European Journal of Pharmaceutics and Biopharmaceutics 73 (2009) 34–42

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

Influences of opioids and nanoparticles on in vitro wound healing models

Nadine B. Wolf [a,1], Sarah Küchler [a,1], Michal R. Radowski [b], Tobias Blaschke [c], Klaus D. Kramer [c], Günther Weindl [a], Burkhard Kleuser [a], Rainer Haag [b], Monika Schäfer-Korting [a,*]

[a] Institut für Pharmazie, Freie Universität Berlin, Berlin, Germany
[b] Institut für Chemie und Biochemie, Freie Universität Berlin, Berlin, Germany
[c] Institut für Physik, Freie Universität Berlin, Berlin, Germany

ARTICLE INFO

Article history:
Received 17 October 2008
Accepted in revised form 25 March 2009
Available online 1 April 2009

Keywords:
Wound healing models
Topical opioids
Solid lipid nanoparticles
Dendritic core-multishell nanotransporters
Skin irritation test
HET-CAM assay

ABSTRACT

For efficient pain reduction in severe skin wounds, topical opioids may be a new option – given that wound healing is not impaired and the vehicle allows for slow opioid release, since long intervals of painful wound dressing changes are intended. We investigated the influence of opioids on the wound healing process via in vitro models, migration assay and scratch test. In fact, morphine, hydromorphone, fentanyl and buprenorphine increased the number of migrated HaCaT cells (spontaneously transformed keratinocytes) twofold. In the scratch test, morphine accelerated the closure of a monolayer wound (scratch). As possible slow release application forms are nanoparticulate systems like solid lipid nanoparticles (SLN) and dendritic core-multishell (CMS) nanotransporters, we evaluated the effect of unloaded nanoparticles on HaCaT cell migration, too. CMS nanotransporters did not inhibit migration, SLN even enhanced it (twofold). Applying morphine plus unloaded nanoparticles reduced morphine effects possibly due to uptake into CMS nanotransporters and adsorption to the surface of SLN. In contrast to SLN, TGF-β1 was taken up by CMS nanotransporters, too. Both nanoparticles are tolerable by skin and eye as derived from Episkin-SM™ skin irritation test and HET-CAM assay. No acute toxic effects were observed either. In conclusion, opioids as well as the investigated nanoparticulate carriers conform the essential conditions for topical pain reduction.

1. Introduction

Agreeable wound management for burn and skin graft (donor site) patients is challenging clinicians. To avoid systemic side effects and the – though limited – risk of dependency, long lasting pain reduction by topical application of opioids appears advantageous. Due to the peripheral expression of (μ) opioid receptors on nociceptive nerve endings [1,2], this should be possible by principle, yet clinical success appears limited [3,4], which may be due to a low amount of opioid reaching the target site over time. Since nanoparticulate carrier systems can increase skin penetration severalfolds [5–8] and can slowly release the loaded drug [9–12], we aimed to investigate the potential of nanoparticulate carriers for a topical application of opioids. First, however, we had to unravel the influence of either drug or carrier on wound healing in order to exclude negative effects. In fact limited data indicate that opioids may even enhance wound healing [13,14].

The wound healing process consists of at least three different phases. The initial inflammatory phase is mainly characterized by a clotting process and chemotaxis of inflammatory cells which help to cleanse the wound. Reepithelialization occurs in the proliferative phase. Stimulated by cytokines, keratinocytes migrate across the wound matrix to close the wound before scar formation is taking place [15]. Enhancement of keratinocyte migration indicates a positive effect on the healing process, delayed migration indicates a negative effect. In the final remodelling phase we observe a reorganization of the scar tissue. Here we studied the effects of opioids on human keratinocyte migration using migration assay and scratch test.

For the use in severely damaged skin the choice of pharmaceutical formulation is very important, as the wound healing process must not be disturbed by the vehicle constituents either. Since the change of the wound dressing is very painful, long lasting analgesic effects conforming a low changing-frequency would be optimal. Aiming for nanocarrier systems for opioid delivery we

Abbreviations: B, buprenorphine; BSA, bovine serum albumin; CMS, dendritic core-multishell nanotransporters; $c_{particle}$, particle concentration; F, fentanyl; FCS, fetal calf serum; H, hydromorphone; HaCaT, human adult low calcium temperature keratinocytes; M, morphine; n.s., not significant; PBS, phosphate buffered saline; r.t., room temperature; SD, standard deviation; SLN, solid lipid nanoparticle(s); TGF-β1, transforming growth factor β1.

* Corresponding author. Institut für Pharmazie, Freie Universität Berlin, Königin-Luise-Str. 2–4, D-14195 Berlin, Germany. Tel.: +49 30 838 53283; fax: +49 30 838 54399.
E-mail address: msk@zedat.fu-berlin.de (M. Schäfer-Korting).

[1] These authors contributed equally to this work.

0939-6411/$ - see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejpb.2009.03.009

compared solid lipid nanoparticles (SLN) [7,8,16,17] and dendritic core-multishell (CMS) nanotransporters which can encapsulate hydrophilic as well as lipophilic substances and transport them to polar and apolar environments [18,19]. Improvement in skin penetration by CMS nanotransporters even can surmount the efficiency of SLN [6]. SLN and CMS nanotransporters were tested for the influence on keratinocyte migration and local tolerability.

While SLN are composed of lipids having GRAS (generally recommended as safe) status, the local tolerability of CMS nanotransporters is by and large unknown. In fact, due to the nanosize, the carriers may show novel properties compared with the bulk material. Since they are enabled to interact with cells [6], subcellular structures, etc., possibly harmful effects have to be excluded [20], which is of crucial importance especially with the intention to apply the nanotransporters onto skin without any or just recovering barrier function.

2. Materials and methods

2.1. Materials

Morphine hydrochloride, buprenorphine hydrochloride, hydromorphone hydrochloride and naloxone hydrochloride dihydrate were purchased from Fagron (Barsbüttel, Germany). Fentanyl citrate and transforming growth factor-β1 (TGF-β1) were obtained from Sigma–Aldrich (Munich, Germany). Test substances were dissolved in phosphate-buffered saline (PBS, pH 7.4 or pH 6.5, respectively) with 0.4% bovine serum albumin (BSA, Sigma-Aldrich).

Compritol® 888 ATO (glyceryl behenate) was a gift from Gattefossé (Weil a. Rh., Germany). Lutrol F68® (poloxamer 188) was obtained from BASF (Ludwigshafen, Germany). Chemicals for the synthesis of the CMS nanotransporters were purchased from Fluka (Seelze, Germany), except for polyglycerol amine (PG_{10000}) which was prepared as described [21]. Reagents for the diazotization reaction were purchased from Promega (Mannheim, Germany). Fibronectin, Giemsa stain solution and all RPMI 1640 medium and supplements for HaCaT cell culture were purchased from Sigma–Aldrich (Munich, Germany). Culture medium for keratinocytes and supplements was obtained from Lonza (Walkersville, MD, USA). Polycarbonate membrane inserts for the migration assays (pore size: 8 μm) were obtained from Biochrom (Berlin, Germany). Isopropanol, sodium dodecylsulfate, hydrochloric acid, acetic acid, sodium hydroxide and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma–Aldrich (Munich, Germany). Texapon® ASV 50 was from Cognis (Düsseldorf, Germany).

HaCaT cells were a gift from Prof. Fusenig from the DKFZ, Heidelberg. For skin irritation test Episkin-SM™ reconstructed human epidermis (0.38 cm^2) was delivered from SkinEthic Laboratories (Nice, France), as well as the maintenance and assay medium. The white leghorn chicken eggs for the HET-CAM test were purchased from Lohmann livestock breeding (Cuxhafen, Germany).

2.2. Particle preparation

SLN and CMS nanotransporters (Table 1) were unloaded. SLN (10% lipid), composed of 10% glycerol behenate and 2.5% surfactant (poloxamer 188) were prepared as described [17]. In short, the lipid was melted, an aqueous solution of poloxamer 188 of the same temperature was added and a preemulsion was formed using an ultra turrax. The premix was homogenized by a Lab 60 high pressure homogenizer (APV Gaulin, Lübeck, Germany) at 500 bar for 2.5 min. SLN were stored at 8 °C and used for migration experiments within 1 month, though stable for almost 3 months [17].

The CMS nanotransporters have the empirical formula $PG_{10000}(\text{-}NH_2)_{0.7}(C_{18}mPEG_6)_{1.0}$ and were synthesized using previously described procedures [19]. For testing, CMS nanotransporter dispersions were used at concentrations of 0.5% and 5%.

Table 1
Average size and particle size distribution of the applied nanoparticles, measured via photon correlation spectroscopy and laser diffraction [6].

	Size (nm)	Polydispersity index (PI)
SLN	150-170	⩽0.250
CMS nanotransporters	20–30	⩽0.250

2.3. Parelectric spectroscopy

Aiming to elucidate possible adsorption or incorporation processes, interactions of the nanoparticles with morphine were studied by parelectric spectroscopy (PS). This method and its underlying theoretical model have been described in detail elsewhere [17,22,23].

2.4. Cell culture

HaCaT cells were maintained in 75 cm^2 flasks (Nunc, Wiesbaden, Germany) with RPMI 1640 medium (pH 7.4) containing 10% fetal calf serum (FCS) and L-glutamine (5 mM). Cells were grown at 37 °C and 5% CO_2 and medium was changed every 2 or 3 days. As soon as grown confluent, cells were split (1:10 or 1:15).

Primary human keratinocytes were isolated from juvenile foreskin, the remainder of circumcision surgeries. Keratinocytes were grown in keratinocyte basal medium supplemented with epidermal growth factor, insulin, gentamicin sulfate, amphotericin B, hydrocortisone and bovine pituitary extract (keratinocyte growth medium, KGM) to a confluence of about 70% [24]. Keratinocytes of the second or third passage were used for the experiments.

2.5. Migration assays

Migration assays were performed with HaCaT cells in modified Boyden chambers as described [25–27]. To measure chemotaxis inserts with polycarbonate membranes (pore size: 8 μm) were coated with fibronectin (3 μg/ml in aqua bidest.) for 1 h at 37 °C and placed into 24-well plates (Nunc, Wiesbaden, Germany) which already contained the test substances (opioids, naloxone) or transforming growth factor β1 (TGF-β1, positive control) in the indicated concentrations in supplemented RPMI 1640 medium (pH 7.4). The pure solvent served as negative control. HaCaT cells (2×10^5 cells/well) were seeded into the upper chamber and cells were allowed to migrate for 5 h at 37 °C towards the lower chamber. Then medium was aspired and remaining cells on the upper surface of the membrane were removed with a Q-tip. Cells that had migrated into the membrane were fixed in ice cold ethanol (96%) for 2 min at −18 °C and stained with Giemsa solution (1:10 in water) for 30 min at 37 °C. To test the inhibitory effect of naloxone, cells were preincubated with growth medium containing naloxone (1 μM) for 15 min in their culture flasks before they were used for the migration assay.

Migration in acidic environment was studied using medium (pH 6.5) acidified with concentrated hydrochloric acid. To test the influence of carriers on the migration of HaCaT cells, suspensions of SLN (40 μl) and CMS nanotransporters (40 μl), respectively were added to the cell suspension, that is, to the upper chamber during the assay.

To measure chemokinesis the chemokine gradient was disrupted by adding the opioids or TGF-β1 to the upper as well as to the lower chamber.

Migrated cells were quantified by light microscopy at a magnification of x150 (Axiovert 135, Carl Zeiss, Göttingen, Germany) by counting the stained cells from 10 randomly selected fields per well. Data presented are derived from at least three independent experiments.

2.6. Wound healing assay

For the in vitro scratch assay [28,29], HaCaT cells were seeded in six-well plates (Nunc, Wiesbaden, Germany) in a density of 5×10^5 cells/well in growth medium. Cells were grown until they had reached a confluence of about 80%. Then a scratch was made through each well using a sterile 200-μl pipet tip. Cells were washed twice with PBS pH 7.4 and the medium was changed. As a positive control TGF-β1 (1 ng/ml) was added to the medium; the test substance was morphine, which was added in a concentration of 100 nM. Scratches were documented under the microscope (x150) immediately after the wounding procedure and once more when kept at 37 °C, 5% CO_2 for 48 h. Pictures were taken exactly at the same position before and after the incubation to document the repair process. The experiments were repeated twice and representative pictures are shown.

2.7. Detection of NO formation

NO formation was measured indirectly by quantifying nitrite (NO_2^-) – one of the two stable decomposition products of NO using a diazotization (Griess) reaction. A dye is formed in the presence of NO_2^- which can be detected by measurement of absorbance [30].

HaCaT cells (5×10^4 cells/well) were seeded in 96-well plates (Nunc, Wiesbaden, Germany), cultivated in growth medium at 37 °C, 5% CO_2 for 24 h and then stimulated with 100 nM morphine for another 24 h without changing the medium. Negative control cells were not stimulated, adding the pure solvent only. To quantify the nitrite concentration medium was removed, centrifuged (1000 g, 5 min, 4 °C) and transferred into a new 96-well plate (50 μl/well). Following the addition of 50 μl of a sulfanilamide solution (1% sulfanilamide in 5% phosphoric acid) and once more following the addition of 50 μl of a NED solution (0.1% N-1-naphthylethylendiamine dihydrochloride in water), samples were incubated for 10 min at room temperature, protected from light. Then absorbance was measured within 30 min in a plate reader with a 540 nm filter (FluoStar OPTIMA, BMG Labtech, Offenburg, Germany). The results shown (absorbance values corrected for blanks) are mean values derived from three independent experiments.

2.8. Viability assay

For cytotoxicity testing, the activity of the cellular mitochondrial dehydrogenase was determined by measuring MTT reduction and conversion into a blue formazan salt [31]. Primary keratinocytes (4×10^4) were seeded into 24-well plates (TPP, Trasadingen, Germany). At the day after seeding the cells were incubated at 37 °C with the nanoparticles for 24 h and 48 h, respectively, before the MTT test was performed as described [24]. SLN (10% lipid) and CMS nanotransporters (5%) were diluted with keratinocyte growth medium (KGM) 1:100; 1:200; 1:1000; 1:2000, respectively. Then 40 μl of MTT solution (5 mg/ml) per well were applied and the cells were incubated for another 3 h. After removing the supernatants, 250 μl dimethylsulfoxide (DMSO) was added to dissolve the formazan salt and its optical density (OD) was measured using the microplate reader setting the excitation 570 nm. Untreated keratinocytes served as reference, the measured fluorescence values were set 100%. Each concentration was tested in triplicate and the experiments were repeated at least twice, respectively. A cell viability $\leqslant$70% predicts cytotoxic effects.

2.9. Skin and eye irritations

Episkin® skin irritation test and HET-CAM test are validated nonclinical methods to evaluate the skin [32] and eye irritation [33] potential of chemicals and preparations. In this study the irritant potential of SLN (10 % lipid) and CMS nanotransporters (5%) were tested, respectively.

2.9.1. Episkin®Skin Irritation Test-^{42hour}

The in vitro skin irritation assay was performed according to the standard operating procedure of the ECVAM Skin Irritation Validation Study [32]. An aqueous solution of SDS (5%) served as positive control, PBS (pH 7.4) as negative control. The test materials (10 μl) were applied to the surface of the reconstructed epidermis for 15 min. Then test substances were rinsed off with PBS and the tissues were postincubated for 42 h. For endpoint assessment cell viability was measured via MTT test as described above. A reduction in cell viability (expressed in %) in treated tissues compared to the negative control predicts an irritant potential and substances with viability $\leqslant$50% are qualified as irritants (R38 according EU classification standards). Direct chemical MTT reduction by the test substance was excluded by a preliminary test [32]. The skin irritation test was performed with two Episkin-SM™ batches using three tissue units per substance.

2.9.2. Hen's egg test on the chorioallantoic membrane (HET-CAM)

Hen's eggs were incubated in an incubator (Bruja, Hammelburg, Germany) with automatic rotation device at 37.5 °C for nine days. By candling the eggs unfertilized eggs were identified and discarded. The fertilized eggs were opened at the blunt end, the visible white egg membrane was moistened with a few drops of distilled water and then removed. The test substances (300 μl) were applied onto the underlying chorioallantoic membrane (CAM) which was then observed using a stereo microscope equipped with a camera (Olympus, Tokyo, Japan). For documentation, pictures of the untreated and treated CAM were taken at three different magnifications. The eye irritation potential of the nanocarriers was tested using six eggs per test material. For test evaluation endpoint assessment was used. After a residence time of 3 min the CAM was inspected for the possible endpoints haemorrhage, intravascular coagulation, extravascular coagulation and vessel-lysis which then were classified as none, weak, moderate or severe reaction [33]. As SLN are non-transparent, the suspensions were carefully rinsed off with 0.9% sodium chloride solution before observation. To afford a semi-quantitative evaluation of the reactions, preliminary tests with reference substances were performed applying three different concentrations of Texapon ASV, sodium hydroxide and acetic acid onto two CAMs, respectively, to distinguish between weak, moderate and severe effects (ECVAM InVitox Protokoll No 96).

2.10. Statistics

The data presented (arithmetic mean values ± standard deviation) resulted from 3–5 independent experiments. Statistical analysis for enhancement over negative control (set in the migration experiment), concentration dependency of opioid effects and the effect of naloxone is based on the Wilcoxon matched pairs test; $p \leqslant 0.05$ is considered to indicate a difference.

3. Results and discussion

Aiming at the topical use of opioids in skin wounds for pain reduction, the influence on wound closure is of high relevance. Inhibition of wound closure will not allow this innovative thera-

peutic approach. Opioid receptors are present in the human skin [34–36], the endogenous ligand β-endorphine induces keratinocyte migration [37,38] and opioid receptor agonists like dalargin, FK-33824, DADLE, met-enkephalin and morphine accelerate wound healing in rats [13,14] – possibly due to a stimulated migration of keratinocytes.

The final aim is to find an application form for opioids, which allows pain reduction for up to 7 days until the first change of wound dressing in burn patients. Sustained release of e.g. vitamin A and glucocorticoids can be realized by loading the drugs onto SLN [9,11,39]. Furthermore, the topical application of SLN can avoid systemic side effects as the permeation of drugs through the skin is reduced [40,41], possibly due to interference of the lipid nanoparticles with the epidermal lipids of the skin surface [42]. Another option for the drug delivery of opioids is CMS nanotransporters, a type of nanocarriers, that can be loaded with lipophilic as well as hydrophilic drugs.

3.1. Migration induction and wound healing

The HaCaT cell is an adequate cell line to study keratinocyte physiology [43] and migration is well in accordance with migration of primary human keratinocytes [25], chemotactic and chemokinetic agents induce HaCaT cell migration [27]. Initial experiments with modified Boyden chambers proved both keratinocyte types to respond to opioids. Inducing the expression of fibronectin receptors, TGF-β1 stimulates keratinocyte migration towards fibronectin [44] covering the membrane. Here, the positive control TGF-β1 (1 ng/ml) consistently duplicated the number of migrated cells counted in the membrane compared to the negative control ($p \leqslant 0.05$). In the chemotactic set-up opioids, too, enhanced HaCaT cell migration ($p \leqslant 0.05$) which was concentration dependant. Maximum opioid effects corresponded to TGF-β1 efficacy. Opioid effects were completely inhibited in the presence of naloxone (1 μM; $p \leqslant 0.05$) whereas the TGF-β1 effect was not influenced by the opioid receptor antagonist (Fig. 1). Therefore opioid-induced migration enhancement is due to a specific receptor interaction.

To investigate whether the effect of the opioids was chemotactic or chemokinetic, drugs were added to the upper as well as to the lower chambers which abolished the gradient in a second set of experiments. TGF-β1 enhanced HaCaT cell migration both chemotactically and chemokinetically, the chemokinetic approach, however, eliminated migration induced by morphine (Fig. 2), fentanyl and hydromorphone (data not shown). This indicates a chemotactic rather than a chemokinetic effect of these opioids on HaCaT cells. In contrast, buprenorphine still enhanced keratinocyte migration to a limited extent ($p \leqslant 0.05$) even after abolishing the gradient (Fig. 2), which suggests that buprenorphine at least partially has a chemokinetic effect on the HaCaT cells. As an acidic pH has to be considered in inflamed wound areas, the efficacy of the opioids in this condition had to be tested, too. Adjusting the cell culture medium to pH 6.5 the opioids still evoked a significant chemotactic stimulation of HaCaT cell migration ($p \leqslant 0.05$) although slightly less than that at physiological pH level (Fig. 3). In the different set-ups of the migration assays the tested opioids proved to evoke almost identical effects. Therefore we decided to focus on morphine as a representative opioid for the following investigations.

Next we aimed to verify the opioid effect using an independent approach. The so called scratch test [28,29] allows to investigate the ability of a substance to accelerate closure of a wound that has been created artificially in a cell monolayer. After a repair period of 48 h at 37 °C, the HaCaT cells had more efficiently proliferated and migrated into the damaged area in the presence of TGF-β1 than without any supplies in the medium. Also the addition of morphine (100 nM) increased the ability of the HaCaT cells to close the damage (Fig. 4). The supply of TGF-β1 or morphine resulted in an interspace clearly less broad than that observed with negative control.

To summarize, using the human keratinocyte derived cell line HaCaT, we found that in fact the opioids tested stimulated cell migration and closure of experimental wounds, enhancement of migration was concentration dependant and could be blocked by the opioid receptor antagonist naloxone, indicating a specific opioid-receptor interaction. Although morphine evoked a chemotactic effect in the 5 h migration assay, its activity in the scratch assay is not surprising because when increasing the test period of the migration assay to 24 h morphine also was active under the "chemokinetic" approach. Surrounded by an acidified environment (pH 6.5), opioids still enhanced HaCaT cell migration indicating that they may be effective, too, when applied onto inflamed tissue like

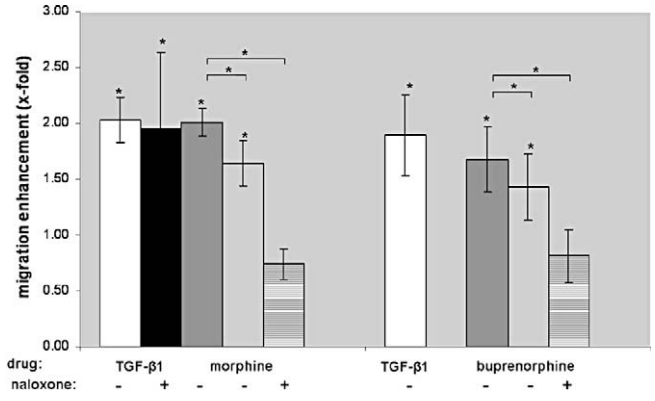

Fig. 1. Chemotactic response of HaCaT cells (mean ± SD enhancement over control, set 1) induced by morphine and buprenorphine. Opioids (100 nM dark grey columns and 1 nM, light grey columns) or TGF-β1 (1 ng/ml, pos. control) were applied for 5 h. TGF-β1 and opioids enhanced cell migration up to twofold over control. The opioid receptor antagonist naloxone (1 μM) abolished the opioid effect (1 μM, striped columns) without influencing the TGF-β1 effect (black column) indicating a specific receptor interaction. The experiments were performed minimum in triplicates ($^{*}p \leqslant 0.05$).

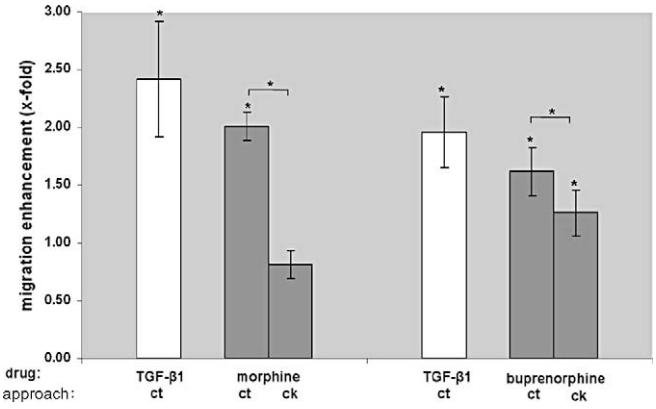

Fig. 2. Opioid-induced migration of HaCaT (mean ± SD enhancement over control, set 1) cells is a chemotactic response. Opioids (100 nM) have been applied for 5 h only in the lower chamber (ct: chemotactic response) and in both chambers (ck: chemokinetic approach), thereby abolishing their gradient. With the latter approach enhanced migration was abolished in the case of morphine, fentanyl and hydromorphone (data not shown) indicating that these opioids act chemotactically, whereas buprenorphine still slightly enhanced keratinocyte migration indicating a partial chemokinetic effect on the HaCaT cells. TGF-β1 (1 ng/ml) enhanced HaCaT cell migration about twofold using the chemokinetic approach, too, indicating a chemotactic and chemokinetic power. The experiments were performed minimum in triplicates ($^{*}p \leqslant 0.05$).

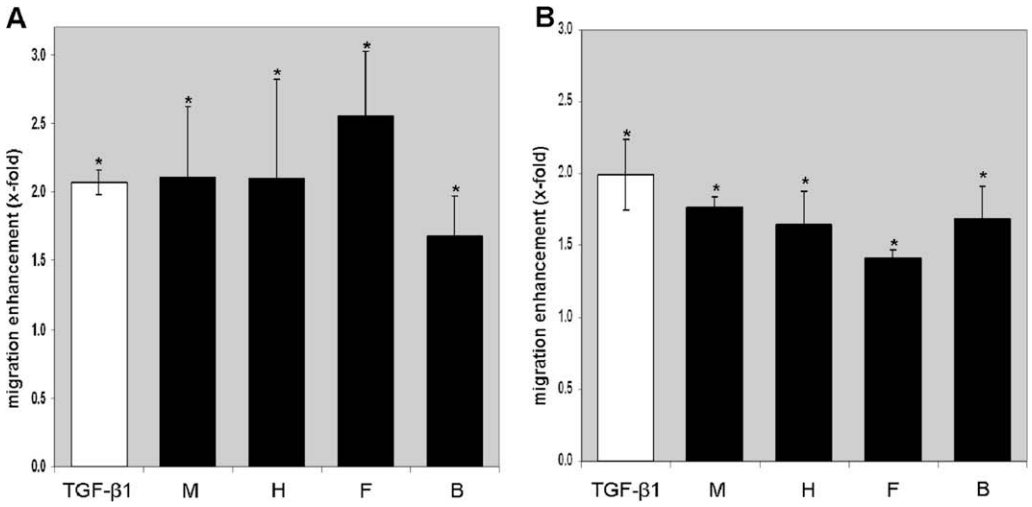

Fig. 3. Enhanced HaCaT cell migration (mean ± SD over control, set 1) in response to morphine (M), hydromorphone (H), fentanyl (F), and buprenorphine (B, each 100 nM) at pH 7.4 (A) and pH 6.5 (B) indicates efficacy in inflamed wound areas (pos. control: TGF-β1, 1 ng/ml). The chemotaxis experiments were performed minimum in triplicates ($^{*}p \leqslant 0.05$).

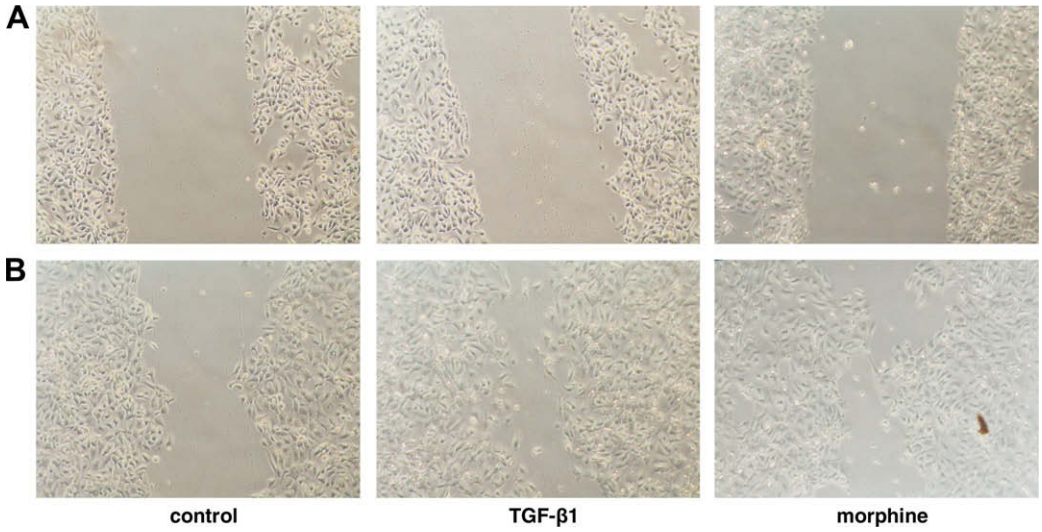

Fig. 4. Scratch test. HaCaT cells were seeded in six-well plates and grown up to 80% confluence. The scratch was made with a pipet tip and pictures were taken immediately (A, 150×). Cells were allowed to close the damage for 48 h when kept at 37 °C, then photo documentation was repeated (B). TGF-β1 (1 ng/ml) and morphine (100 nM) facilitated wound closure. The experiment was repeated twice and similar results were obtained.

burn wounds. Next opioid effects on wound healing and the influence of carrier systems have to be studied in vivo.

3.2. NO formation

Among other molecules, nitric oxide (NO), produced via the inducible NO synthase (iNOS) or the endothelial NO synthase (eNOS) is of importance for the wound healing process, e.g. a functionally active iNOS is fundamental for an adequate formation of granulation tissue [45]. The NO in the wound area can modulate cytokines and therefore stimulate angiogenesis, cell proliferation [46] and epithelial cell migration [47]. Human keratinocytes express the inducible nitric oxide synthase (iNOS) and the endothelial nitric oxide synthase (eNOS) [48]. In the absence of both eNOS and iNOS wound healing is delayed, which has been demonstrated via iNOS and eNOS knockout mice [30,49]. A positive effect on wound healing via an activation of keratinocyte migration has been shown for sphingosin 1-phosphate (S1P), too [27,50]. In fact S1P and its structural analogue FTY720-phosphate activate the eNOS [51,52]. Furthermore it has been shown that both NOS isoforms can be induced by opioids in murine skin cells [13] and a stimulating effect of endomorphins on the iNOS of inflammatory cells has been described [53].

Therefore we studied, if opioids induce HaCaT cell migration via NO-formation as already described for epithelial cells, which switch from a stationary to a locomoting phenotype shortly after wounding [47]. Following stimulation with 100 nM morphine for 24 h, dye formation increased significantly ($p \leqslant 0.05$; Fig. 5) indicating that morphine induces the formation of nitric oxide in HaCaT cells. Thus keratinocyte migration can be due to NO-synthase activation. Since the Griess reaction-based assay measures the stable endproduct of oxidation of NO, nitrite, NO formation may be even underestimated due to e.g. nitrate formation.

3.3. Influence of nanoparticles on keratinocyte migration

As SLN and CMS nanotransporters are possible carrier systems for the opioids, it had to be tested whether these carriers had

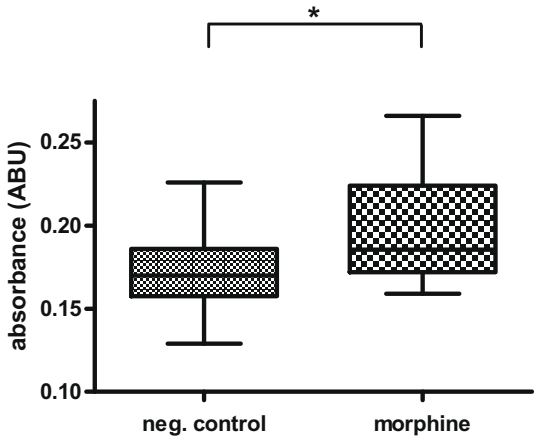

Fig. 5. Morphine (100 nM for 24 h) enhanced NO-formation of HaCaT cells ($^{*}p \leqslant 0.05$; $n = 3$) as measured by the Griess reaction.

any effect on cell migration, native and morphine induced. This was studied by adding the unloaded nanoparticles to the cell suspension during the migration assays. Fig. 6 shows that CMS nanotransporters did not influence migration, whereas non-loaded SLN strongly induced HaCaT cell migration ($p \leqslant 0.05$), efficiency was close to TGF-β1 effect. Free fatty acids from glycerol behenate by lipase cleavage [54] subsequently bind a tumor suppressor [55], which prevents cell migration. In fact p53 null cells displayed an enhanced cell spreading, which is a crucial component of cell migration [56,57]. HaCaT cells lack functional p53, yet express the highly homologous tumor suppressor p63 [58,59], and knockdown of p63 in HaCaT cells leads to stimulation of cell migration via up-regulation of several genes promoting motility [60,61]. The binding of free fatty acids and p63 leads to a state comparable with p63 knockdown and therefore explains our observation of induced migration.

The addition of the hydrophilic agent TGF-β1 together with the non-loaded particles may even improve the boost induced by SLN (Fig. 6; $p \leqslant 0.05$). Therefore TGF-β1 is not taken up and deactivated by the lipidic carrier during the test period of 5 h. In contrast, TGF-β1 activity declines when administered together with CMS nanotransporters. Therefore there appears some diffusion into the carrier matrix, which can incorporate hydrophilic agents [18]. Obviously this was also true for the addition of the lipophilic agent morphine together with CMS nanotransporters. In fact, the addition of morphine together with the CMS nanotransporters enhanced migration over the untreated control and empty nanotransporters (Fig. 6; $p \leqslant 0.05$) – yet less compared to the case when testing morphine alone (Fig. 1) indicating diffusion of morphine into the lipophilic shell of CMS nanotransporters. The addition of morphine together with SLN, however, was no more active than SLN alone. This effect should be explained by the ability of SLN to adsorb lipophilic agents like morphine, which reduces the concentration of freely available substance.

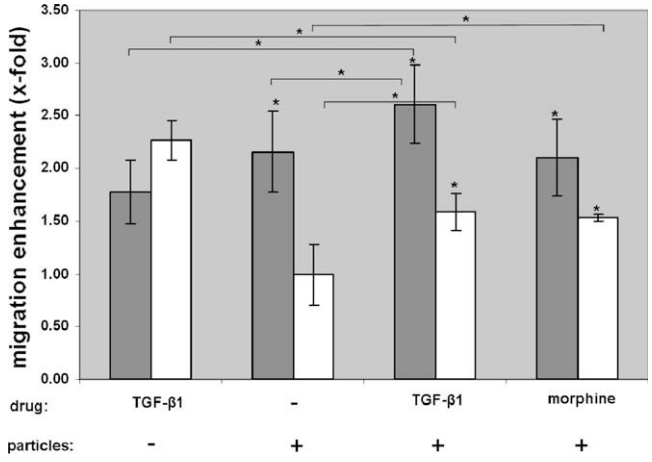

Fig. 6. Chemotaxis of HaCaT cells (mean ± SD enhancement over control, set 1) in response to morphine (100 nM) and TGF-β1 (1 ng/ml) in presence of SLN (grey columns) and CMS nanotransporters (white columns) in the upper chamber. Migration time was 5 h. Non-loaded SLNs induced HaCaT cell migration about twofold, CMS nanotransporters did not influence migration. TGF-β1 effects were partially enhanced by SLN and reduced by CMS nanotransporters. Also morphine effects declined partially in the presence of CMS nanotransporters ($^{*}p \leqslant 0.05$).

In conclusion, when added to the cell suspension in an unloaded form, CMS nanotransporters did not impair cell migration, thus should not interfere with opioid-induced pain reduction and accelerated wound closure. Importantly, unloaded SLN can even induce HaCaT cell migration. For future investigation of the intended long lasting opioid release from the eventual carrier system, the cutaneous freeze injury method [62] appears to be an appropriate pain model, as it allows to evaluate the hyperalgesia of a defined skin wound up to 72 h.

3.4. Interactions of morphine with SLN and CMS nanotransporters

Since CMS nanotransporters appeared to take up TGF-ß1 and morphine during 5 h of the migration assay (Fig. 6), the interactions were followed in a cell-free test system using parelectric spectroscopy. This technique unravelled nile red uptake in previous experiments [6]. In this experimental procedure we could prove that probes which contained morphine hydrochloride together with the particles – SLN or CMS nanotransporters, respectively – showed a scheme of mobility which was completely different from those probes containing morphine hydrochloride alone dissolved in PBS with 0.4% BSA. The combined increase in dipole density ($\Delta\varepsilon$) and decrease in dipole mobility (f_0) of the nanoparticles dispersions (columns 2 and 3) as depicted in Table 2 results from the larger masses of the carriers (SLN, CMS) with their higher dipole moments. Columns 4–6 show the reverse effect, the higher dipole moment of morphine hydrochloride is reduced by the addition of the carriers. This fact indicates an interaction between morphine and nanoparticles when being applied together, which can be either an attachment to the particle surface or an incorporation of morphine into the particle matrix. Concerning the CMS nanotransporters, we presume that the drug is incorporated into the particle matrix, an event which is well in analogy to the results with CMS nanoparticles and nile red [6] as well as carotene and the hydrophilic dye congo red [19]. Looking at drug-particle-interactions with the SLN and morphine however, opioid attachment to the surface appears more likely as compared to incorporation into the solid lipid matrix.

3.5. Cell viability

The local tolerability of the carrier systems is very important for topical applications. Dendritic structures are of particular concern

Table 2
Results from Parelectric Spectroscopy depicting dipole mobility (f_0) and dipole density ($\Delta\varepsilon$) [22]. All measurements have been carried out using 0.4% BSA in PBS, carriers are dispersed in the vehicle, morphine is dissolved in the vehicle. The standard deviations as caused by the method are ±0.005 for f_0 and ±0.05 for $\Delta\varepsilon$ in all formulations.

Dispersion	0.4% BSA	SLN dispersion	CMS	Morphine solution	Morphine solution + SLN	Morphine solution + CMS
$\Delta\varepsilon$ x10^5	1.60	1.85	2.12	9.00	3.60	3.35
f_0/MHz	0.160	0.127	0.114	0.030	0.068	0.072

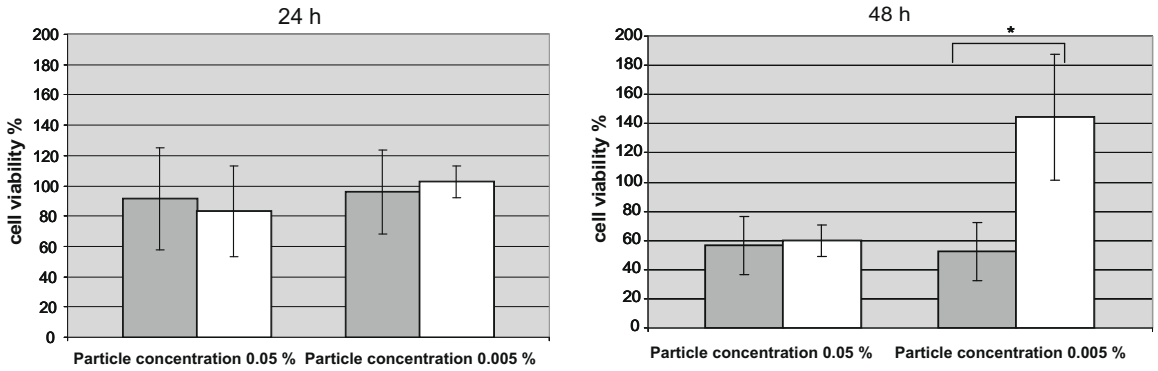

Fig. 7. Keratinocytes were incubated 24 and 48 h with SLN (grey column) and CMS nanotransporters (white column), respectively. The cell viability was measured by the MTT assay. A cell viability ⩽70% predicts cytotoxicity. $^{*}p \leqslant 0.05$ indicates differences in the tolerability of SLN and CMS nanotransporters.

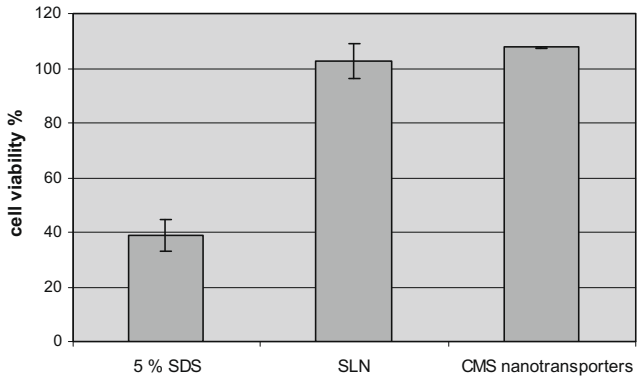

Fig. 8. Episkin® Skin irritation Test$^{-42hour}$: No irritant potential was found after application of SLN and CMS nanotransporters. As to be expected, 5% SDS solution (positive control) reduced cell viability to <40% indicating a valid result. The experiment was repeated and the results were confirmed.

as many structures induce unacceptable toxicity or immunogenicity [63]. Especially charged nanoparticles impair cell viability by inappropriate interactions with cellular membranes [64]. Amine-terminated dendrimers can form nanoscale holes upon lipid bilayers [65], negatively charged liposomes can activate human complement system [66]. Therefore the synthesis of uncharged nanocarriers is an important step to generate biocompatible drug carriers. In contrast, SLN show good local tolerability – given that adequate surfactants are used for stabilization of the dispersion [7,67–69]. This holds true with non-ionic surfactants and block-polymers, like the poloxamer 188 used here [22,68,70].

To detect possible cytotoxic effects on viable keratinocytes, MTT tests were performed with SLN and CMS nanotransporters. The effects of both nanocarriers were dependant on exposure time and concentration. When applying identical amounts (0.05% and 0.005% carrier) for 24 h, both SLN and CMS nanotransporters were non-toxic (keratinocyte viability > 70%). No significant alterations of cell viability were observed after application of both carrier sys-

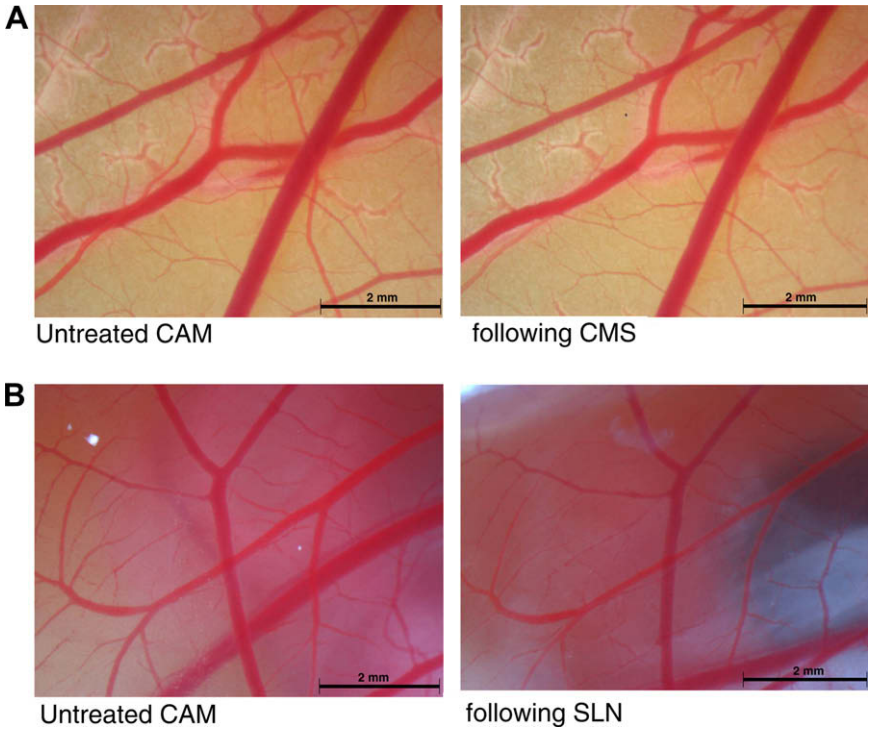

Fig. 9. Photo documentation of the untreated CAM (magnification 50×) following the application of the carrier systems for 3 min. CMS nanotransporters (A) and SLN (B) did not induce haemorrhage, vessel lysis or coagulation of the chorioallantoic membrane.

tems. After 48 h this was only true with the more diluted CMS nanotransporters ($p \leqslant 0.05$), although the increased viability (140%) may be a first hint of toxicity as seen with the higher concentration [71,72]. Such an increase in cell viability may be due to an enhanced cell proliferation just before the die off of the cells as already observed with other cell lines [71,72].

The higher concentration of CMS nanotransporters and both SLN dilutions decreased cell viability significantly (Fig. 7).

In summary, both nanocarrier systems show good compatibility over a period of 24 h, CMS nanotransporters even do not impair cell viability over a longer time (Fig. 7). The good tolerability of the CMS nanotransporters is due to the uncharged dendritic core surrounded by a monomethoxy poly(ethylene glycol) shell [19].

3.6. Skin and eye irritations

Aiming at the topical use, we had to assess the skin irritation potential of the nanocarrier systems, too. In this study, a validated *in-vitro* protocol based on the use of Episkin-SM™ was followed which can replace the respective experiment in the rabbit in regulatory toxicology [32]. Rabbit data overestimate the effects and, thus, are not directly assignable to human skin [73]. For the prediction and classification of the skin irritation potential of SLN and CMS nanotransporters, the Episkin® Skin Irritation Test-^{42hours} was performed. In contrast to keratinocyte monolayer cultures, the Episkin-SM™ matrix builds up a horny layer which impairs percutaneous absorption, though less than normal human skin [74]. Preceding direct MTT reduction tests showed no chemical interaction between the test substances and MTT which means that no modification of the test was necessary. The assay acceptance criteria were fulfilled as the optical density of the negative control was >0.6 and 5% SDS solution reduced the mean viability by $\geqslant$60%, respectively, standard deviation was $\leqslant$20% in both cases. Data evaluation revealed no irritant potential according to EU classification R38 for the tested carrier systems, cell viability was approximately 100% for SLN and CMS nanotransporters (Fig. 8).

Since accidental or intentional exposure to the eyes can result in slight reactions like redness or even severe reactions like the loss of vision [73], an evaluation of the eye irritation potential is crucial for topical dermatics [75], too. So the HET-CAM test was performed to assess the eye irritation potential of SLN and CMS nanotransporters. To objectify response evaluation, Texapone ASV, sodium hydrochloride and acetic acid at different concentrations were tested in parallel for reference which allowed for a differentiation between none, weak, moderate and severe reactions of the possible endpoints haemorrhage, coagulation and vessel lysis. The endpoint method was chosen because of the non-transparency of SLN. CAM pictures at different magnifications of the treated and untreated CAMs were taken and compared. SLN showed slight mucoadhesive features. We excluded major conjunctiva irritation using the HET-CAM test protocol [33]. Once more, an irritant reaction was not observed for the carrier systems (Fig. 9B and D).

Therefore, no changes in the CAM-morphology were observed after application of SLN or CMS carriers, respectively. As differentiation between non-irritant and slightly irritant substances for skin and eye currently is not possible with the *in-vitro* approach, *in-vivo* tests are still required.

4. Conclusion

In the future, opioids loaded to SLN or CMS nanotransporters may be suitable for topical pain reduction and improved wound healing since both opioids and even unloaded carriers can improve keratinocyte migration and wound closure. Major unwanted side effects are not induced by the nanoparticulate carrier systems as to be derived from standardized *in vitro* tests.

References

[1] M. Bigliardi-Qi, L.T. Sumanovski, S. Buchner, T. Rufli, P.L. Bigliardi, Mu-opiate receptor and Beta-endorphin expression in nerve endings and keratinocytes in human skin, Dermatology 209 (2004) 183–189.

[2] J.B. Nissen, M. Lund, K. Stengaard-Pedersen, K. Kragballe, Enkephalin-like immunoreactivity in human skin is found selectively in a fraction of CD68-positive dermal cells: increase in enkephalin-positive cells in lesional psoriasis, Arch. Dermatol. Res. 289 (1997) 265–271.

[3] C. Stein, M. Schafer, A.H. Hassan, Peripheral opioid receptors, Ann. Med. 27 (1995) 219–221.

[4] R.K. Twillman, T.D. Long, T.A. Cathers, D.W. Mueller, Treatment of painful skin ulcers with topical opioids, J. Pain Symptom Manage. 17 (1999) 288–292.

[5] R. Alvarez-Roman, A. Naik, Y.N. Kalia, R.H. Guy, H. Fessi, Enhancement of topical delivery from biodegradable nanoparticles, Pharm. Res. 21 (2004) 1818–1825.

[6] S. Küchler, M.R. Radowski, T. Blaschke, M. Dathe, J. Plendl, R. Haag, M. Schäfer-Korting, K.D. Kramer, Nanoparticles for skin penetration enhancement - A comparison of a dendritic core-multishell-nanotransporter and solid lipid nanoparticles, Eur. J. Pharm. Biopharm. 71 (2009) 243–250.

[7] C. Santos Maia, W. Mehnert, M. Schaller, H.C. Korting, A. Gysler, A. Haberland, M. Schäfer-Korting, Drug targeting by solid lipid nanoparticles for dermal use, J. Drug Target. 10 (2002) 489–495.

[8] J. Stecova, W. Mehnert, T. Blaschke, B. Kleuser, R. Sivaramakrishnan, C.C. Zouboulis, H. Seltmann, H.C. Korting, K.D. Kramer, M. Schäfer-Korting, Cyproterone acetate loading to lipid nanoparticles for topical acne treatment: particle characterisation and skin uptake, Pharm. Res. 24 (2007) 991–1000.

[9] V. Jenning, M. Schäfer-Korting, S. Gohla, Vitamin A-loaded solid lipid nanoparticles for topical use: drug release properties, J. Control. Release 66 (2000) 115–126.

[10] W. Mehnert, K. Mäder, Solid lipid nanoparticles: production, characterization and applications, Adv. Drug Deliv. Rev. 47 (2001) 165–196.

[11] S.A. Wissing, O. Kayser, R.H. Müller, Solid lipid nanoparticles for parenteral drug delivery, Adv. Drug Deliv. Rev. 56 (2004) 1257–1272.

[12] A. zur Mühlen, C. Schwarz, W. Mehnert, Solid lipid nanoparticles (SLN) for controlled drug delivery–drug release and release mechanism, Eur. J. Pharm. Biopharm. 45 (1998) 149–155.

[13] T. Poonawala, B.K. Levay-Young, R.P. Hebbel, K. Gupta, Opioids heal ischemic wounds in the rat, Wound Repair Regen. 13 (2005) 165–174.

[14] V.A. Vinogradov, S.E. Spevak, K.N. Iarygin, N.V. Korobov, A.I. Solov'eva, Opioid activity of peptides and wound healing of the skin, Bull. Eksp. Biol. Med. 104 (1987) 89–91.

[15] G. Broughton 2nd, J.E. Janis, C.E. Attinger, Wound healing: an overview, Plast. Reconstr. Surg. 117 (2006) 1e-S–32e-S.

[16] V. Jenning, A. Gysler, M. Schäfer-Korting, S.H. Gohla, Vitamin A loaded solid lipid nanoparticles for topical use: occlusive properties and drug targeting to the upper skin, Eur. J. Pharm. Biopharm. 49 (2000) 211–218.

[17] S. Lombardi Borgia, M. Regehly, R. Sivaramakrishnan, W. Mehnert, H.C. Korting, K. Danker, B. Röder, K.D. Kramer, M. Schäfer-Korting, Lipid nanoparticles for skin penetration enhancement-correlation to drug localization within the particle matrix as determined by fluorescence and parelectric spectroscopy, J. Control. Release 110 (2005) 151–163.

[18] R. Haag, Supramolecular drug-delivery systems based on polymeric core-shell architectures, Angew. Chem. Int. Ed. Engl. 43 (2004) 278–282.

[19] M.R. Radowski, A. Shukla, H. von Berlepsch, C. Böttcher, G. Pickaert, H. Rehage, R. Haag, Supramolecular aggregates of dendritic multishell architectures as universal nanocarriers, Angew. Chem. Int. Ed. Engl. 46 (2007) 1265–1269.

[20] SCCP, The Scientific Committee on Cosmetic Products (SCCP) Opinion on safety of nanomaterials in vosmetic products, 2007. Available from: <http://eceuropaeu/health/ph_risk/committees/04_sccp/docs/sccp_o_123pdf>.

[21] A. Sunder, M. Kramer, R. Hanselmann, R. Mulhaupt, H. Frey, Molecular nanocapsules based on amphiphilic hyperbranched polyglycerols, Angew. Chem. Int. Ed. Engl. 38 (1999) 3552–3555.

[22] T. Blaschke, L. Kankate, K.D. Kramer, Structure and dynamics of drug-carrier systems as studied by parelectric spectroscopy, Adv. Drug Deliv. Rev. 59 (2007) 403–410.

[23] R. Sivaramakrishnan, C. Nakamura, W. Mehnert, H.C. Korting, K.D. Kramer, M. Schäfer-Korting, Glucocorticoid entrapment into lipid carriers – characterisation by parelectric spectroscopy and influence on dermal uptake, J. Control. Release 97 (2004) 493–502.

[24] A. Gysler, K. Lange, H.C. Korting, M. Schäfer-Korting, Prednicarbate biotransformation in human foreskin keratinocytes and fibroblasts, Pharm. Res. 14 (1997) 793–797.

[25] T. Bartolmäs, T. Heyn, M. Mickeleit, A. Fischer, W. Reutter, K. Danker, Glucosamine-glycerophospholipids that activate cell–matrix adhesion and migration, J. Med. Chem. 48 (2005) 6750–6755.

[26] B. Sauer, R. Vogler, K. Zimmermann, M. Fujii, M.B. Anzano, M. Schäfer-Korting, A.B. Roberts, B. Kleuser, Lysophosphatidic acid interacts with transforming growth factor-beta signaling to mediate keratinocyte growth arrest and chemotaxis, J. Invest. Dermatol. 123 (2004) 840–849.

[27] R. Vogler, B. Sauer, D.S. Kim, M. Schäfer-Korting, B. Kleuser, Sphingosine-1-phosphate and its potentially paradoxical effects on critical parameters of cutaneous wound healing, J. Invest. Dermatol. 120 (2003) 693–700.
[28] E. Hintermann, M. Bilban, A. Sharabi, V. Quaranta, Inhibitory role of alpha 6 beta 4-associated erbB-2 and phosphoinositide 3-kinase in keratinocyte haptotactic migration dependent on alpha 3 beta 1 integrin, J. Cell Biol. 153 (2001) 465–478.
[29] L. Koivisto, G. Jiang, L. Hakkinen, B. Chan, H. Larjava, HaCaT keratinocyte migration is dependent on epidermal growth factor receptor signaling and glycogen synthase kinase-3alpha, Exp. Cell Res. 312 (2006) 2791–2805.
[30] P.C. Lee, A.N. Salyapongse, G.A. Bragdon, L.L. Shears 2nd, S.C. Watkins, H.D. Edington, T.R. Billiar, Impaired wound healing and angiogenesis in eNOS-deficient mice, Am. J. Physiol. 277 (1999) H1600–H1608.
[31] C. Faller, M. Bracher, N. Dami, R. Roguet, Predictive ability of reconstructed human epidermis equivalents for the assessment of skin irritation of cosmetics, Toxicol. In Vitro 16 (2002) 557–572.
[32] H. Spielmann, S. Hoffmann, M. Liebsch, P. Botham, J.H. Fentem, C. Eskes, R. Roguet, J. Cotovio, T. Cole, A. Worth, J. Heylings, P. Jones, C. Robles, H. Kandarova, A. Gamer, M. Remmele, R. Curren, H. Raabe, A. Cockshott, I. Gerner, V. Zuang, The ECVAM international validation study on in vitro tests for acute skin irritation: report on the validity of the EPISKIN and EpiDerm assays and on the Skin Integrity Function Test, Altern. Lab. Anim. 35 (2007) 559–601.
[33] H. Spielmann, M. Liebsch, F. Moldenhauer, H.G. Holzhutter, D.M. Bagley, J.M. Lipman, W.J. Pape, H. Miltenburger, O. de Silva, H. Hofer, W. Steiling, IRAG working group 2. CAM-based assays. Interagency Regulatory Alternatives Group, Food Chem. Toxicol. 35 (1997) 39–66.
[34] M. Bigliardi-Qi, C. Gaveriaux-Ruff, K. Pfaltz, P. Bady, T. Baumann, T. Rufli, B.L. Kieffer, P.L. Bigliardi, Deletion of mu- and kappa-opioid receptors in mice changes epidermal hypertrophy, density of peripheral nerve endings, and itch behavior, J. Invest. Dermatol. 127 (2007) 1479–1488.
[35] M. Bigliardi-Qi, C. Gaveriaux-Ruff, H. Zhou, C. Hell, P. Bady, T. Rufli, B. Kieffer, P. Bigliardi, Deletion of delta-opioid receptor in mice alters skin differentiation and delays wound healing, Differentiation 74 (2006) 174–185.
[36] S. Salemi, A. Aeschlimann, N. Reisch, A. Jungel, R.E. Gay, F.L. Heppner, B.A. Michel, S. Gay, H. Sprott, Detection of kappa and delta opioid receptors in skin–outside the nervous system, Biochem. Biophys. Res. Commun. 338 (2005) 1012–1017.
[37] P.L. Bigliardi, S. Buchner, T. Rufli, M. Bigliardi-Qi, Specific stimulation of migration of human keratinocytes by mu-opiate receptor agonists, J. Recept. Signal Transduct. Res. 22 (2002) 191–199.
[38] P.L. Bigliardi, L.T. Sumanovski, S. Buchner, T. Rufli, M. Bigliardi-Qi, Different expression of mu-opiate receptor in chronic and acute wounds and the effect of beta-endorphin on transforming growth factor beta type II receptor and cytokeratin 16 expression, J. Invest. Dermatol. 120 (2003) 145–152.
[39] H. Yuan, L.L. Wang, Y.Z. Du, J. You, F.Q. Hu, S. Zeng, Preparation and characteristics of nanostructured lipid carriers for control-releasing progesterone by melt-emulsification, Colloids Surf. B Biointerf. 60 (2007) 174–179.
[40] J.Y. Fang, P.F. Liu, C.M. Huang, Decreasing systemic toxicity via transdermal delivery of anticancer drugs, Curr. Drug Metab. 9 (2008) 592–597.
[41] J. Liu, W. Hu, H. Chen, Q. Ni, H. Xu, X. Yang, Isotretinoin-loaded solid lipid nanoparticles with skin targeting for topical delivery, Int. J. Pharm. 328 (2007) 191–195.
[42] J. Kuntsche, H. Bunjes, A. Fahr, S. Pappinen, S. Ronkko, M. Suhonen, A. Urtti, Interaction of lipid nanoparticles with human epidermis and an organotypic cell culture model, Int. J. Pharm. 354 (2008) 180–195.
[43] N. Schürer, A. Kohne, V. Schliep, K. Barlag, G. Goerz, Lipid composition and synthesis of HaCaT cells, an immortalized human keratinocyte line, in comparison with normal human adult keratinocytes, Exp. Dermatol. 2 (1993) 179–185.
[44] G. Zambruno, P.C. Marchisio, A. Marconi, C. Vaschieri, A. Melchiori, A. Giannetti, M. De Luca, Transforming growth factor-beta 1 modulates beta 1 and beta 5 integrin receptors and induces the de novo expression of the alpha v beta 6 heterodimer in normal human keratinocytes: implications for wound healing, J. Cell Biol. 129 (1995) 853–865.
[45] T.R. Howdieshell, W.L. Webb, Sathyanarayana, P.L. McNeil, Inhibition of inducible nitric oxide synthase results in reductions in wound vascular endothelial growth factor expression, granulation tissue formation, and local perfusion, Surgery 133 (2003) 528–537.
[46] A. Schwentker, Y. Vodovotz, R. Weller, T.R. Billiar, Nitric oxide and wound repair: role of cytokines?, Nitric Oxide 7 (2002) 1–10
[47] E. Noiri, T. Peresleni, N. Srivastava, P. Weber, W.F. Bahou, N. Peunova, M.S. Goligorsky, Nitric oxide is necessary for a switch from stationary to locomoting phenotype in epithelial cells, Am. J. Physiol. 270 (1996) C794–C802.
[48] Y. Shimizu, M. Sakai, Y. Umemura, H. Ueda, Immunohistochemical localization of nitric oxide synthase in normal human skin: expression of endothelial-type and inducible-type nitric oxide synthase in keratinocytes, J. Dermatol. 24 (1997) 80–87.
[49] K. Yamasaki, H.D. Edington, C. McClosky, E. Tzeng, A. Lizonova, I. Kovesdi, D.L. Steed, T.R. Billiar, Reversal of impaired wound repair in iNOS-deficient mice by topical adenoviral-mediated iNOS gene transfer, J. Clin. Invest. 101 (1998) 967–971.
[50] T. Herzinger, B. Kleuser, M. Schäfer-Korting, H.C. Korting, Sphingosine-1-phosphate signaling and the skin, Am. J. Clin. Dermatol. 8 (2007) 329–336.
[51] J. Igarashi, T. Michel, S1P and eNOS regulation, Biochim. Biophys. Acta 1781 (2008) 489–495.
[52] M. Tölle, B. Levkau, P. Keul, V. Brinkmann, G. Giebing, G. Schonfelder, M. Schafers, K. von Wnuck Lipinski, J. Jankowski, V. Jankowski, J. Chun, W. Zidek, M. Van der Giet, Immunomodulator FTY720 Induces eNOS-dependent arterial vasodilatation via the lysophospholipid receptor S1P3, Circ. Res. 96 (2005) 913–920.
[53] A. Saric, T. Balog, S. Sobocanec, T. Marotti, Endomorphin 1 activates nitric oxide synthase 2 activity and downregulates nitric oxide synthase 2 mRNA expression, Neuroscience 144 (2007) 1454–1461.
[54] C. Olbrich, R.H. Müller, Enzymatic degradation of SLN-effect of surfactant and surfactant mixtures, Int. J. Pharm. 180 (1999) 31–39.
[55] H. Iijima, N. Kasai, H. Chiku, S. Murakami, F. Sugawara, K. Sakaguchi, H. Yoshida, Y. Mizushina, The inhibitory action of long-chain fatty acids on the DNA binding activity of p53, Lipids 41 (2006) 521–527.
[56] B. Cooper, N. Brimer, S.B. Vande Pol, Human papillomavirus E6 regulates the cytoskeleton dynamics of keratinocytes through targeted degradation of p53, J. Virol. 81 (2007) 12675–12679.
[57] L. Roger, G. Gadea, P. Roux, Control of cell migration: a tumour suppressor function for p53?, Biol Cell 98 (2006) 141–152.
[58] K. Harms, S. Nozell, X. Chen, The common and distinct target genes of the p53 family transcription factors, Cell Mol. Life Sci. 61 (2004) 822–842.
[59] M.A. Vigano, J. Lamartine, B. Testoni, D. Merico, D. Alotto, C. Castagnoli, A. Robert, E. Candi, G. Melino, X. Gidrol, R. Mantovani, New p63 targets in keratinocytes identified by a genome-wide approach, EMBO J. 25 (2006) 5105–5116.
[60] C.E. Barbieri, L.J. Tang, K.A. Brown, J.A. Pietenpol, Loss of p63 leads to increased cell migration and up-regulation of genes involved in invasion and metastasis, Cancer Res. 66 (2006) 7589–7597.
[61] T. Ichikawa, Y. Suenaga, T. Koda, T. Ozaki, A. Nakagawara, DeltaNp63/BMP-7-dependent expression of matrilin-2 is involved in keratinocyte migration in response to wounding, Biochem. Biophys. Res. Commun. 369 (2008) 994–1000.
[62] C. Chassaing, J. Schmidt, A. Eschalier, J.M. Cardot, C. Dubray, Hyperalgesia induced by cutaneous freeze injury for testing analgesics in healthy volunteers, Br. J. Clin. Pharmacol. 61 (2006) 389–397.
[63] R. Duncan, L. Izzo, Dendrimer biocompatibility and toxicity, Adv. Drug Deliv. Rev. 57 (2005) 2215–2237.
[64] N.A. Stasko, C.B. Johnson, M.H. Schoenfisch, T.A. Johnson, E.L. Holmuhamedov, Cytotoxicity of polypropylenimine dendrimer conjugates on cultured endothelial cells, Biomacromolecules 8 (2007) 3853–3859.
[65] S. Hong, A.U. Bielinska, A. Mecke, B. Keszler, J.L. Beals, X. Shi, L. Balogh, B.G. Orr, J.R. Baker Jr., M.M. Banaszak Holl, Interaction of poly(amidoamine) dendrimers with supported lipid bilayers and cells: hole formation and the relation to transport, Bioconjug. Chem. 15 (2004) 774–782.
[66] S.M. Moghimi, J. Szebeni, Stealth liposomes and long circulating nanoparticles: critical issues in pharmacokinetics, opsonization and protein-binding properties, Prog. Lipid Res. 42 (2003) 463–478.
[67] N. Schöler, H. Hahn, R.H. Müller, O. Liesenfeld, Effect of lipid matrix and size of solid lipid nanoparticles (SLN) on the viability and cytokine production of macrophages, Int. J. Pharm. 231 (2002) 167–176.
[68] N. Schöler, C. Olbrich, K. Tabatt, R.H. Müller, H. Hahn, O. Liesenfeld, Surfactant, but not the size of solid lipid nanoparticles (SLN) influences viability and cytokine production of macrophages, Int. J. Pharm. 221 (2001) 57–67.
[69] W. Weyenberg, P. Filev, D. Van den Plas, J. Vandervoort, K. De Smet, P. Sollie, A. Ludwig, Cytotoxicity of submicron emulsions and solid lipid nanoparticles for dermal application, Int. J. Pharm. 337 (2007) 291–298.
[70] R.H. Müller, D. Rühl, S. Runge, K. Schulze-Forster, W. Mehnert, Cytotoxicity of solid lipid nanoparticles as a function of the lipid matrix and the surfactant, Pharm. Res. 14 (1997) 458–462.
[71] F. Focher, S. Spadari, Thymidine phosphorylase: a two-face Janus in anticancer chemotherapy, Curr. Cancer Drug Targets 1 (2001) 141–153.
[72] A. Richartz, Ein neues Target zur Therapie von Basaliom und spinozellulärem Karzinom - Inhibition der humanen DNA Polymerase alpha. Institut für Pharmazie, vol. Dr., Freie Universität, Berlin, 2007, p. 126.
[73] M.P. Vinardell, M. Mitjans, Alternative methods for eye and skin irritation tests: an overview, J. Pharm. Sci. 97 (2008) 46–59.
[74] M. Schäfer-Korting, U. Bock, W. Diembeck, H.J. Düsing, A. Gamer, E. Haltner-Ukomadu, C. Hoffmann, M. Kaca, H. Kamp, S. Kersen, M. Kietzmann, H.C. Korting, H.U. Krächter, C.M. Lehr, M. Liebsch, A. Mehling, C. Müller-Goymann, F. Netzlaff, F. Niedorf, M.K. Rübbelke, U. Schäfer, E. Schmidt, S. Schreiber, H. Spielmann, A. Vuia, M. Weimer, The use of reconstructed human epidermis for skin absorption testing: results of the validation study, Altern. Lab. Anim. 36 (2008) 161–187.
[75] C. Eskes, S. Bessou, L. Bruner, R. Curren, J. Harbell, P. Jones, R. Kreiling, M. Liebsch, P. McNamee, W. Pape, M.K. Prinsen, T. Seidle, P. Vanparys, A. Worth, V. Zuang, Eye irritation, Altern. Lab. Anim. 33 (Suppl 1) (2005) 47–81.