International Journal of Pharmaceutics 428 (2012) 171–177

Contents lists available at SciVerse ScienceDirect

International Journal of Pharmaceutics

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

Pharmaceutical Nanotechnology

Design of cyclic RKKH peptide-conjugated PEG liposomes targeting the integrin $\alpha_2\beta_1$ receptor

Nina Ø. Knudsen[a,b], Raymond M. Schiffelers[c], Lene Jorgensen[a], Jens Hansen[b], Sven Frokjaer[a], Camilla Foged[a,*]

[a] Department of Pharmacy, Faculty of Health and Medical Sciences, University of Copenhagen, Universitetsparken 2, DK-2100 Copenhagen Ø, Denmark
[b] LEO Pharma A/S, Industriparken 55, DK-2750 Ballerup, Denmark
[c] Laboratory Clinical Chemistry and Haematology, University Medical Center Utrecht, P.O. Box 85500, 3508 GA Utrecht, The Netherlands

ARTICLE INFO

Article history:
Received 8 December 2011
Received in revised form 19 February 2012
Accepted 26 February 2012
Available online 5 March 2012

Keywords:
Liposomes
Integrin alpha 2
Targeting
Calcipotriol
Drug delivery
Nanomedicine

ABSTRACT

Peptide conjugation to the surface of stealth liposomes has been studied for liposomal drug targeting to cells expressing specific receptors to provide a site-specific drug delivery. This study investigated the potential of peptide-conjugated liposomes for targeting cells expressing the human integrin $\alpha_2\beta_1$ receptor. A 12 amino acid head-to-tail cyclic peptide derived from the Jararhagin protein containing the Arg-Lys-Lys-His (RKKH)-specific binding site was conjugated to the distal ends of poly(ethylene glycol) (PEG) chains on PEGylated liposomes. Epithelial cells expressing the receptor showed increased cellular association and uptake of peptide-conjugated liposomes at 4 °C, compared to liposomes conjugated with a non-specific peptide. The interaction between cells and peptide-conjugated liposomes was significantly increased at 37 °C suggesting that a possible uptake mechanism might be energy-dependent endocytosis. In keratinocyte cell cultures, the ligand-conjugated liposomes loaded with the vitamin D_3 analogue calcipotriol induced transcription of the gene encoding the antimicrobial peptide cathelicidin, which is activated through the vitamin D_3 receptor upon binding of vitamin D_3 analogues. This suggests that the liposomes are internalized and that calcipotriol is delivered intracellularly and released in an active form. In conclusion, the 12 amino acid head-to-tail cyclic RKKH peptide seems promising for targeting of liposomes to the integrin $\alpha_2\beta_1$ receptor.

1. Introduction

Integrin receptors are transmembrane glycoproteins that mediate cell–cell interactions and anchor cells to the extracellular matrix. In man, 24 different integrin receptor subtypes have been identified, which have been further divided into four subgroups based on their ligand recognition pattern. Different integrin receptors play important and distinct roles for the progression of several diseases, e.g. psoriasis, and consequently, the expression of specific subtypes of integrin receptors is regulated with respect to cell type, differentiation status and diseased state (Call-Culbreath and Zutter, 2008). The tissue-specific expression of specific integrin receptors can be exploited for the site-specific delivery of drugs (Dunehoo et al., 2006). An example is the human integrin $\alpha_2\beta_1$ receptor that belongs to the collagen-binding integrin subfamily, and it is the only collagen I integrin receptor expressed on keratinocytes in the basal layer of the skin (Parks, 2007). A drug delivery system specifically binding to the integrin $\alpha_2\beta_1$ receptor is therefore an interesting vehicle for targeting of drugs to the lower epidermis.

The interaction between the integrin $\alpha_2\beta_1$ receptor and collagen I is mediated through the extracellular insert domain of the integrin α_2 subunit (α2I-domain) (Tuckwell et al., 1995), which can be inhibited by the Jararhagin protein isolated from the venom of *Bothrops Jararaca* (DeLuca et al., 1995; Ivaska et al., 1999). A small nine amino acid peptide has been derived from Jararhagin (Cys-Thr-Arg-Lys-Lys-His-Asp-Asn-Ala-Gln-Cys, C^{241}TRKKHDNAQ^{249}C) with an internal disulfide bond, also denoted (RKK9, Fig. 1). It

Abbreviations: α2 I-domain, extracellular insert domain of the integrin α_2 subunit; CAMP, cathelicidin antimicrobial peptide; DiD, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate; DMEM, Dulbecco's modified Eagle's medium; DSPC, 1,2-distearoyl-sn-glycero-3-phosphatidylcholine; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HEKa, adult human epidermal keratinocytes; HPLC, high pressure liquid chromatography; HRP, horse radish peroxidase; LMVs, large multilamellar vesicles; Mal-PEG_{2000}-DSPE, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide-poly(ethylene glycol)2000]; PDI, polydispersity index; PEG, poly(ethylene glycol); PEG_{2000}-DSPE, (carbonyl-methoxypoly(ethylene glycol)-2000)-1,2-distearoyl-sn-glycero-3-phosphoethanol-amine; SATA, N-succinimidyl-S-acetyl thiolacetate; SUVs, small unilamellar vesicles; UPLC, ultra performance liquid chromatography.

* Corresponding author. Tel.: +45 3533 6402.
E-mail address: cfo@farma.ku.dk (C. Foged).

0378-5173/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2012.02.043

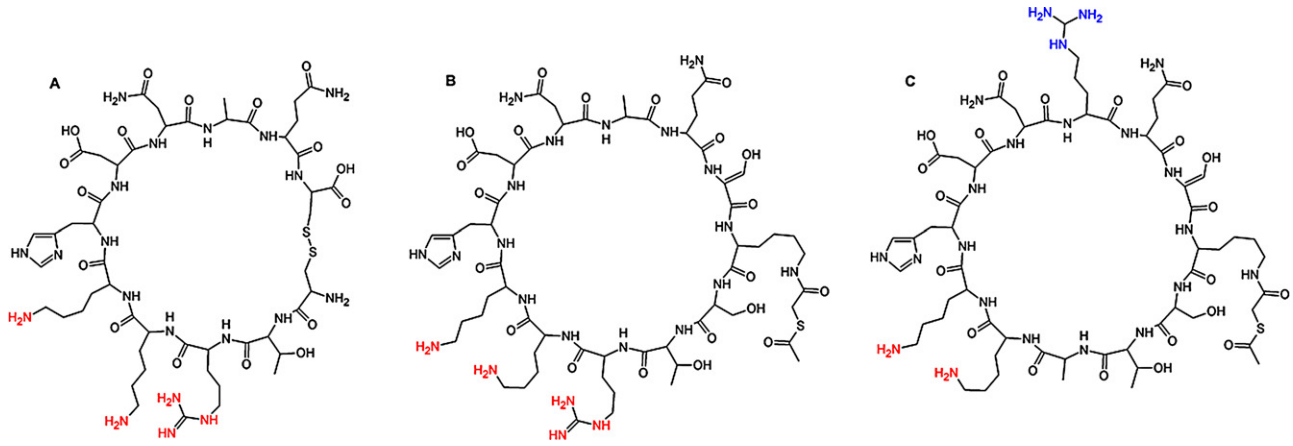

Fig. 1. Structure of peptides derived from the Jararhagin protein. (A) Cyclic CTRKKHDNAQC C1-C11 peptide (RKK9). (B) Head-to tail cyclized H-KHDNAQS-(SATA)KSTRK-OH peptide (RKK12). (C) Head-to tail cyclized H-KHDNRQS-(SATA)KSTAK-OH control peptide (AKK12). The binding domains are marked with red. For the AKK12 control peptide, an arginine was substituted for an alanine in the binding site. The opposite substitution was performed outside the binding domain to preserve the overall amino acid composition for both peptides (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

binds to the α2I-domain and inhibits the interaction with collagen I (Ivaska et al., 1999). The Arg-Lys-Lys-His (RKKH) part of the peptide is important for the interaction between the α2I-domain and the RKK9 peptide (Ivaska et al., 1999; Lambert et al., 2008). The specific ring structure of the peptide mimics the loop in Jararhagin, and it is essential for the α2I-domain recognition of collagen I (Ivaska et al., 1999). Isothermal titration calorimetry and nuclear magnetic resonance examinations of the interaction between RKK9 and the α2I-domain confirm that the peptide binds directly to the α2I-domain in close proximity to the metal ion-dependent adhesion site without introducing any major conformational changes in the α2I-domain (Lambert et al., 2008). The peptide is suggested to lock the α2I-domain in a closed conformation, and thereby inhibits the binding to collagen I (Lambert et al., 2008).

Liposomes are commonly applied drug delivery vehicles used to improve drug stability and to increase the therapeutic index of drugs by targeting of the liposomes to specific tissues (Musacchio and Torchilin, 2011). One way to increase liposomal drug targeting is by conjugation of receptor-specific ligands to the outer surface of liposomes that can facilitate a selective targeting to cells expressing specific receptors and increase the cellular uptake (Musacchio and Torchilin, 2011). Ligand targeting to integrin receptors has been studied intensively, e.g. applying the small cyclic peptide with the amino acid sequence Arg-Gly-Asp (RGD) for targeting of drug-loaded liposomes to integrin receptors from the RGD-binding subfamily including the integrin $\alpha_v\beta_3$ and $\alpha_v\beta_5$ receptors (Schiffelers et al., 2003; Xiong et al., 2005; Schiffelers and Storm, 2008). However, the improved therapeutic efficacy of integrin-targeted liposomes is not believed to be a result of an increased accumulation of liposomes at the target site, but is rather a consequence of enhanced cellular uptake of drug via binding of liposomes to internalizing surface integrin receptors (Schiffelers et al., 2003; Xiong et al., 2005).

Calcipotriol is a widely used drug for topical treatment of psoriasis (Su and Fang, 2008). It inhibits proliferation and stimulates differentiation of keratinocytes in the lower epidermis expressing the integrin $\alpha_2\beta_1$ receptor (Jensen et al., 1998; Watt, 2002). Calcipotriol is a vitamin D_3 analogue that binds to the vitamin D_3 receptor and activates the transcription of the gene encoding the cathelicidin antimicrobial peptide (*CAMP*) (Bury et al., 2001; Weber et al., 2005). In healthy human skin, low levels of cathelicidin are constitutively expressed by keratinocytes in the basal layer, but during psoriasis the expression of cathelicidin is upregulated, and topical treatment of psoriatic plaques with calcipotriol further arguments the transcription of cathelicidin (Peric et al., 2009).

This study aimed to examine the potential of ligand-conjugated liposomes intended for topical delivery of the antipsoriatic vitamin D analogue calcipotriol for targeting to kerationocytes expressing the human integrin $\alpha_2\beta_1$ receptor during psoriasis. A novel 12 amino acid peptide ligand derived from Jararhagin including the RKKH-binding site was coupled to the distal ends of poly(ethylene glycol) (PEG) chains on PEGylated liposomes for liposomal targeting to the integrin $\alpha_2\beta_1$ receptor. To evaluate the effect of the new peptide, the liposomes were labeled with fluorescence, and the cellular association and internalization were evaluated. In addition, calcipotriol was intercalated in the lipid bilayer of the liposomes (Knudsen et al., 2011). The biological effect of peptide-conjugated liposomes loaded with calcipotriol was evaluated.

2. Materials and methods

2.1. Materials

1,2-Distearoyl-sn-glycero-3-phosphatidylcholine (DSPC) (≥99% purity) and N-[carbonyl-methoxypoly(ethylene glycol)-2000]-1,2-distearoyl-sn-glycero-3-phosphoethanol-amine (PEG_{2000}-DSPE), sodium salt (≥98% purity), were obtained from Lipoid GmbH (Ludwigshafen, Germany). Sodium cholate (≥99% purity) was provided by Acros Organics (Geel, Belgium). 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide-poly(ethylene glycol)2000] (Mal-PEG_{2000}-DSPE), ammonium salt, was purchased from Avanti Polar Lipids (Alabaster, AL, US). Calcipotriol monohydrate (≥94% purity, Mw = 430.6 Da, log *P* = 4.9) was obtained from LEO Pharma A/S (Ballerup, Denmark). 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate (DiD) was provided by Molecular Probes (Eugene, OR, USA). All other general chemicals and reagents were obtained commercially at analytical grade.

2.2. Peptide synthesis

The head-to-tail cyclized peptides were synthesized with an N-succinimidyl-S-acetyl thiolacetate (SATA) group by JPT Peptide Technologies GmbH (Berlin, Germany) according to the manufacturer's regular procedures using solid phase synthesis with Fmoc-protected amino acids. The head-to-tail cyclized targeting peptide and the control peptide had the sequences H-KHDNAQS-(SATA)KSTRK-OH (denoted RKK12) and H-KHDNRQS-(SATA)KSTAK-OH (denoted AKK12), respectively (Fig. 1). Both

peptides contained a Lys(SATA) group used for subsequent conjugation to the distal ends of PEG chains on the surface of the liposomes (Kok et al., 2002).

2.3. Preparation of DiD-labeled liposomes

The liposomal formulations were prepared by the thin film method as described by Bangham et al. (1965). Briefly, DSPC (62 mol%), PEG_{2000}-DSPE (2.5 mol%), Mal-PEG_{2000}-DSPE (2.5 mol%) and cholesterol (33 mol%) were dissolved in chloroform:methanol (9:1, w/w). A volume of 1 ml DiD (200 μg/ml in ethanol) was added for labeling purposes. The organic solvent was evaporated, and the lipid film was flushed with N_2 for 5 min. The lipid film was hydrated for 1 h at 55 °C with HBS-E buffer (10 mM HEPES, 136 mM NaCl and 1 mM EDTA, pH = 7.4) to a final concentration of 15 mM lipid to form large multilamellar vesicles (LMVs). To obtain small unilamellar vesicles (SUVs), the LMVs were extruded ten times through two stacked 100 nm filters from Whatman (GE Healthcare, Little Chalfont, United Kingdom) using a Lipex extruder from Northern Lipids Inc. (Burnaby, BC, Canada), and kept in the dark at 4 °C.

2.4. Preparation of calcipotriol-loaded liposomes

The liposomal formulations loaded with calcipotriol were prepared by the thin film method essentially as described above with the following exceptions: The formulations consisted of DSPC (84 mol%), cholate (11 mol%), PEG_{2000}-DSPE (2.5 mol%) and Mal-PEG_{2000}-DSPE (2.5 mol%). Calcipotriol was dissolved in chloroform and added to the chloroform phase before evaporation of the organic phase, and the resulting lipid films were hydrated at 65 °C with HBS-E for 1 h. The hydrated lipid dispersions contained a final lipid concentration of 8.5 mM and a calcipotriol concentration of 50 μg/ml (0.121 mM). The LMVs were extruded twice through two stacked 200 nm filters (Whatman), followed by eight extrusions through two 100 nm filters.

2.5. Peptide conjugation of liposomes

The conjugation of peptides to the maleimide groups on the distal ends of the PEG chains on the liposomal outer surface was carried out as described previously (Schiffelers et al., 2003). The cyclic acetyl-protected SATA-peptides were deacetylated in an aqueous solution of 0.5 M HEPES, 0.5 M hydroxylamine-HCl and 25 mM EDTA (pH 7.0) for 30 min at room temperature. The liposomes were incubated with the activated SATA-peptide on a roller bench overnight at 4 °C and separated from non-conjugated peptide by ultracentrifugation. The dispersions of liposomes labeled with DiD were diluted 1:9 with HBS buffer (10 mM HEPES and 136 mM NaCl, pH = 7.4) and pelleted by centrifugation at 60,000 rpm for 1 h at 4 °C (Beckman LE-80K Ultracentrifuge fixed angle rotor Type 70.1 Ti). The pellet was resuspended in 10 ml HBS buffer and recentrifuged. The liposomes were resuspended in HBS buffer without EDTA to avoid depletion of Mg^{2+} ions in subsequently experiments, since the presence of Mg^{2+} ions is required for the interaction between the RKKH-binding site and the integrin receptor. Liposomes with calcipotriol were diluted 1:3 with tris buffer (13 mM tris, pH = 8.5) and precipitated by centrifugation at 50,000 rpm for 1 h at 4 °C (Beckman L-80 XP-ULTRA fixed angle rotor type 50.2 Ti). The pellet was resuspended in 25 ml tris buffer and recentrifuged. The tris buffer was used for calcipotriol-containing liposomes, since calcipotriol is less stable at the lower pH provided by the HEPES buffer. Finally, all liposomal dispersions were diluted to a final lipid concentration of 15 mM and stored in the dark at 4 °C. The amount of unconjugated peptides was determined by ultra performance liquid chromatography (UPLC) using a BEH300 C18 column (1.7 μm, 2.1 mm × 500 mm) from Waters (Milford, MA, USA). The eluent gradient was set from 100% acetonitrile:water:trifluoroacetic acid (5:95:0.1, v/v) to acetonitrile:trifluoroacetic acid (100:0.1, v/v) over 12 min. The peptides were detected by absorbance at 210 nm, and the amount of conjugated peptides was calculated as the total amount of peptides minus the amount of uncoupled peptides.

2.6. Characterization of liposomes

The final concentration of calcipotriol in the liposomal formulations was quantified by high pressure liquid chromatography (HPLC) using a Sunfire C18, 3.5 μm, 150 mm × 4.6 mm column (Waters) with acetonitrile:water (60:40, v/v) as the mobile phase. The detection was performed at 264 nm. The final lipid concentrations of liposomes with DiD were assessed by the Rouser determination method (Rouser et al., 1970). Brief, approximately 50 nmol of phospholipid was heated to 180 °C. After complete evaporation of liquid, 300 μl perchloric acid was added. The samples were incubated at 180 °C for 45 min, cooled to room temperature, and 1 ml water, 0.5 ml molybdate and 0.5 ml freshly prepared 5% (v/v) ascorbic acid was added. The samples were incubated in a 100 °C water bath for 5 min, cooled to room temperature, and the absorbance was measured at 797 nm. The lipid concentrations for the calcipotriol-containing liposomes were assessed using the colorimetric Phospholipids B enzymatic assay from MTI-Diagnostics GmbH (Idstein, Germany) as previously described (Grohganz et al., 2003). Briefly, the liposomal dispersions were diluted 1:40, 2.5% (v/v) Triton X-100 was added, and the samples were heated above the T_m for 20 min. A volume of 45 μl of all samples was transferred to a microtiter plate, 180 μl of the coloring reagent solution was added, and the plates were incubated at 37 °C. After 1 h, the absorbance at 490 nm was measured using a VICTORTM X3 Multilabel Plate Reader from Perkin Elmer (Waltham, MA, USA).

The average particle size distribution and polydispersity index (PDI) were determined by dynamic light scattering using the photon correlation spectroscopy technique on samples diluted 1:40 in HBS or tris buffer. The surface charge of the particles was estimated by analysis of the zeta-potential (Laser-Doppler Electrophoresis) on samples diluted 1:40 in water. The measurements were repeated three times per sample ($n = 1$). Both types of measurements were performed at 25 °C using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) equipped with a 633 nm laser and 173° detection optics. For viscosity and refractive index the values of pure water were used. Malvern DTS v.5.10 software was used for data acquisition and analysis.

2.7. Cellular uptake of liposomes labeled with DiD

The human epidermoid carcinoma cell line A431 was a kind gift from Dr. Paul M.P. van Bergen en Henegouwen (Dept. Biology, Faculty of Science, Utrecht University, The Netherlands). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 3.7 g/l sodium bicarbonate and 4.5 g/l glucose from PAA Laboratories (Pasching, Austria), supplemented with antimicrobial agents, 2 mM L-glutamine and 7.5% (v/v) fetal bovine serum (FBS) at 37 °C in a humidified atmosphere containing 5% CO_2. Nearly confluent monolayers of A431 cells were washed with PBS, and the cells were detached using 1 mM tris-EDTA in PBS. The cells were subsequently suspended in cold PBS supplemented with 1.26 mM $CaCl_2$ and 0.81 mM $MgSO_4$ at 4 °C and isolated by centrifugation. The cells were counted, and 1×10^5 cells in 100 μl medium were incubated with 100 μl (2–100 nmol total lipid) liposomes diluted in PBS supplemented with 1.26 mM $CaCl_2$ and 0.81 mM $MgSO_4$ for 1 h at 4 °C or at 37 °C. At the end of the incubation period, the cells were washed three times with PBS supplemented with 1% (v/v) BSA (PBS–BSA) at 4 °C, and resuspended in 200 μl PBS–BSA. Afterwards, the cells were analyzed using a FACScalibur flow cytometer from

Becton Dickinson (San Jose, CA, USA), and the geometric mean fluorescence intensity was determined using the BD FACDiva software version 6.1.1.

2.8. Confocal laser scanning microscopy

A431 cells were plated on collagen-coated 16-well Lab-Tex chamber slides from Nalgene Nunc (Rochester, NY, USA). After two days, the cells were incubated with 500 μM DiD-labeled RKK12-conjugated liposomes for 1 h at 37 °C. At the end of the incubation period, the cells were washed three times in PBS–BSA at 4 °C and fixed with 4% (v/v) paraformaldehyde for 30 min at room temperature. Afterwards, the cells were mounted with Fluorosave from Calbiochem (Merck, Darmstadt, Germany) and covered with a glass slide. Fluorescence microscopy was performed on a Zeiss LSM510 confocal laser scanning microscope (Carl Zeiss, Jena, GmbH, Germany) equipped with an argon laser (458 and 488 nm) and a HeNe laser (543 nm) using the LSM 510 software (Carl Zeiss).

2.9. Keratinocyte interaction with peptide-conjugated liposomes loaded with calcipotriol

Adult human epidermal keratinocytes (HEKa) were obtained from Cascade Biologics (Portland, OR, USA) and cultured in EpiLife Medium (Cascade Biologics) containing human keratinocyte growth supplement and gentamicin/amphotericin B (Cascade Biologics) at 37 °C in a humidified atmosphere with 5% CO_2. For the cell interaction assays, 2×10^5 exponentially growing keratinocytes were seeded one day prior to treatment. On the following day (at 80% confluency), the medium was renewed, and the cell culture medium was supplied with 15 μl of liposome suspension to a final concentration of 0.10 μM calcipotriol, 15 μl placebo liposomal suspension, or calcipotriol in 0.1% (v/v) DMSO solution to a final concentration of 0.10 μM calcipotriol and incubated for 1 h at 37 °C. The liposomes were conjugated with either RKK12 or AKK12. After 1 h, the cells were washed three times with PBS–BSA, and 1.5 ml medium was added followed by incubation for 24 h at 37 °C. Subsequently, the medium was removed, and mRNA was extracted using the RNeasy kit from Qiagen (Hilden, Germany). An amount of 80 ng mRNA was reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription kit from Applied Biosystems (Foster City, CA, USA). For qPCR, cDNA was amplified in triplicates according to manufacturer's protocol (Applied Biosystems, CAMP Hs00189038_m1 and GAPDH Hs99999905_m1). The expression level of *CAMP* was normalized to the expression level of the housekeeping gene Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The relative change in the expression of *CAMP* was quantified using the comparative Ct method ($2^{-\Delta\Delta Ct}$) (Livak and Schmittgen, 2001).

2.10. Integrin $\alpha_2\beta_1$ expression

The human keratinocyte cell line HaCaT was provided by Switch Biotech (Conway, AR, USA) and cultured in DMEM supplemented with antimicrobial agents, 10% FBS and 2 mM L-glutamine. Adherent cells were washed with PBS, followed by incubation with SDS-lysis buffer (50 mM Tris–HCl, 10 mM b-glycerophosphate, 10 mM NaF, 0.1 mM orthovanadate, 2.5% (w/v) SDS and 10% (v/v) glycerol) for 10 min on ice, and the lysate was cleared by centrifugation. A cell extract of a skin biopsy from a transgenic human integrin $\alpha_2\beta_1$ mouse was prepared as described previously (Teige et al., 2010). Primary keratinocytes were isolated from tissues removed by human breast reduction at Herlev Hospital (Herlev, Denmark). The primary keratinocytes were isolated from the epidermis by incubation with trypsin at 37 °C for 15 min. Cells were cultured in DMEM supplied with 10% (v/v) calf serum. The cells were lysed as described above for the HaCaT cells. A431 cell extracts were prepared as described previously (Oliveira et al., 2007). The protein concentration in the cell extracts was determined using the BCA Protein Assay kit (Pierce Biotechnology, Rockford, IL, US) according to the manufacturer's protocol. The samples (7.5 μg each) were analyzed by electrophoresis on a 4–12% criterion XT Bis-tris gel from Bio-Rad (Hercules, CA, USA). The protein bands were transferred to a hydrophobic polyvinylidene difluoride membrane (Hybond-P, Amersham, GE healthcare), and the membrane was probed with the anti-human integrin alpha 2/CD49b antibody MAB12331 from R&D Systems (Minneapolis, MN, USA) diluted 1:500, followed by incubation with a 1:2000 dilution of rabbit anti-rat immunoglobulin horse radish peroxidase (HRP) from DAKO (Glostrup, Denmark). The immunostained bands were visualized using the SuperSignal Western Pico western blotting detection system (Thermo science, IL, US). The membrane was incubated for 1 h with a 0.8% (v/v) mercaptoethanol solution at 50 °C, and washed before re-probing with anti-actin from Sigma–Aldrich (St. Louis, MO, USA) diluted 1:2500, followed by rabbit anti-mouse immunoglobulin HRP from DAKO diluted 1:2000, and the immunostained bands were visualized.

2.11. Statistics

The *t*-test comparing two variations assuming equal variation was used for analysis of the cell interaction between DiD-loaded liposomes and A431 cells ($p < 0.05$ was considered significant). The statistical significance of the expression of *CAMP* was determined using the Parametric test Lima using StatMiner version 4.2 (Intergromics®, Granada, Spain) ($p < 0.05$ was considered significant).

3. Results and discussion

3.1. Peptide structure

The novel head-to-tail cyclic RKK12 peptide had a sequence very similar to the original RKK9 peptide described in the literature (Fig. 1). The RKK12 peptide is conserved in the RKKH part of the peptide compared to the RKK9 peptide, since this region is known to be essential for the interaction between the α2 I-domain and the RKK9 peptide (Ivaska et al., 1999; Lambert et al., 2008). For the control peptide (AKK12), this region was modified by replacing an arginine with an alanine in the binding site. The opposite substitution was performed outside the binding domain to preserve the overall amino acid composition. Furthermore, the total length of the bonds within the ring structure of the peptides was similar to the total length of the bonds in the RKK9 peptide. The head-to-tail cyclic design of the peptides was preferred to the previously reported construct cyclized via a disulfide bond, because the disulfide bond is less stable under reducing conditions, and may therefore cause scrambling during synthesis and conjugation of the peptide to the surface of the liposomes via maleimide coupling and thioether bond formation. Finally, a peptidic linker connects the N- and the C-terminal of the backbone of the new peptides, which in general decreases their susceptibility to enzymatic degradation in biological tissues (Lovelace et al., 2006).

3.2. Peptide conjugation to PEG-chains on the surface of PEGylated liposomes

The ligands were conjugated to the surface of the PEGylated liposomes after preparation. In the present study, the targeting ligands were conjugated to the distal ends of the PEG chains on PEGylated liposomes due to the fact that introduction of a PEG spacer between the liposomal bilayer and the peptide ligand has been

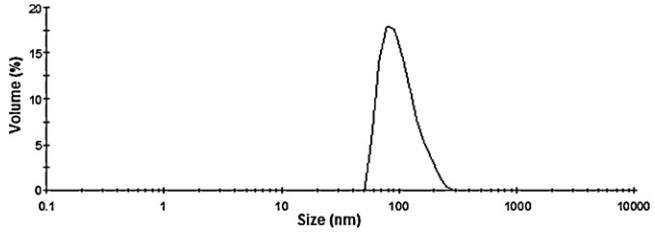

Fig. 2. A representative volume-based particle size distribution of the RKK12-conjugated liposomes.

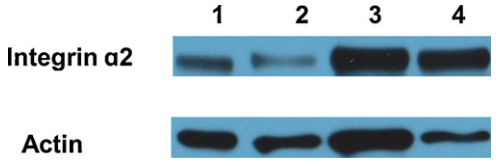

Fig. 3. Protein expression of integrin $\alpha_2\beta_1$ receptor. Lane 1, lysate of a skin biopsy from a transgenic human integrin $\alpha_2\beta_1$ mouse. Lane 2, cell extract prepared from the human epidermoid carcinoma cell line A431. Lane 3, cell extract from primary keratinocytes isolated from a human donor. Lane 4, cell extract from the keratinocyte cell line HaCaT. The expression of actin was used as a reference.

found to increase the affinity of the ligand for the target receptor (Lee and Low, 1994). In a pilot study, we tested the effect of PEG grafting density, and liposomes containing 2 mol% PEG (1 mol% Mal-PEG-DSPE/1 mol% PEG-DSPE) were compared with liposomes containing 5 mol% PEG (2.5 mol% MAL-PEG-DSPE/2.5 mol% PEG-DSPE). The increased liposomal surface concentration of MAL-PEG resulted in a seven-fold increase in the cellular association of the ligand-conjugated liposomes for 5 mol% PEGylated liposomes, compared to the 2 mol% PEGylated liposomes. In addition, our unpublished studies have shown that grafting of the liposomes with 0.5, 1 and 5 mol% PEG does not affect the liposomal lipid penetration into the skin. Consequently, a liposomal composition containing 5 mol% PEG-conjugated lipid was used in the present study.

The amount of conjugated ligand was calculated from the amount of unconjugated ligand measured by UPLC. The amount of conjugated peptide was 1.6–2.1 mol% for the liposomes labeled with DiD, whereas the amount of conjugated peptide was 0.8–1.1 mol% for liposomes containing calcipotriol. This corresponds to approximately 640–840 ligands per 100 nm vesicle for liposomes labeled with DiD, and 320–440 ligands per 100 nm vesicle for calcipotriol-containing liposomes, respectively, under the assumption of 80,000 phospholipids molecules per one 100 nm-sized liposome (Kirpotin et al., 1997). This is in the same order of magnitude as reported for previous investigations of integrin-targeted, RGD-peptide decorated PEG-liposomes. The reason for the reduced coupling efficiency of calcipotriol-loaded liposomes is at present unknown. The drug molecule does not contain chemical groups that would quench maleimide reactivity, but it might be possible that the presence of calcipotriol in the membrane changes the distribution of PEG-lipid leading to reduced possibilities for interaction of the peptide with the maleimide group on PEG.

The ligand-conjugated liposomes showed a tendency toward a small increase in zeta-potential, compared to liposomes without conjugated peptide (Table 1). This further confirms the successful conjugation, since the peptide contains basic amino acids that contribute with positive charge to the zeta-potential resulting in a net reduction of the negative zeta-potential. Previous studies have shown a similar change in zeta-potential upon conjugation of positively charged antibodies to the surface of PEGylated liposomes (Nassander et al., 1995). A small increase in size was also observed upon conjugation of the peptide to the surface of the liposomes (Table 1 and Fig. 2).

3.3. The integrin $\alpha_2\beta_1$ receptor is expressed in epithelial cells

The expression of the integrin α_2 receptor was confirmed in the epidermoid carcinoma cell line A431, in the keratinocyte cell line HaCaT as well as in the primary keratinocytes isolated from a human donor (Fig. 3). The protein expression level of the integrin α_2 receptor was higher in the keratinocytes compared to the epidermoid cell line. This correlates with the high expression levels reported for the integrin $\alpha_2\beta_1$ receptor in proliferating keratinocytes (Watt, 2002).

3.4. Ligand conjugation increases liposomal interaction with epithelial cells

The cellular association and uptake of DiD-labeled liposomes were examined by flow cytometry. At 4 °C, the association with A431 cells was increased for the RKK12-conjugated liposomes, compared to AKK12-conjugated liposomes or non-conjugated liposomes at all examined lipid concentrations (Fig. 4A). At 37 °C, the effect of the RKK conjugation to the liposomes was even more pronounced (Fig. 4B). This suggests a certain degree of temperature dependency of the association and a specific internalization, since endocytosis is generally inhibited at 4 °C (McKeown et al., 1990). The cellular internalization of liposomes was examined further by confocal microscopy: After 1 h of incubation with DiD-labeled, RKK12-conjugated liposomes at 37 °C, the fluorescence was mainly localized intracellularly, possibly in the cytoplasm (Fig. 5). A similar

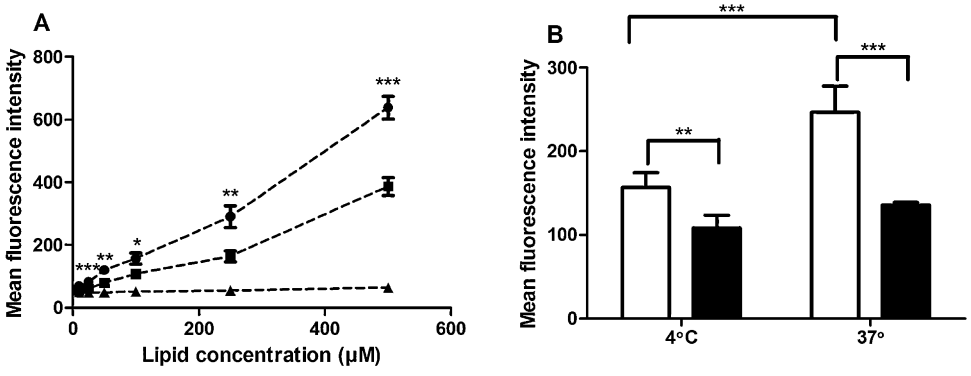

Fig. 4. Cellular interaction determined by flow cytometry. (A) Binding of RKK12-conjugated liposomes (●), AKK12-conjugated liposomes (■) and liposomes without peptide conjugated to the surface (▲) to the surface of A431 cells at different lipid concentrations. (B) Differences between binding of RKK12-conjugated liposomes (empty bars) and AKK12-conjugated liposomes (black bars) to the surface of cells at 4 °C and the cellular uptake of liposomes at 37 °C after incubation of liposomes (100 μM). Results denote mean ± SD ($n = 3$). Significant differences in mean fluorescence intensity are indicated: $p < 0.05^*$, $p < 0.01^{**}$ and $p < 0.001^{***}$.

Table 1
Particle size, PDI and zeta-potential of ligand-conjugated liposomes. Each formulation was measured three times, and results denote mean ± SD of the measurements ($n = 1$).

Liposomal composition[a]	Calcipotriol conc. (μg/ml)	Size (μm)	PDI	Zeta-potential (mV)
RKK12-Mal-PEG_{2000}-DSPE (2.5 mol%)	50	0.11 ± 0.01	0.10 ± 0.01	−38.1 ± 0.42
AKK12-Mal-PEG_{2000}-DSPE (2.5 mol%)	50	0.11 ± 0.01	0.13 ± 0.02	−36.5 ± 1.6
Mal-PEG_{2000}-DSPE (2.5 mol%)	50	0.10 ± 0.003	0.09 ± 0.01	−41.0 ± 2.1
RKK12-Mal-PEG_{2000}-DSPE (2.5 mol%)	0	0.11 ± 0.01	0.08 ± 0.02	−39.5 ± 1.9
Mal-PEG_{2000}-DSPE (2.5 mol%)	0	0.11 ± 0.01	0.07 ± 0.01	−42.0 ± 1.5

[a] Additionally, all liposomal formulations contained DSPC (84 mol%), cholate (11 mol%) and PEG_{2000}-DSPE (2.5 mol%).

increase in cellular uptake upon interaction with integrin-targeted liposomes at 37 °C has been demonstrated previously, where visualization confirmed that liposomal endocytosis occurred at 37 °C upon coupling of integrin-targeted peptides to the surface of liposomes (Xiong et al., 2005; Koning et al., 2006). These results therefore suggest that liposomes conjugated with the RKK12 peptide are effectively bound and internalized by epithelial cells in vitro.

3.5. Liposome-intercalated calcipotriol stimulates the expression of cathelicidin in keratinocytes

The drug delivery potential of RKK12-conjugated liposomes loaded with calcipotriol was evaluated in keratinocytes, and the formulation increased significantly the expression of cathelicidin compared to the RKK12-conjugated liposomal placebo formulation (Fig. 6). This confirms that calcipotriol is delivered into the cells and reaches its target site (the vitamin D_3 receptor) in an active form. Increased levels of cathelicidin have previously been observed upon incubation of HaCaT cells with calcipotriol (Weber et al., 2005). The AKK12-conjugated and non-conjugated liposomes were also tested, but there was no significant difference between the formulations (results not shown). The increase in cathelicidin expression verifies that calcipotriol maintains its biological activity upon delivery into the cells when it is formulated in liposomes. The confocal images of the fluorescently labeled liposomes furthermore suggest that the liposomes are targeted to the cytoplasm of the cells. The vitamin D_3 receptor is located in the cytoplasm as well as in the nucleus of keratinocytes (Barsony et al., 1997). These results might therefore suggest that calcipotriol is delivered to the cytoplasm of keratinocytes, where it can bind to the vitamin D_3 receptor before it translocates to the nucleus and induces the transcription of *CAMP*.

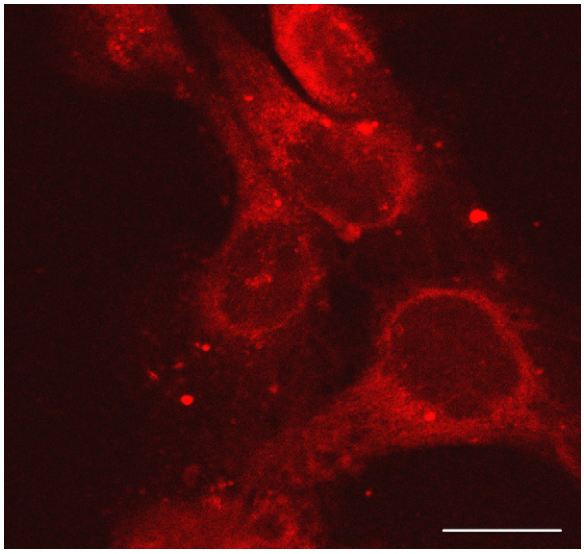

Fig. 5. Confocal microscopy of cells incubated with DiD-labeled, RKK12-conjugated liposomes for 1 h at 37 °C. DiD is mainly distributed in the cytoplasm. Scale bar = 20 μm.

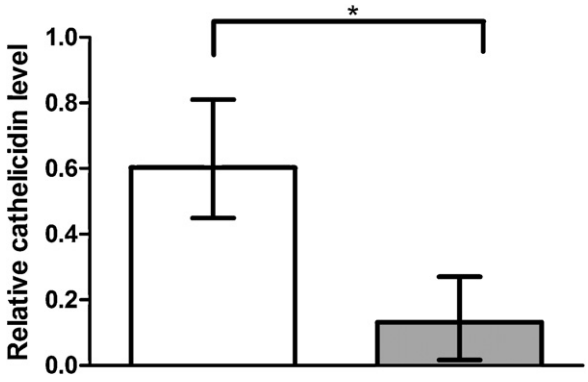

Fig. 6. Biological effect of calcipotriol-loaded, RKK12-conjugated liposomes (empty bar) compared to placebo RKK12-conjugated liposomes (grey bar) in keratinocytes. The levels of cathelicidin were detected by qPCR and normalized to the level of GAPDH. The relative change in the gene expression level was calculated using the comparative Ct method, using 0.1 μM calcipotriol in a DMSO solution as a reference (set to 1). Bars denote the relative mean ΔCt ± SD ($n = 3$). The level of cathelicidin was significantly increased after incubation of RKK12-conjugated liposomes loaded with calcipotriol compared to incubation with placebo RKK12-conjugated liposomes ($p < 0.05$*).

4. Conclusions

The novel RKK12 peptide designed to target the integrin $\alpha_2\beta_1$ receptor increased the cellular association between RKK12-conjugated liposomes and cells expressing the integrin $\alpha_2\beta_1$ receptor. The increased association was accomplished by increased cellular uptake, most likely mediated by receptor-specific endocytosis. A preserved biological activity of liposome-intercalated calcipotriol was demonstrated in vitro, since the RKK12-conjugated liposomes loaded with calcipotriol enhanced the transcription of cathelicidin in keratinocytes. The RKK12 peptide is therefore a promising candidate for targeting of liposomes to the integrin $\alpha_2\beta_1$ receptor, but further studies are required to evaluate the effect of active targeting under in vivo conditions.

Role of the funding source

The project was financially supported by the Danish Ministry of Science, Technology and Innovation (N.K.). The funding sources had no involvement in the study design or the collection, analysis and interpretation of data, just as they had no involvement in the writing of the report and the decision to submit the paper for publication.

Conflicts of interests

N.K. is co-inventor of a filed patent covering the use of the cyclic peptide for keratinocyte targeting.

Acknowledgements

We are grateful to Karlijn Wilschut for help with the cell culture and flow cytometric analyses, to Inge van Rooy for assistance with the UPLC analyses, Karen Engell for assistance with the HPLC analyses, Lili Rohde for technical assistance with the cell culture and Sidsel Boesen for technical assistance with qPCR of cathelicidin. We also thank Charlotte Vermehren and Hanne Norsgaard for valuable scientific discussions.

References

Bangham, A.D., Standish, M.M., Watkins, J.C., 1965. Diffusion of univalent ions across the lamellae of swollen phospholipids. J. Mol. Biol. 13, 238–252.

Barsony, J., Renyi, I., McKoy, W., 1997. Subcellular distribution of normal and mutant vitamin D receptors in living cells. Studies with a novel fluorescent ligand. J. Biol. Chem. 272, 5774–5782.

Bury, Y., Ruf, D., Hansen, C.M., Kissmeyer, A.M., Binderup, L., Carlberg, C., 2001. Molecular evaluation of vitamin D3 receptor agonists designed for topical treatment of skin diseases. J. Invest. Dermatol. 116, 785–792.

Call-Culbreath, K.D., Zutter, M.M., 2008. Collagen receptor integrins: rising to the challenge. Curr. Drug Targets 9, 139–149.

DeLuca, M., Ward, C.M., Ohmori, K., Andrews, R.K., Berndt, M.C., 1995. Jararhagin and Jaracetin – novel snake venom inhibitors of the integrin collagen receptor alpha(2)beta(1). Biochem. Biophys. Res. Commun. 206, 570–576.

Dunehoo, A.L., Anderson, M., Majumdar, S., Kobayashi, N., Berkland, C., Siahaan, T.J., 2006. Cell adhesion molecules for targeted drug delivery. J. Pharm. Sci. 95, 1856–1872.

Grohganz, H., Ziroli, V., Massing, U., Brandl, M., 2003. Quantification of various phosphatidylcholines in liposomes by enzymatic assay. AAPS PharmSciTech 4, E63.

Ivaska, J., Kapyla, J., Pentikainen, O., Hoffren, A.R., Hermonen, J., Huttunen, P., Johnson, M.S., Heino, J., 1999. A peptide inhibiting the collagen binding function of integrin alpha I-2 domain. J. Biol. Chem. 274, 3513–3521.

Jensen, A.M., Llado, M.B., Skov, L., Hansen, E.R., Larsen, J.K., Baadsgaard, O., 1998. Calcipotriol inhibits the proliferation of hyperproliferative CD29 positive keratinocytes in psoriatic epidermis in the absence of an effect on the function and number of antigen-presenting cells. Br. J. Dermatol. 139, 984–991.

Kirpotin, D., Park, J.W., Hong, K., Zalipsky, S., Li, W.L., Carter, P., Benz, C.C., Papahadjopoulos, D., 1997. Sterically stabilized anti-HER2 immunoliposomes: design and targeting to human breast cancer cells in vitro. Biochemistry 36, 66–75.

Knudsen, N.O., Jorgensen, L., Hansen, J., Vermehren, C., Frokjaer, S., Foged, C., 2011. Targeting of liposome-associated calcipotriol to the skin: effect of liposomal membrane fluidity and skin barrier integrity. Int. J. Pharm. 416, 478–485.

Kok, R.J., Schraa, A.J., Bos, E.J., Moorlag, H.E., Asgeirsdottir, S.A., Everts, M., Meijer, D.K., Molema, G., 2002. Preparation and functional evaluation of RGD-modified proteins as alpha(v)beta(3) integrin directed therapeutics. Bioconjug. Chem. 13, 128–135.

Koning, G.A., Schiffelers, R.M., Wauben, M.H.M., Kok, R.J., Mastrobattista, E., Molema, G., ten Hagen, T.L.M., Storm, G., 2006. Targeting of angiogenic endothelial cells at sites of inflammation by dexamethasone phosphate-containing RGD peptide liposomes inhibits experimental arthritis. Arthritis Rheum. 54, 1198–1208.

Lambert, L.J., Bobkov, A.A., Smith, J.W., Marassi, F.M., 2008. Competitive interactions of collagen and a jararhagin-derived disintegrin peptide with the integrin alpha 2-I domain. J. Biol. Chem. 283, 16665–16672.

Lee, R.J., Low, P.S., 1994. Delivery of liposomes into cultured KB cells via folate receptor-mediated endocytosis. J. Biol. Chem. 269, 3198–3204.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) method. Methods 25, 402–408.

Lovelace, E.S., Armishaw, C.J., Colgrave, M.L., Wahlstrom, M.E., Alewood, P.F., Daly, N.L., Craik, D.J., 2006. Cyclic MrIA: a stable and potent cyclic conotoxin with a novel topological fold that targets the norepinephrine transporter. J. Med. Chem. 49, 6561–6568.

McKeown, M., Knowles, G., McCulloch, C.A., 1990. Role of the cellular attachment domain of fibronectin in the phagocytosis of beads by human gingival fibroblasts in vitro. Cell Tissue Res. 262, 523–530.

Musacchio, T., Torchilin, V.P., 2011. Recent developments in lipid-based pharmaceutical nanocarriers. Front. Biosci. 16, 1388–1412.

Nassander, U.K., Steerenberg, P.A., de Jong, W.H., van Overveld, W.O., te Boekhorst, C.M., Poels, L.G., Jap, P.H., Storm, G., 1995. Design of immunoliposomes directed against human ovarian carcinoma. Biochim. Biophys. Acta 1235, 126–139.

Oliveira, S., Fretz, M.M., Hogset, A., Storm, G., Schiffelers, R.M., 2007. Photochemical internalization enhances silencing of epidermal growth factor receptor through improved endosomal escape of siRNA. Biochim. Biophys. Acta 1768, 1211–1217.

Parks, W.C., 2007. What is the alpha2beta1 integrin doing in the epidermis? J. Invest. Dermatol. 127, 264–266.

Peric, M., Koglin, S., Dombrowski, Y., Gross, K., Bradac, E., Buchau, A., Steinmeyer, A., Zugel, U., Ruzicka, T., Schauber, J., 2009. Vitamin D analogs differentially control antimicrobial peptide/alarmin expression in psoriasis. PLoS One 4, e6340.

Rouser, G., Fkeischer, S., Yamamoto, A., 1970. Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. Lipids 5, 494–496.

Schiffelers, R.M., Koning, G.A., ten Hagen, T.L.M., Fens, M.H.A.M., Schraa, A.J., Janssen, A.N.P.C., Kok, R.J., Molema, G., Storm, G., 2003. Anti-tumor efficacy of tumor vasculature-targeted liposomal doxorubicin. J. Control. Release 91, 115–122.

Schiffelers, R.M., Storm, G., 2008. Liposomal nanomedicines as anticancer therapeutics: beyond targeting tumor cells. Int. J. Pharm. 364, 258–264.

Su, Y.H., Fang, J.Y., 2008. Drug delivery and formulations for the topical treatment of psoriasis. Exp. Opin. Drug Deliv. 5, 235–249.

Teige, I., Backlund, A., Svensson, L., Kvist, P.H., Petersen, T.K., Kemp, K., 2010. Induced keratinocyte hyper-proliferation in alpha2beta1 integrin transgenic mice results in systemic immune cell activation. Int. Immunopharmacol. 10, 107–114.

Tuckwell, D., Calderwood, D.A., Green, L.J., Humphries, M.J., 1995. Integrin alpha 2 I-domain is a binding site for collagens. J. Cell Sci. 108, 1629–1637.

Watt, F.M., 2002. Role of integrins in regulating epidermal adhesion, growth and differentiation. EMBO J. 21, 3919–3926.

Weber, G., Heilborn, J.D., Jimenez, C.I.C., Hammarsjo, A., Torma, H., Stahle, M., 2005. Vitamin D induces the antimicrobial protein hCAP18 in human skin. J. Invest. Dermatol. 124, 1080–1082.

Xiong, X.B., Huang, Y., Lu, W.L., Zhang, X., Zhang, H., Nagai, T., Zhang, Q., 2005. Intracellular delivery of doxorubicin with RGD-modified sterically stabilized liposomes for an improved antitumor efficacy: in vitro and in vivo. J. Pharm. Sci. 94, 1782–1793.