$ SDs (N = 3). (Fold of control. Statistical analysis compared with oxazolone-only group, a: $p < .05$, b: $p < .01$, c: $p < .001$).

		Oxazolone						
	Control		Dictamnine 0.2 μ g/mL	Dictamnine 2 μ g/mL	DD 2 μ g/mL	Dictamnine 0.2 μ g/mL	Dictamnine 2 μ g/mL	DD 2 μ g/mL
TNF- α	1 $\pm$ 0	3.86 $\pm$ 0.69	1.09 $\pm$ 0.28 ^C	0.88 $\pm$ 0.22 ^C	1.43 $\pm$ 0.94 ^b	1.03 $\pm$ 0.15 ^C	1.20 $\pm$ 0.2 ^b	1.13 $\pm$ 0.35 ^C
IL-1 β	1 $\pm$ 0	12.84 $\pm$ 1.91	1.08 $\pm$ 0.06 ^C	1.03 $\pm$ 0.07 ^C	1.10 $\pm$ 0.18 ^b	1.15 $\pm$ 0.12 ^C	1.28 $\pm$ 0.09 ^C	0.98 $\pm$ 0.04 ^b

approximately 186 $\pm$ 30 nm and a polydispersity index (PDI) of approximately 0.146 $\pm$ 0.072 (Fig. 1B). Furthermore, the stability of lyophilized Dic-PLGA-NC preserved in dry-box in room temperature, revealed almost identical PDI and averaged particle size after 3 months storage. SEM examination revealed uniform spherical nanoparticles at the fresh prepared status and broken nanoparticles at the released experiment at day 3 (Fig. 1B). HPLC results indicate that the dictamnine encapsulation efficiency and loading capacity were approximately 93.7% and 51.8%, respectively (Fig. 1C). The cumulative drug release profile was examined by immersing Dic-PLGA-NC in PBS at 30 °C (Fig. 1D); drug release was approximately 72.8% on day 9.

3.5. Anti-inflammatory effect of Dic-PLGA-NC in mice treated with oxazolone

To evaluate the efficacy of dictamnine administration for AD therapy, we applied naked dictamnine and Dic-PLGA-NC to mice with oxazolone-induced AD. ICR mice were used to establish an AD model by applying oxazolone to the skin, after which drug administration was initiated on day 17 (Fig. 2A). Healing efficacy was evaluated using gross pictures and histological examination on day 31. Based on gross picture observation (Fig. 2B), the animals who received oxazolone without subsequent drug administration exhibited severe skin inflammation, swelling, sparse hairs, and red-colored skin. Animals that received oxazolone followed by naked dictamnine administration exhibited reduced skin inflammation and red-colored skin swelling but limited hair regrowth. The animals treated with oxazolone followed by Dic-PLGA-NC administration exhibited apparent amelioration of skin inflammation and considerable hair regrowth; this result was almost identical to that of the normal reference mouse. Three individuals assessed the skin healing appearance, which was classified into five grades from 0 (no healing) to 4 (complete healing) as described previously [33]. The animals that received oxazolone alone had a score of 0.333, indicating almost no healing. Animals that received naked dictamnine administration had a higher healing grade of 2.667, and the healing grade of animals that received Dic-PLGA-NC treatment was 3.778. Therefore, Dic-PLGA-NC appeared to relieve AD symptoms induced by oxazolone.

3.6. Histological examination of mouse skin treated with oxazolone and Dic-PLGA-NC penetration

To observe the overall inflammation scenario, we sectioned animal skins for hematoxylin and eosin (H&E) staining. IHC staining was performed using CD3, CD45 antibody, and toluidine blue to observe T cells, white blood cells, and mast cells, respectively. Moreover, COX-2 signals were assessed to examine the downstream factor expression of the MAPK pathway *in situ* (Fig. 5A). In the oxazolone-induced AD sections, H&E staining revealed significant epidermal tissue proliferation; picket-like dense aggregates; acanthosis-like neotissue; and apparent CD3,

CD45, COX-2, and mast cell signal deposition (dotted line). These findings demonstrate the establishment of a critical AD model. Compared with the oxazolone-induced group, the group receiving naked dictamnine exhibited increased epidermal tissue proliferation and relieved CD3, CD45, COX-2, and mast cell signals. Notably, the group receiving Dic-PLGA-NC exhibited apparent epidermal regeneration and diminished CD3, CD45, COX-2, and mast cell signals, thereby confirming the superior healing observed in gross pictures. In the histomorphometric analysis of tissue sections, the Dic-PLGA-NC group exhibited reductions in acanthosis-like epidermal tissue proliferation and CD3, CD45, COX-2, and mast cell signals of approximately 18.4%, 21.9%, 26.2%, 12.9%, and 30.4%, respectively, relative to the naked dictamnine group. This demonstrates that the effect of Dic-PLGA-NC was considerably stronger than that of naked dictamnine (Fig. 5B).

The skin penetration capability of the naked and nanoformulated drugs was examined to determine their healing efficacy in the AD model. Therefore, dictamnine blended with fluorescent dyes and prepared as naked form or prepared as Dic-PLGA-NC were applied to the AD mouse skin; the fluorescent signals were intravitaly observed 24 h after administration. Through transverse and three-dimensional reconstruction in the naked dictamnine group, sparse fluorescence was observed on the superficial epidermis, but most fluorescence was noted on residual hairs (Fig. 6). Conversely, in the Dic-PLGA-NC group, apparent and dense fluorescent signals were detected at approximately 300 μ m (Fig. 6), demonstrating that the nanoformulated dictamnine reached the dermal tissue layer and possessed superior skin penetration capability for AD therapy compared with naked dictamnine.

3.7. Mitigation of inflammatory cytokine mRNA expression by Dic-PLGA-NC treatment in an oxazolone-induced mouse model

To examine the inflammatory scenario *in vivo* elicited by oxazolone and subsequent recovery, we collected the spleens of the animals for comparison with body weight. Based on gross pictures, oxazolone treatment alone resulted in spleen enlargement compared with naked dictamnine and Dic-PLGA-NC treatment (Fig. 7A). Quantitative data further demonstrate that the spleen-to-body weight ratio was elevated after treatment with oxazolone alone and reduced in the Dic-PLGA-NC group (Fig. 7B). This demonstrates the anti-inflammatory efficacy of nanoformulated dictamnine.

To analyze inflammatory cytokine expression in the skin, we extracted and performed qRT-PCR analysis on total RNA. Oxazolone treatment alone without drug administration significantly increased TSLP, IL-1 β , and TNF- α mRNA expression (Fig. 8). Naked dictamnine administration in the AD model reduced TSLP and IL-1 β expression but not TNF- α mRNA expression, which might elicit complex immune responses; further analysis is required. Dic-PLGA-NC administration reduced TSLP (Fig. 8A), IL-1 β (Fig. 8B), and TNF- α (Fig. 8C) mRNA expression. The TSLP and TNF- α mRNA measurements of the Dic-PLGA-NC and naked dictamnine differed significantly.

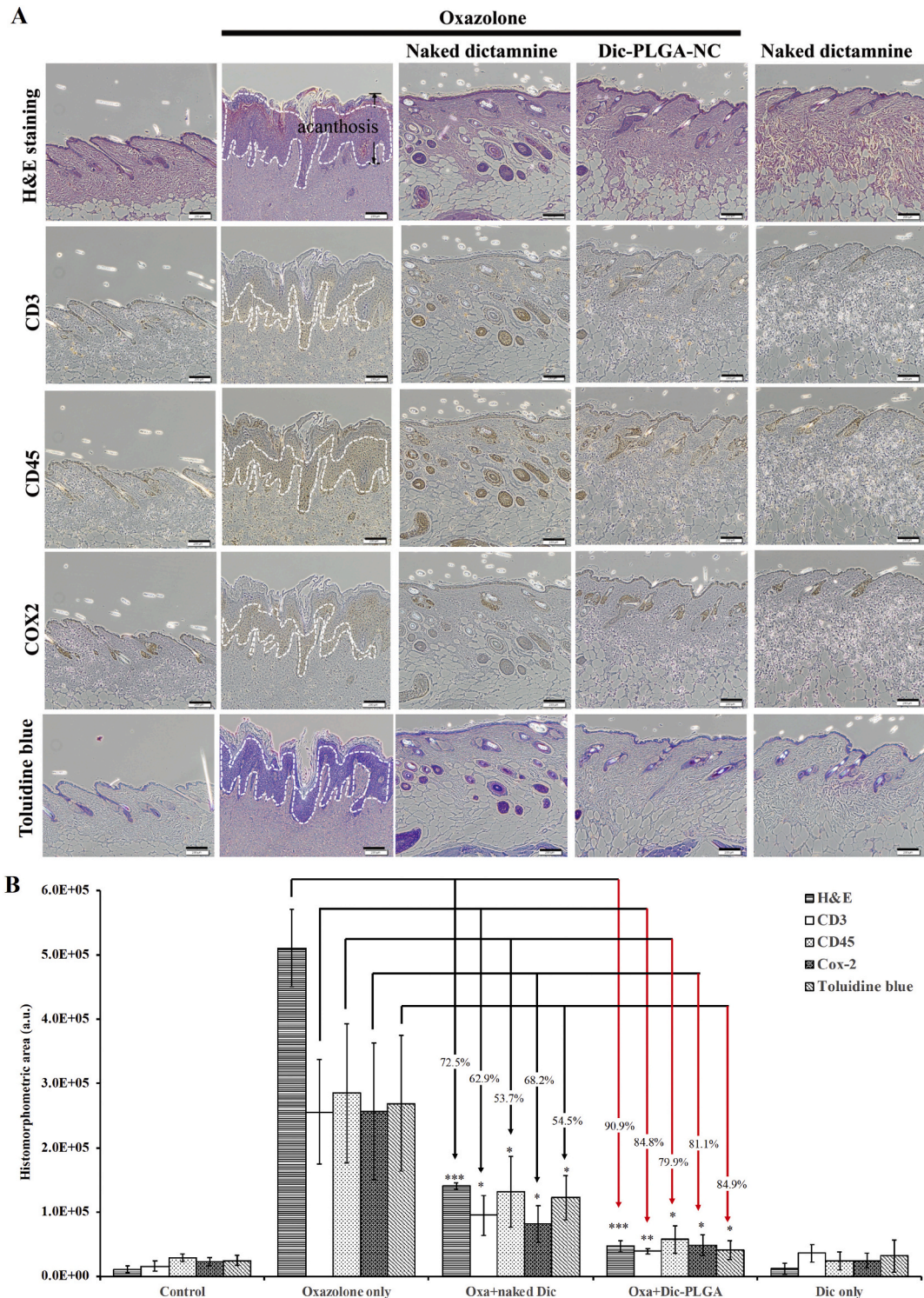


Fig. 5. Histological examination of mouse skin. (A) Skin pathology was measured using H&E staining, showed representative images. Hyperparakeratosis, epidermal thickening (hyperplasia), acanthosis, CD3+ or CD45+ cell infiltration into the dermis, and the development of the proinflammatory component COX-2. Scale bar, 200 μ m. (B) Histomorphometric analysis. Data represented as means $\pm$ SDs of three animals (N = 3); * p < .05, ** p < .01, *** p < .001 compared with the oxazolone-only group. COX2: cyclooxygenase 2; CD: cluster of differentiation.

4. Discussion

AD is one of the most prevalent skin diseases, and because no primary medication is available for AD therapy, scientists and pharmacologists worldwide have explored therapeutic strategies. The rate of AD during recent decades has increased sharply, which may not be

completely attributed to hereditary factors. Increasing data have demonstrated that environmental exposure may play another vital role in skin inflammations such as AD [34]. In the present study, oxazolone was selected to induce AD in ICR mice. This hapten-induced murine model had multiple features identical to those of AD in humans, such as a Th2-like hypersensitivity, increased IL-4 expression, elevated serum

Oxazolone

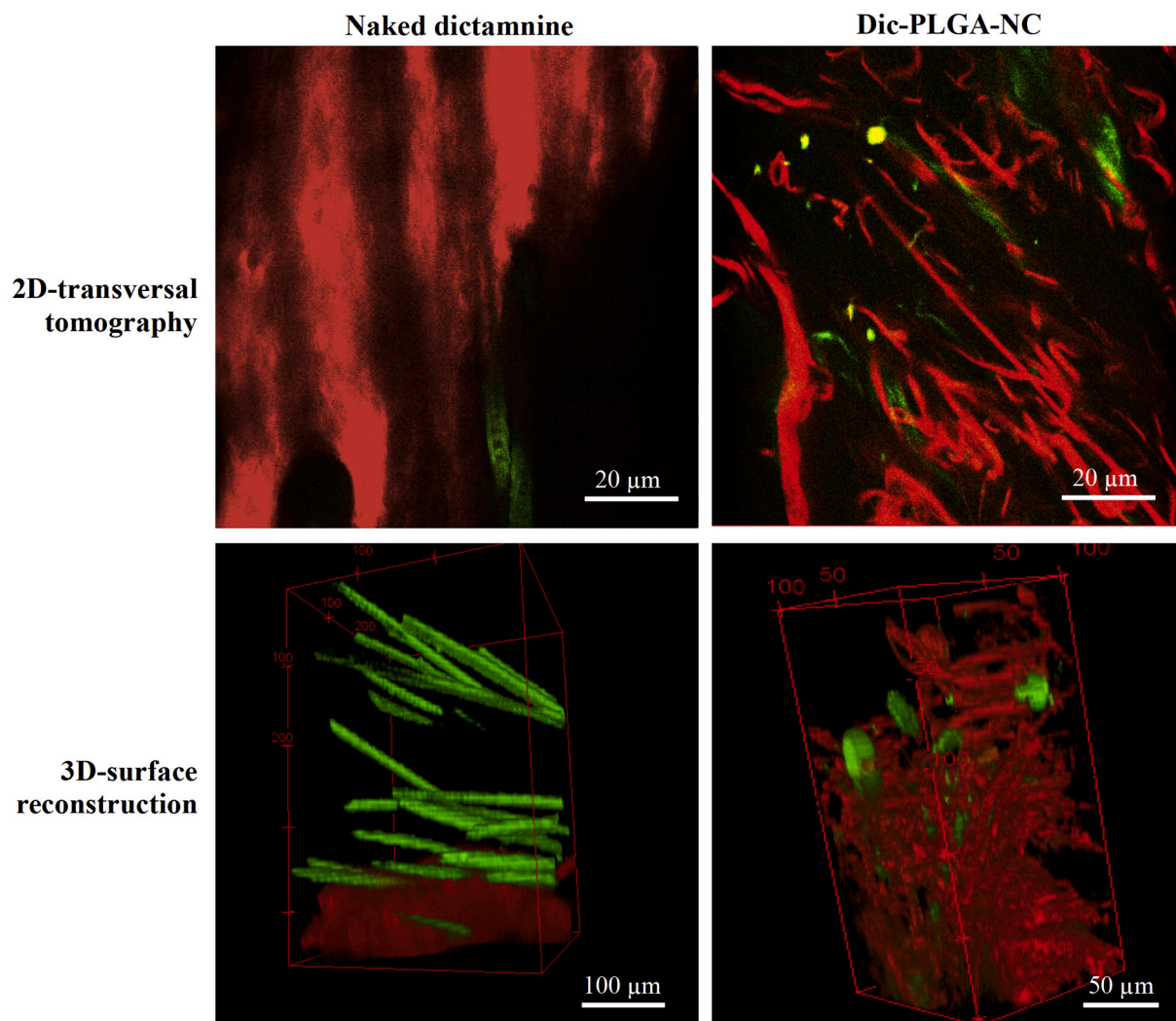


Fig. 6. Representative images show two-photon microscopic evaluation of mouse skin after drug administration.

IgE levels, and abnormal permeability barrier function [18]. We administered oxazolone at a higher dose to induce acute immune responses during the initial 3 days and then downgraded to a lower dose to induce chronic inflammation. The skin appearance and pathological characteristics were similar to those of AD in humans elicited by environmental exposure.

Topical spreading is the most convenient delivery method for the treatment of skin-related diseases because of its noninvasive and patient-compliant characteristics [35], which minimize the systemic side effects that result from invasive drug administration. Nanoencapsulation is a popular and well-developed technique for skin disease therapy and cosmetics. In this approach, nanoformulations are used to carry and protect active ingredients for therapy, detection, and prevention [36]. Nanoformulated drugs for the treatment of skin disease have many benefits, such as protecting the drugs in a sophisticated container, controlling the drug release rate and area, delivering lipophilic drugs, sustaining therapeutic efficacy, and ameliorating drug permeability into skin. However, the materials and manufacturing techniques used for nanocarrier preparation, including physical parameters and the selection of dispersants, greatly influence the drug release profile [35]. To examine the effects of dictamnine on AD therapy, consider its photosensitivity, and extend its protection and release, we encapsulated dictamnine in a nanocarrier by using a U-SiM

bioreactor.

In the mouse AD model, nanocarrier-encapsulated dictamnine exhibited superior drug release efficacy and epidermal tissue penetration capability to those of naked dictamnine (Figs. 2B, C, 5A, B, and 6). Based on skin appearance, Dic-PLGA-NC appeared to alleviate significant AD symptoms, such as hair loss, swelling, debris, and scarring. Moreover, Dic-PLGA-NC reduced CD3, CD45, and COX-2 expression and mast cell infiltration in the histological examination (Fig. 5A). Drug encapsulation provided sustained release capability and protection efficacy for the photosensitive drug; therefore, its therapeutic efficiency in the AD animal model was strengthened.

Antisolvent precipitation is an ultrarapid and promising manufacturing process for the controlled production of organic nanoparticles. In this technique, nanoparticle size and stability depend on the biocompatible block copolymer selected for production, which protects the drug within its nanostructure. A confined impinging jet mixer (CIJM) is a manufacturing instrument that utilizes the antisolvent precipitation principle to produce nanoformulated drugs. It is conventionally used for the nanoencapsulation of sensitive drugs, but it relies on identical momenta and an equivalent volumetric flow rate, both of which limit the supersaturation level inside the mixer [37]. To modify the CIJM, we merged dispersion with sonication to provide turbulent fluctuations and produce fine particles, and we adopted a larger dilution

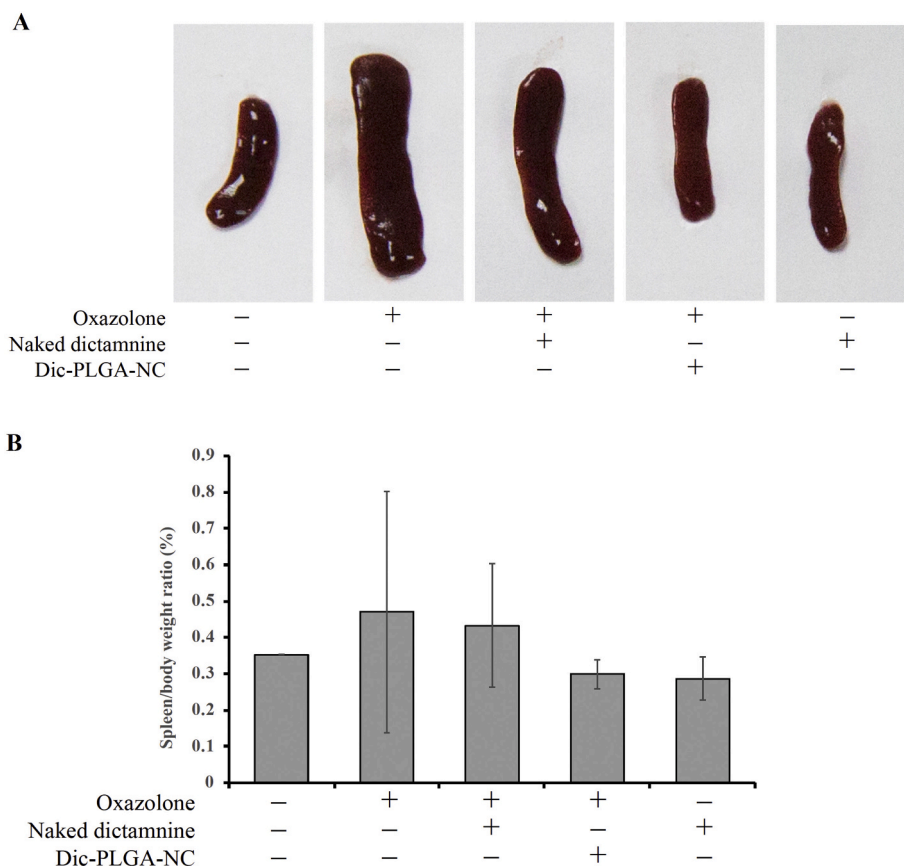


Fig. 7. Spleen-to-body-weight ratio. (A) Representative gross picture of the spleen collected from the animal on day 31. (B) Spleen-to-body-weight ratio. Data represented as means $\pm$ SDs of three animals (N = 3).

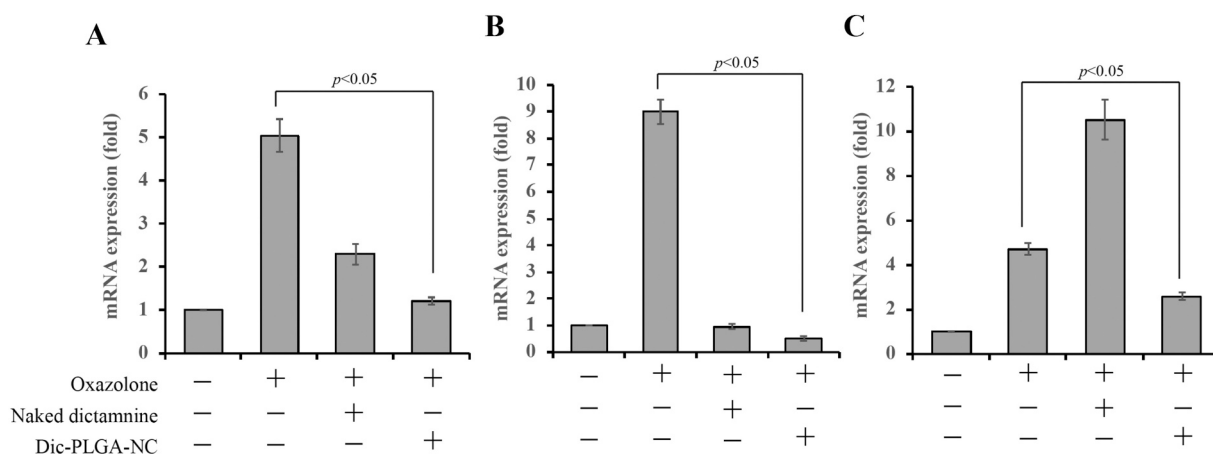


Fig. 8. qRT-PCR of mouse skin tissues. (A) TSLP. (B) IL-1 β . (C) TNF- α . Data represented as means $\pm$ SDs (N = 3).

tank with the aid of amphiphilic homopolymers as stabilizers [38]. This system featured a fine-tuned stream flow rate that was 9-fold that of the drug stream. This difference was reflected in the different diameters of the injection tubes. Moreover, the two streams impinged and completed at the same times, resulting in an efficient nanoformulated drug with high encapsulation efficiency. The drug particle size produced by the U-SiM bioreactor was controlled at approximately 186 nm. The manufacturing process was affordable and simple, making it suitable for the large-scale production of photosensitive and water-immiscible drugs. Furthermore, we selected the PLGA (85:15) block copolymer for the production of nanocarriers because PLA has a longer degradation

time than does PGA, resulting in a sustained drug release profile. Our bioreactor included a second dilution step immediately following the impingement of the injected streams, which was aimed at increasing the supersaturation of nanoparticles and minimizing the recrystallization of solutes. Moreover, the bioreactor had advantages of being manually operable and easy to scale up.

Skin provides a natural barrier to prevent the invasion of pathogens or the penetration of outside particles. Studies have hypothesized that nanoparticles with a size of >10 nm are impeded by the stratum corneum layer in epidermal tissue and accumulate in the cavities of hair follicles. However, nanoparticle absorption into epidermal and dermal

tissues is significantly enhanced in aged, damaged, and diseased skin, which provides an ideal environment for drug nanoformulations to achieve sustained release [39]. In our acute AD model induced by high dose of oxazolone, which may disrupt the regular microstructure orientation in the epidermal and dermal tissues, created an optimal situation for nanoformulated dictamnine absorption and sustained release of dictamnine from PLGA carriers. Moreover, tortuous aqueous and lipidic channels in the microstructure of the diseased and intact skin differ substantially, and penetration of the particle matrix is significantly increased in damaged and diseased skin [39]. The pharmaceutical mechanism of dictamnine nanoformulation (Dic-PLGA-NC) we used in this study may need to be further explored to understand the mechanism of Dic-PLGA-NC penetration. Our method was used to load dictamnine into nanocarriers, which protected the drug inside a polymeric nanostructure (Fig. 1); these nanocarriers penetrated to at least 300 μm deep to reach the superficial dermal or viable epidermal layer [40], as shown in intravital two-photon microscopy (Fig. 6). The nanocarriers then persistently released the drug for >216 h in a cumulative release test. Compare to the naked dictamnine administration in the oxazolone induced AD model, Dic-PLGA-NC administration showed effective skin repair efficacy (Fig. 2B), and minimal inflammatory responses in the epidermal microstructure (Fig. 5A), which may attribute from the slow and sustained release of dictamnine from PLGA carriers.

5. Conclusion

The treatment of keratinocytes with oxazolone induces cytosolic and mitochondrial ROS overexpression, resulting in the dissociation of I κ B from NF κ B, the release of cellular COX-2 and TNF- α , and NLRP3 inflammasome aggregation. This leads to the activation of caspase-1 and the overexpression of IL-1 β . Moreover, overexpression of COX-2, TNF- α and IL-1 β stimulates mast cells and leukocytes to aggregate in inflammatory tissue and stimulates activated T cells to elicit cascaded inflammatory scenarios similar to those in AD. In a mouse model, nanoformulated dictamnine, which was manufactured using a highly efficient bioreactor, significantly alleviated AD symptoms and mechanistic cytokine expression in the AD inflammatory scenario induced by oxazolone. The nanoformulated dictamnine penetrated the dermal tissue according to intravital observation and achieved sustained drug release. The methodology developed in this study was an efficient and simple manufacturing process for the production of nanoformulated dictamnine, which is promising for the treatment of AD.

Oxazolone.

	Control	Dictamnine 0.2 $\mu\text{g}/\text{mL}$	Dictamnine 2 $\mu\text{g}/\text{mL}$	DD 2 $\mu\text{g}/\text{mL}$	Dictamnine 0.2 $\mu\text{g}/\text{mL}$	Dictamnine 2 $\mu\text{g}/\text{mL}$	DD 2 $\mu\text{g}/\text{mL}$
TNF- α	1 $\pm$ 0	3.86 $\pm$ 0.28 ^C	0.88 $\pm$ 0.22 ^C	1.43 $\pm$ 0.94 ^b	1.03 $\pm$ 0.15 ^C	1.20 $\pm$ 0.2 ^b	1.13 $\pm$ 0.35 ^C
IL-1 β	1 $\pm$ 0	12.84 $\pm$ 0.06 ^C	1.03 $\pm$ 0.07 ^C	1.10 $\pm$ 0.18 ^b	1.15 $\pm$ 0.12 ^C	1.28 $\pm$ 0.09 ^C	0.98 $\pm$ 0.04 ^b

Acknowledgement

This study was financially supported by China Medical University (CMU108-Z-01) and the Ministry of Science and Technology, Taiwan (Grant Nos. MOST 108-2314-B-039-050, MOST 108-2622-E-039-002-CC1 and MOST 108-2221-E-039-006-MY3).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jconrel.2020.10.007>.

References

- [1] R.R. Wickett, M.O. Visscher, Structure and function of the epidermal barrier, *Am. J. Infect. Control* 34 (2006) S98–S110.
- [2] A. Baroni, E. Buommino, V. De Gregorio, E. Ruocco, V. Ruocco, R. Wolf, Structure and function of the epidermis related to barrier properties, *Clin. Dermatol.* 30 (2012) 257–262.
- [3] K. Abuabara, A.M. Yu, J.P. Okhovat, I.E. Allen, S.M. Langan, The prevalence of atopic dermatitis beyond childhood: a systematic review and meta-analysis of longitudinal studies, *Allergy* 73 (2018) 696–704.
- [4] J.N. Mayba, M.J. Gooderham, Review of atopic dermatitis and topical therapies, *J. Cutan. Med. Surg.* 21 (2017) 227–236.
- [5] J.A. Odhiambo, H.C. Williams, T.O. Clayton, C.F. Robertson, M.I. Asher, I.P.T.S. Group, Global variations in prevalence of eczema symptoms in children from ISAAC Phase Three, *J. Allergy Clin. Immunol.* 124 (2009) 1251–1258 (e1223).
- [6] T. Bieber, Atopic dermatitis, *N. Engl. J. Med.* 358 (2008) 1483–1494.
- [7] E.J. Yang, K.M. Beck, S. Sekhon, T. Bhutani, J. Koo, The impact of pediatric atopic dermatitis on families: a review, *Pediatr. Dermatol.* 36 (2019) 66–71.
- [8] S. Weidinger, N. Novak, Atopic dermatitis, *Lancet* 387 (2016) 1109–1122.
- [9] M.R. Mack, J.R. Brestoff, M.M. Berrien-Elliott, A.M. Trier, T.B. Yang, M. McCullen, P.L. Collins, H. Niu, N.D. Bodet, J.A. Wagner, E. Park, A.Z. Xu, F. Wang, R. Chibnall, M.L. Council, C. Heffington, F. Kreisel, D.J. Margolis, D. Sheinbein, P. Lovato, E. Vivier, M. Cella, M. Colonna, W.M. Yokoyama, E.M. Oltz, T. A. Fehniger, B.S. Kim, Blood natural killer cell deficiency reveals an immunotherapy strategy for atopic dermatitis, *Sci. Transl. Med.* 12 (2020).
- [10] B. Yang, H.B. Lee, S. Kim, Y.C. Park, K. Kim, H. Kim, Decoction of Dictamnus Dasycaepus Turcz. Root bark ameliorates skin lesions and inhibits inflammatory reactions in mice with contact dermatitis, *Pharmacogn. Mag.* 13 (2017) 483–487.
- [11] T.M. Chang, T.Y. Yang, Y.L. Niu, H.C. Huang, The extract of *D. dasycaepus* Ameliorates oxazolone-induced skin damage in Mice by anti-inflammatory and antioxidant mechanisms, *Antioxidants (Basel)* 7 (2018).
- [12] G. Yang, D. Chen, Alkaloids from the roots of *Zanthoxylum nitidum* and their antiviral and antifungal effects, *Chem. Biodivers.* 5 (2008) 1718–1722.
- [13] X. Gao, P.H. Zhao, J.F. Hu, Chemical constituents of plants from the genus *Dictamnus*, *Chem. Biodivers.* 8 (2011) 1234–1244.
- [14] J.Y. Wang, Z. Wang, M.Y. Li, Z. Zhang, C. Mi, H.X. Zuo, Y. Xing, Y.L. Wu, L.H. Lian, G.H. Xu, L.X. Piao, J. Ma, X. Jin, Dictamnine promotes apoptosis and inhibits epithelial-mesenchymal transition, migration, invasion and proliferation by downregulating the HIF-1 α and Slug signaling pathways, *Chem. Biol. Interact.* 296 (2018) 134–144.
- [15] C.M. Schempp, B. Simon-Haerhaus, R. Krieger, J.C. Simon, Solar simulator-induced phototoxicity of the furoquinoline alkaloid dictamnine compared to 8-methoxypsoralen and 5-methoxypsoralen, *Planta Med.* 72 (2006) 941–943.
- [16] S. Mashiko, S. Bouguermouh, M. Rubio, N. Baba, R. Bissonnette, M. Sarfati, Human mast cells are major IL-22 producers in patients with psoriasis and atopic dermatitis, *J. Allergy Clin. Immunol.* 136 (2015) 351–359 (e351).
- [17] M.K. Oyoshi, R. He, L. Kumar, J. Yoon, R.S. Geha, Cellular and molecular mechanisms in atopic dermatitis, *Adv. Immunol.* 102 (2009) 135–226.
- [18] M.Q. Man, Y. Hatano, S.H. Lee, M. Man, S. Chang, K.R. Feingold, D.Y. Leung, W. Holleran, Y. Uchida, P.M. Elias, Characterization of a hapten-induced, murine model with multiple features of atopic dermatitis: structural, immunologic, and biochemical changes following single versus multiple oxazolone challenges, *J. Invest. Dermatol.* 128 (2008) 79–86.
- [19] Y. Jiang, W. Ma, Assessment of neutrophil-to-lymphocyte ratio and platelet-to-lymphocyte ratio in atopic dermatitis patients, *Med. Sci. Monit.* 23 (2017) 1340–1346.
- [20] T. Werfel, J.P. Allam, T. Biedermann, K. Eyerich, S. Gilles, E. Guttman-Yassky, W. Hoetzenecker, E. Knol, H.U. Simon, A. Wollenberg, T. Bieber, R. Lauener, P. Schmid-Grendelmeier, C. Traidl-Hoffmann, C.A. Akdis, Cellular and molecular immunologic mechanisms in patients with atopic dermatitis, *J. Allergy Clin. Immunol.* 138 (2016) 336–349.
- [21] F.T. Liu, H. Goodarzi, H.Y. Chen, IgE, mast cells, and eosinophils in atopic dermatitis, *Clin. Rev. Allergy Immunol.* 41 (2011) 298–310.
- [22] J. Zhao, S. Jia, P. Xie, G.A. Arenas, R.D. Galiano, S.J. Hong, T.A. Mustoe, Topical application of *Dermatophagoides farinae* or oxazolone induces symptoms of atopic dermatitis in the rabbit ear, *Arch. Dermatol. Res.* 309 (2017) 567–578.
- [23] L. Miao, C.M. Lin, L. Huang, Stromal barriers and strategies for the delivery of nanomedicine to desmoplastic tumors, *J. Control. Release* 219 (2015) 192–204.
- [24] M.M. Lubtow, L.C. Nelke, J. Seifert, J. Kuhnemundt, G. Sahay, G. Dandekar, S. L. Nietzer, R. Luxenhofer, Drug induced micellization into ultra-high capacity and stable curcumin nanoformulations: Physico-chemical characterization and evaluation in 2D and 3D in vitro models, *J. Control. Release* 303 (2019) 162–180.
- [25] J. Han, Z. Zhu, H. Qian, A.R. Wohl, C.J. Beaman, T.R. Hoye, C.W. Macosko, A simple confined impingement jets mixer for flash nanoprecipitation, *J. Pharm. Sci.* 101 (2012) 4018–4023.
- [26] L. Sun, Z. Liu, L. Wang, D. Cun, H.H.Y. Tong, R. Yan, X. Chen, R. Wang, Y. Zheng, Enhanced topical penetration, system exposure and anti-psoriasis activity of two particle-sized, curcumin-loaded PLGA nanoparticles in hydrogel, *J. Control. Release* 254 (2017) 44–54.
- [27] J. Sun, C. Bi, H.M. Chan, S. Sun, Q. Zhang, Y. Zheng, Curcumin-loaded solid lipid nanoparticles have prolonged in vitro antitumor activity, cellular uptake and improved in vivo bioavailability, *Colloids Surf B Biointerfaces* 111 (2013) 367–375.
- [28] H. Jin, R. He, M. Oyoshi, R.S. Geha, Animal models of atopic dermatitis, *J. Invest. Dermatol.* 129 (2009) 31–40.

- [29] P. Uysal, S. Avcil, B.I. Abas, C. Yenisey, Evaluation of oxidant-antioxidant balance in children with atopic dermatitis: a case-control study, *Am. J. Clin. Dermatol.* 17 (2016) 527–537.
- [30] B.J. Wang, H.M. Sheu, Y.L. Guo, Y.H. Lee, C.S. Lai, M.H. Pan, Y.J. Wang, Hexavalent chromium induced ROS formation, Akt, NF-kappaB, and MAPK activation, and TNF-alpha and IL-1alpha production in keratinocytes, *Toxicol. Lett.* 198 (2010) 216–224.
- [31] G.L. Johnson, R. Lapadat, Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases, *Science* 298 (2002) 1911–1912.
- [32] M.A. Read, M.Z. Whitley, S. Gupta, J.W. Pierce, J. Best, R.J. Davis, T. Collins, Tumor necrosis factor alpha-induced E-selectin expression is activated by the nuclear factor-kappaB and c-JUN N-terminal kinase/p38 mitogen-activated protein kinase pathways, *J. Biol. Chem.* 272 (1997) 2753–2761.
- [33] S.W. Huang, Y.J. Chen, S.T. Wang, L.W. Ho, J.K. Kao, M. Narita, M. Takahashi, C. Y. Wu, H.Y. Cheng, J.J. Shieh, Azithromycin impairs TLR7 signaling in dendritic cells and improves the severity of imiquimod-induced psoriasis-like skin inflammation in mice, *J. Dermatol. Sci.* 84 (2016) 59–70.
- [34] Y.L. Lee, C.W. Li, F.C. Sung, H.S. Yu, H.M. Sheu, Y.L. Guo, Environmental factors, parental atopy and atopic eczema in primary-school children: a cross-sectional study in Taiwan, *Br. J. Dermatol.* 157 (2007) 1217–1224.
- [35] M.R. Prausnitz, R. Langer, Transdermal drug delivery, *Nat. Biotechnol.* 26 (2008) 1261–1268.
- [36] A.H. Mota, P. Rijo, J. Molpeceres, C.P. Reis, Broad overview of engineering of functional nanosystems for skin delivery, *Int. J. Pharm.* 532 (2017) 710–728.
- [37] B.K. Johnson, R.K. Prud'homme, Chemical processing and micromixing in confined impinging jets, *AIChE J.* 49 (2003) 2264–2282.
- [38] K.M. Koczur, S. Mourdikoudis, L. Polavarapu, S.E. Skrabalak, Polyvinylpyrrolidone (PVP) in nanoparticle synthesis, *Dalton Trans.* 44 (2015) 17883–17905.
- [39] T.W. Prow, J.E. Grice, L.L. Lin, R. Faye, M. Butler, W. Becker, E.M. Wurm, C. Yoong, T.A. Robertson, H.P. Soyer, M.S. Roberts, Nanoparticles and microparticles for skin drug delivery, *Adv. Drug Deliv. Rev.* 63 (2011) 470–491.
- [40] R. Schulz, K. Yamamoto, A. Klossek, R. Flesch, S. Honzke, F. Rancan, A. Vogt, U. Blume-Peytavi, S. Hedtrich, M. Schafer-Korting, E. Ruhl, R.R. Netz, Data-based modeling of drug penetration relates human skin barrier function to the interplay of diffusivity and free-energy profiles, *Proc. Natl. Acad. Sci. U. S. A.* 114 (2017) 3631–3636.