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RU 58841-myristate – prodrug development for topical treatment of acne and androgenetic alopecia

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Acne and androgenetic alopecia are linked to androgen effects and therefore should improve following topical application of antiandrogens. We present a new antiandrogen prodrug, RU 58841–myristate (RUM) for topical therapy. Almost devoid of affinity to the androgen receptor, as derived from investigations in the mouse fibroblast cell line 29 +/GR +, RUM is rapidly metabolised to the potent antiandrogen RU 58841 by cultured human foreskin fibroblasts and keratinocytes, male occipital scalp skin dermal papilla cells, and by cells of the sebaceous gland cell line SZ95. In order to improve a specific targeting of the hair follicle, RUM was loaded on solid lipid nanoparticles (SLN), which are already known to support dermal targeting effects. Physically stable RUM loaded SLN were produced by hot homogenization. Penetration/permeation studies carried out using the Franz diffusion cell proved only negligible permeation of reconstructed epidermis and excised porcine skin within 6 h, implying a more topical action of the drug. Targeting to the hair follicle using SLN was visualised by fluorescence microscopy, following the application of Nile Red labelled SLN to human scalp skin. Transmission electron microscopy (TEM) allowed to detect intact silver labelled SLN in porcine hair follicles of preparations applied to the skin for 24 h. RUM loaded SLN should be considered for topical antiandrogen therapy of acne and androgenetic alopecia.

1. Introduction

Androgens stimulate the proliferation of human sebocytes (Akamatsu et al. 1992) and sebum production. Affecting androgen sensitive hair follicles, androgens cause baldness in genetically predisposed men and women (Hamilton 1942; Ludwig 1977). The presence of 5α-reductase type I in sebaceous glands (Fritsch et al. 2001) and 5α-reductase type II in androgen sensitive dermal papilla cells (Ando et al. 1999) indicates that local transformation of testosterone to the more potent dihydrotestosterone (DHT) is pivotal in the pathogenesis of acne and androgenetic alopecia. Innovative therapeutic options include strictly locally acting 5α-reductase inhibitors or androgen receptor antagonists.

So far topical application of finasteride in androgenetic alopecia is not established despite of its systemic activity. Due to the fear of side effects the well documented reduction of sebum production (Leyden 1997) by antiandrogens is not used for topical treatment of men. The aim of our investigation was to create a new therapeutic option for acne and androgenetic alopecia by a topical antiandrogen targeted to the diseased skin. Since lipophilic drug carriers enhance follicular uptake (Masini et al. 1993; Lauer et al. 1995; Bernard et al. 1997) we tried to incorporate the potent nonsteroidal antiandrogen RU 58841 (Battmann et al. 1994) into solid lipid nanoparticles (SLN) (Mehnert and Mäder 2001; Müller et al. 2002).

2. Investigations and results

2.1. RUM synthesis and SLN production

First we tried to incorporate RU 58841 into SLN. The solubility of RU 58841 in melted commonly used lipids for SLN production (Compritol®, Precirol®), however, was very low currently excluding the production of stable preparations.

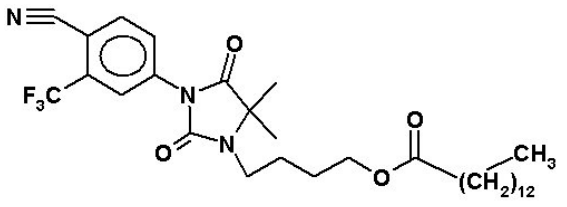

Table 1: Results of laser diffractometry (LD) and photon correlation spectroscopy (PCS) measurements up to 16 days after SLN production

		day 3 (4)				day 16			
		LD		PCS	PI	LD		PCS	PI
Lipid		LD50 (μm)	LD95 (μm)	(μm)		LD50 (μm)	LD95 (μm)	(μm)	
Compritol	RUM	0.214	0.563	0.255	0.292	0.211	0.640	n.d.	n.d.
	Contr.	0.249	0.586	0.259	0.314	0.260	0.615	n.d.	n.d.
Precirol	RUM	0.199	1.963	0.214	0.247	0.181	1.544	0.214	0.201
	Contr.	0.151	0.843	0.247	0.256	0.185	1.877	0.224	0.234
Nano-emulsion	RUM	0.171	0.920	0.325	0.131	0.149	0.149	n.d.	n.d.

0.1% RUM was incorporated into SLN, drug free SLN as well as a nanoemulsion (Miglyol) served for control. LD50 (95): 50 (95)% of particles are smaller than the given value; PCS: mean diameter; PI: Polydispersity Index; n.d. not determined.

To improve the drug incorporation into SLN, we esterfied RU 58841 with myristylchloride, generating RUM. The identity of the ester was confirmed as described under Experimental. The melting point of RUM was 70.7–72.4 °C. In contrast to RU 58841, RUM could be formulated with Compritol® and Precirol® to colloidal drug loaded carriers. The physical stability of these products was proved for at least 16 days after production (Table 1). The particle sizes were good with respect to the Compritol®-SLN and acceptable with respect to the Precirol® based formulation. The Polydispersity Indices (PI) of the nanoemulsion and both SLN preparations ranging from 0.131 to 0.314 indicated acceptable particle size distribution. A clear melting peak was detected indicating solid particle formation of the Precirol® and the Compritol® preparation. Light microscopy excluded visible drug crystal formation with the Precirol® based particles even 10 days after production, while with the Compritol® based SLN a few drug crystals were detected. SLN and nanoemulsion production was repeated twice at different days.

2.2. Receptor binding experiments

Whether RUM may antagonise androgen effects by itself or acts as a prodrug by releasing the potent antiandrogen RU 58841, was investigated by receptor binding experiments. RUM was compared to DHT and RU 58841 (Fig. 1). EC_{50} values were derived from DHT competition experiments as follows: DHT, 0.21 ± 0.10 nM; RU 58841, 88.1 ± 69.3 nM and RUM, 7458.3 ± 4687.8 nM. The K_d of DHT was 2×10^{-10} M. Binding calculated for RUM to 1% of RU 58841 binding, however, may not only be due to the ester itself, since 3.1% of the ester is transformed to RU 58841 under binding experiment conditions.

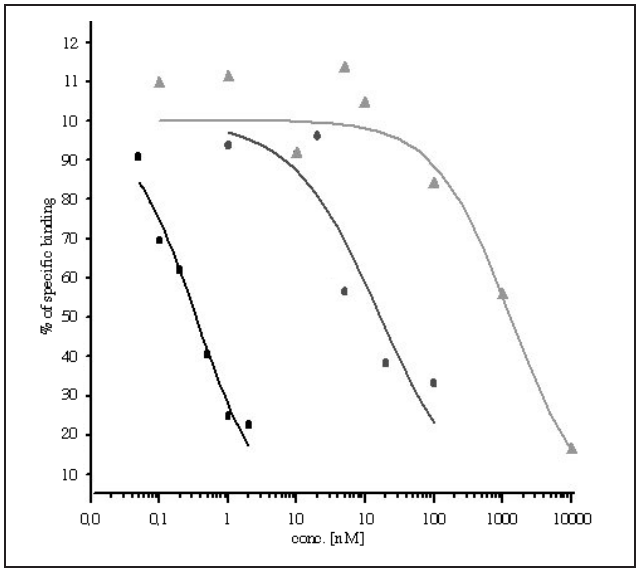

Fig. 1: Androgen competition. 29 +/GR + cells were incubated with ^{3}H-DHT 5×10^{-10} M in the presence of nonlabelled DHT (■), RU 58841 (●) and RUM (△) for 1 h under standard conditions. Results of one representative experiment (mean, n = 3) are depicted. Experiments were repeated twice (each n = 3).

Table 2: Hydrolysis of RUM in cell cultures

Cell type	RU 58841 formation	
	pmol/μg protein	% per well
DPC	177.9 ± 23.4	49.6 ± 6.5
SZ95	76.3 ± 4.5	41.2 ± 2.4
FB (2 pools)	162.7 ± 20.5	45.1 ± 5.6
KC (2 pools)	21.9 ± 1.1	23.6 ± 1.4

DPC, dermal papilla cells; SZ95, human sebaceous gland cell line; FB, fibroblasts; KC, keratinocytes. Cells were incubated with RUM (10^{-5} M) for 24 h under standard conditions. Dried chloroform extracts of the culture media were subjected to HPLC. Data are expressed as mean ± SD. Experiments were repeated twice (each n = 3)

2.3. Metabolism of RUM and RU 58841 in skin

RU 58841 was no subject of oxidative/reductive drug metabolism in foreskin keratinocytes and fibroblasts, male occipital dermal papilla cells, and in cells of the human sebocyte cell line SZ95.
RUM (10^{-5} M), however, was hydrolysed to RU 58841 to a large extent (Table 2). Esteratic activity decreased in the following order: male occipital scalp dermal papilla cells ≈ foreskin fibroblasts > sebocytes (SZ95) >> foreskin keratinocytes.

2.4. Cellular toxicity

Neither RU 58841 nor RUM (10^{-6}–10^{-4} M) reduced cell viability in any investigated cell type (dermal papilla cells were not tested for RU 58841 and RUM 10^{-4} M). Therefore underestimation of cutaneous metabolism due to cellular toxicity is excluded.

2.5. Permeation experiments

Because of varying and dissatisfying extraction rates of RUM from FCS containing acceptor fluids drug permeation had to be followed after esteratic cleavage via RU 58841 detection. Yet, neither RUM nor RU 58841 was

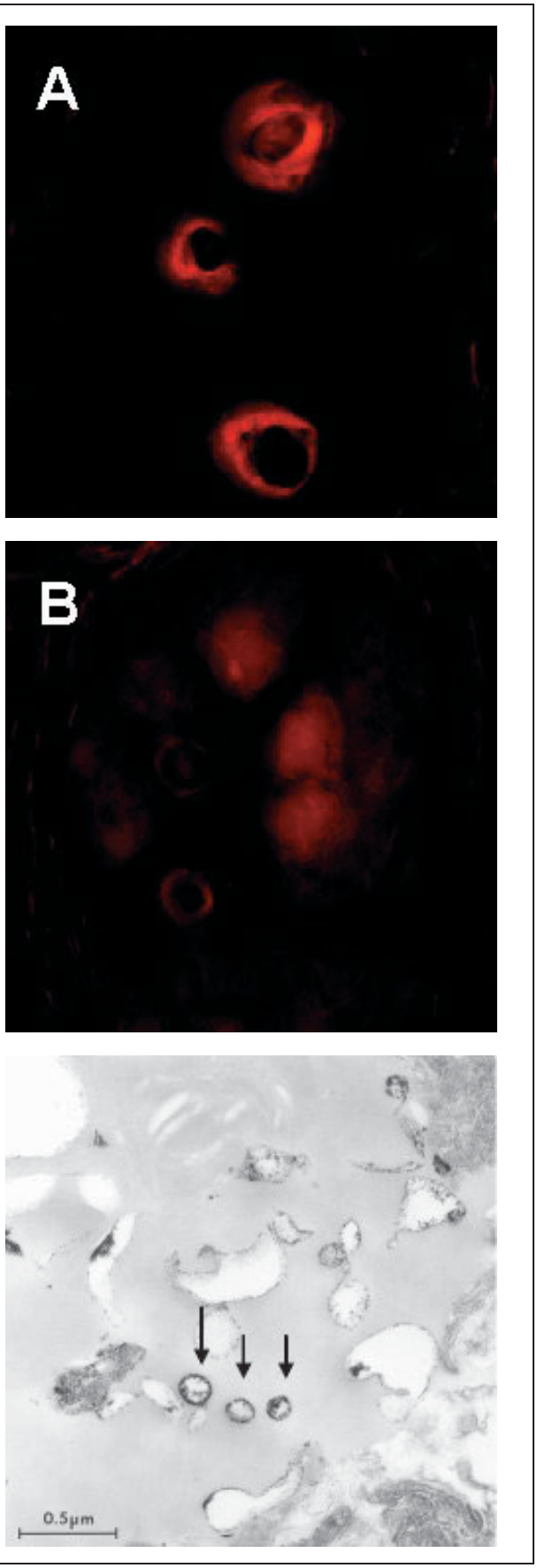

Fig. 2: Drug targeting to the hair follicle following topical application of Nile Red (A, B) or silver (C) labelled SLN for 24 h. A, B) Fluorescence microscopy of human hair follicles, depth 200 µm (A) and 650 µm (B). C) Electron microscopy of porcine hair follicles, arrays indicate the presence of intact silver loaded particles.

detected in any type of acceptor fluid. This holds true with all preparations tested (0.1% and 1% cream preparation, 0.1% nanoemulsion, and 0.1% SLN) and both with full thickness porcine skin and with reconstructed epidermis.

2.6. Characterization of the follicular penetration

To demonstrate follicular penetration, Nil Red labelled Compritol based SLN (LD50: 153 nm LD95: 484 nm), Miglyol based nanoemulsion (LD50: 189 nm, LD95: 489 nm) and cream were applied to human scalp skin for 24 h. Fluorescence microscopy revealed an intense fluorescence within the hair follicles of all investigated preparations. Fig. 3A demonstrates for Nil Red labelled SLN intense fluorescence within the hair follicle up to about 200 µm depth. From 400–900 µm skin depth the fluorescence decreased within the hair follicle but simultaneously appeared within the sebaceous glands (Fig. 3B). No fluorescence was detectable in deeper skin layers.
To evaluate if intact SLN may reach the hair follicle 1% silver sulfadiazine labelled Compritol® based SLN (LD50: 151 nm, LD95: 543 nm) were applied to porcine skin for 24 h. TEM allowed to detect intact SLN in the deeper layer of the hair follicle (Fig. 3C).

3. Discussion

Since RU 58841 was not lipophilic enough to be loaded onto SLN, the myristate ester was synthesized. Its pronounced lipophilicity did not only facilitate drug association with SLN but should also enable drug targeting to the hair follicle. Esterification of RU 58841, however, reduced its antiandrogenic potency. Receptor binding experiments in fact proved the almost complete loss of activity. Since cell incubation experiments demonstrated the ability of the skin cells to release the active antiandrogen, RUM should be a potent prodrug. In contrast to our observations with glucocorticoid ester hydrolysis (Gysler et al. 1997), keratinocytes appeared less active than fibroblasts to cleave RUM. In contrast to *in vivo* studies in rats (Cousty-Berlin et al. 1994), metabolism of RU 58841 to RU 59416 or RU 56279 was not detectable within human skin.
Although RUM extraction problems occurred with FCS containing acceptor fluids, permeation studies were possible. Overnight incubation with porcine liver esterase resulted in complete ester cleavage which allowed to measure the RUM concentration indirectly by RU 58841 detection. Most interestingly, RUM permeation of full thickness pig skin and reconstructed epidermis was not observed, irrespective of the applied vehicle or the type of acceptor fluid. RU 58841 permeation, which might result from ester cleavage within the skin followed by permeation into the acceptor fluid was also not detected. Probably the antiandrogens bind with high affinity to cutaneous proteins, e.g. keratins. The fact that RUM did not permeate through reconstructed epidermis which consists of only a few cell layers, however, was primarily unexpected since glucocorticoids permeate reconstructed epidermis by about 2–6% of the applied dose (Santos Maia et al. 2002). With respect to systemic side effects in men the poor permeation appears most important, although the predictability of this *in vitro* method with respect to the uptake in man awaits further investigation.
In order to follow the fate of SLN in the skin, we produced Nile Red and silver labelled SLN. Indeed, fluorescence microscopy demonstrated an extensive follicular up-

take. This result is well in accordance with previous studies showing that lipophilicity not only of the drug (Millikan 2000) but also of the carrier (Masini et al. 1993; Lauer et al. 1995; Bernard et al. 1997) improves drug uptake by the hair follicle. Yet, a Nile Red nanoemulsion and a cream did not appear inferior with respect to the hair follicle targeting. Since TEM allowed even to detect intact silver-labelled particles in the hair follicle when applied for 24 h, SLN may act as a slow release system and thus add to low systemic drug levels.

In conclusion, because of its activation in the skin to a potent antiandrogen and the low systemic availability, RUM offers the chance for topical antiandrogen treatment of acne and androgenetic alopecia. Because of their targeting effect, SLN-based preparations should further be developed with respect to control unwanted effects.

4. Experimental

4.1. Chemicals

RU 58841 (4-(4,4-dimethyl-2,5-dioxo-3-(4-hydroxybutyl)-1-imidazolydinyl)-2-(trifluoromethyl)-benzonitrile), RU 59416 (4-(4,4-dimethyl-2,5-dioxo-3-(3-carboxypropyl)-1-imidazolydinyl)-2-(trifluoromethyl)-benzonitrile), RU 56279 (4-(4,4-dimethyl-2,5-dioxo-1-imidazolydinyl)-2-(trifluoromethyl)-benzonitrile) and RU 57073 (4-(4,4-dimethyl-3-(2-hydroxyethyl)-5-oxo-2-thioxo-1-imidazolidinyl)-2-trifluoromethyl-benzonitril) were provided by Roussel-Uclaf (Romainville Cedex, France). Dihydrotestosterone (DHT), myristoylchloride, chloroform absolute, ethyl acetate, triethylamine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), glycerol, tris-base, Nile Red, silver sulfadiazine, porcine liver esterase, IMDM (Iscove's modified Dulbecco's medium), DMEM (Dulbecco's modified Eagle's medium), KBM (keratinocyte basal medium), supplements consisting of hEGF (human epidermal growth factor), insuline, hydrocortisone and BPE (bovine pituitary extract), glutamine, fetal calf serum were from Sigma (Taufkirchen, Germany). Acetonitrile HPLC grade, methanol, potassium dihydrogenophosphate and dimethylsulfoxide were purchased from Merck (Darmstadt, Germany). Sebomed® basal medium, amphotericin B and gentamicin were from Biochrom (Berlin, Germany). Compritol® 888 ATO, Precirol® ATO 5 were obtained from Gattefossé (Saint-Priest, France). Miglyol® 812 was obtained from Caelo (Minden, Germany), Poloxamer® 188 (Lutrol F68) from BASF (Ludwigshafen, Germany) and sucrose ester S1670 from Mitsubishi-Kagaku Foods Corporation (Tokyo, Japan). [1,2,6,7]-^{3}H-DHT was delivered by Amersham (Freiburg, Germany).

4.2. RUM synthesis

RUM was obtained by esterification of RU 58841 with myristoylchloride. Identity of the product was confirmed by ^{1}H NMR-spectroscopy, ^{13}C NMR-spectroscopy, H–H cosy-spectroscopy, C–H cosy-spectroscopy (all 400 MHz; Bruker WM 400, Rheinstetten, Germany; tetramethylsilan served as internal standard), mass analysis (EI-MS, ThermoFinniganMat, Bremen, Germany) and C–H–N analysis (Elementaranalyzer 240 B, 240 C, Perkin-Elmer, Rodgau-Jügesheim, Germany). A digital technique served for melting point determination of the ester (Elektrothermal, Hannover, Germany).

4.3. SLN-production and characterisation

SLN and nanoemulsions were produced by means of hot homogenisation (Mehnert and Mäder 2001) using an EmulsiFlex-B3 homogenizer (Avestin, Ontario, Canada) or alternatively a LAB 40 homogenizer (APV Gaulin, Lübeck, Germany). SLN preparations had the following composition: RUM, 0.1%; lipid, 5% (Compritol®, Precirol®); tensid (Poloxamer 188®), 1.25%; water, 93.65%. Drug free samples as well as a nanoemulsion containing Miglyol® instead of a solid lipid served for control. Silver sulfadiazine labelled SLN had the following composition: silver sulfadiazine, 1%; Compritol®, 10%; sucrose ester S1670, 4%; water, 86.5%. Nile Red labelled SLN were composed of Nile Red, 0.004%; Compritol®, 10%; Poloxamer 188®, 2.5%; water, 87.1%.

SLN preparations were characterized by laser diffractometry (LD; Coulter-LS, Coulter, Miami, Florida) and photon correlation spectroscopy (PCS; Malvern Zetasizer 4, Uniqema, UK) (Mehnert and Mäder 2001). Differential scanning calorimetry (DSC; Mettler DSC 821, Mettler Toledo, Gießen, Germany) served to prove formation of solid particles. Preparations were also microscopically and macroscopically checked for crystal formation.

4.4. Production of creams

"Nichtionische hydrophile Creme NRF" was added to RUM and Nile Red. Each addition was followed by repeated homogenisation using a pistill. Final concentrations were RUM 0.1% and 1%; Nile Red 0.004%.

4.5. Receptor binding studies

29 +/GR + cells were grown to confluence in 24 well plates. To remove endogenous steroids growth medium was changed to basal medium three days before the binding experiments. To determine the K_d of DHT, cells were incubated with ^{3}H-DHT (10^{-10} M to 10^{-8} M) for 1 h under standard conditions (5% CO_2, 37 °C). Then the medium was removed, cells were washed three times with phosphate buffered saline (PBS) and lysed in buffer containing 2% sodium dodecyl sulfate (SDS), 10% glycerol and 10 mM Tris HCl (pH 6.8). Bound ^{3}H-DHT was quantified by liquid scintillation counting (1450 Microbeta Plus Liquid Scintillation Counter, Wallac, Turku, Finland). Nonspecific binding was determined in the presence of a 500-fold excess of nonlabelled DHT. Four independent experiments, each run in triplicate, were carried out.

To quantify the relative binding affinities 29 +/GR + cells were incubated with 5×10^{-10} M ^{3}H-DHT alone and in the presence of nonlabelled DHT (5×10^{-11} M to 2×10^{-9} M), RU 58841 (10^{-9} M to 10^{-6} M) or RUM (10^{-10} M to 10^{-4} M) for 1 h. The EC_{50} values were calculated (Microsoft Origin 6.0) summarizing three independent experiments, each run in triplicate. Relevant RUM metabolism during binding experiments was investigated as described below, DHT metabolism as described elsewhere (Münster et al. 2003).

4.6. Cutaneous metabolism of RU 58841 and RUM

Skin cells and the cell line SZ95 were isolated and cultured as previously described (Gysler et al. 1997; Chen et al. 1998; Zouboulis et al. 1999). Cells were incubated with RU 58841 or RUM 10^{-5} M added as ethanolic solution for 24 h under standard conditions (5% CO_2, 37 °C, six well plates; Nunc, Roskilde, Denmark). 0.5 ml of the culture media were extracted with 0.5 ml chloroform and the organic phases were transferred to fresh tubes. The extraction was repeated twice and the combined chloroform extracts were dried under vacuum (Universal Vacuum System, Life Science, Frankfurt, Germany), redissolved in 100 µl acetonitril and subjected to HPLC. Cell free medium served for negative control.

4.7. Estimation of androgen cytotoxicity

To exclude artificially reduced drug metabolism due to cytotoxicity occipital scalp dermal papilla cells were incubated with RU 58841 and RUM 10^{-6}–10^{-5} M, other cells with 10^{-6}–10^{-4} M, for 24 h. Then the MTT-test was performed (Gysler et al. 1997).

4.8. Permeation studies

Permeation studies were carried out using the Franz diffusion cell (Gysler et al. 1997). 50 µl RUM preparations (1% "nichtionische hydrophile Creme NRF", 0.1% "nichtionische hydrophile Creme NRF", 0,1% nanoemulsion and 0.1% SLN) were applied onto reconstructed epidermis (SkinEthic®, SkinEthic, Nice, France) and full thickness porcine skin for 6 h. Acceptor fluid (PBS or alternatively DMEM supplemented with glutamine, amphotericin, gentamicin and 10% FCS) perfusing the lower site of the reconstructed epidermis at a rate of 6 ml/h was collected.

Incubation of acceptor medium with 2 U porcine liver esterase for 24 h at 37 °C served for complete ester cleavage. RU 58841 was extracted as described above. Two independent experiments (different skin batches of reconstructed epidermis or donor pigs) were run in duplicate.

4.9. Antiandrogene analytics (HPLC)

The HPLC system consisted of a Hitachi pump L-7100 operated at a flow rate of 1 ml/min, a Hitachi autosampler L-7200 equipped with a 100 µl loop (20 µl sample volume were injected onto the column using the cut loop method) and a Hitachi UV detector L-7400 operated at 260 nm. A Kromasil C 8 (100–5) 250 × 4.6 mm analytical column together with a C 8 (100–5) 4 × 4.6 mm pre-column (CS-chromatographics, Langerwehe, Germany) served to separate RU 58841, RU 59416, RU 56279, RUM and the internal standard RU 57073. A mobile phase consisting of acetonitrile/50 mM potassium dihydrogenophosphate buffer adjusted to pH 5.2 (50 : 50) was used for the first 15 min. The composition was changed to acetonitrile/water (50/50) over 2 min and then maintained for another 3 min. From minute 21–24: acetonitrile was increased to 100% and then maintained for another 5 min. 30 min after the start the composition was stepwise returned to acetonitrile/buffer by a change to acetonitrile/water (50/50) over 1 min and change to acetonitrile/buffer (50/50) at min 36 which served until the end of a run (min 55). Retention times were for RU 59416 7.27 min, RU 58841 8.06 min, RU 56279 8.72 min, RU 57073 10.05 min, and for RUM 32.09 min. The limit of detection and limit of quantification were 8×10^{-12} mol and 4×10^{-11} mol, respectively.

4.10. Fluorescence microscopy

After human scalp skin was incubated with Nile Red labelled SLN, nanoemulsion and cream for 24 h excessive material was removed with a paper towel and skin was cut into 30 µm slices top down to the hair root using a microtome (2800 Frigocut N, Reichert-Jung, Nußloch, Germany). Skin slices were inspected using a fluorescence microscope (Axioplan microscope, Zeiss, Oberkochen, Germany) and pictures were taken using a SensiCam 12-bit CCD camera (PCO Computer Optics, Kelheim, Germany). Controls (unlabelled SLN) were set black.

4.11. Transmission electron microscopy (TEM)

TEM-pictures (EM 902, Zeiss, Oberkochen, Germany) of silver labelled SLN were taken as previously described for silver labelled liposomes (Schaller et al. 1997).

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