

Abstracts

Wissenschaftliche Posterausstellung



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Wissenschaftliche Posterausstellung: Poster 1

Quantification of nanoparticle uptake into hair follicles - in vitro in vivo correlation

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The hair follicle represents an interesting target site for drug delivery, as the stratum corneum (SC) is absent in the lower third of the hair follicle facilitating the absorption of topically delivered compounds. Nanoparticles (NPs) penetrate more efficiently into hair follicles than solutions (1), making them ideal carriers for drug delivery. Based on the differential stripping technique (2) we developed a fully quantitative, validated method to determine the exact dose of NPs delivered to hair follicles.

As model carriers NPs prepared from poly(D,L-lactide-co-glycolide) (PLGA) and chitosan coated PLGA NPs (Chit.-PLGA) (hydrodynamic diameter around 165 nm, PDI \leq 0.2 for both types of NPs) were used. The NPs were labeled covalently with a fluorescent dye for detection (3). Both polymers are widely used for drug carriers. Their opposite surface charge (PLGA-NPs -30 mV, Chit.-PLGA-NPs +26 mV) allows investigating the influence of charge on follicular uptake. The investigation was performed on pig ear skin in vitro and on the hairy outer forearm of 11 human volunteers in vivo (6 male, 5 female, skin type II-IV, ethical approval by Ärztekammer des Saarlandes and written informed consent by the volunteers). The NPs were applied, incubated and sampled by differential stripping with tape strips and cyanoacrylate biopsies according to a standardized protocol. Subsequent extraction of tape-strips and cyanoacrylate casts of particle filled follicles allowed quantification of the amount of NPs that had penetrated into the hair follicles.

For exact quantification of follicular uptake the efficacy of removing NPs from the skin surface is essential. This was proven by confocal microscopy detecting NP associated fluorescence on the removed tape strips, in follicle casts and on the skin surface, and by environmental scanning electron microscopy using metal core model nanoparticles (screen MAG chitosan coated nanoparticles, 100 nm, chemicell GmbH, Berlin, Germany). Thus we could demonstrate complete removal of NPs from the skin surface by tape stripping.

We found less than 5% of the applied NPs penetrated into the hair follicles, while the major fraction remained on the skin surface. This result was independent of the NPs used; indicating



that surface charge had no effect on follicular penetration of these particular NPs. Furthermore follicular uptake was very comparable in vivo in the human volunteers and in vitro on the pig ears. Plotting the mean amount recovered from skin surface, the hair follicles and total recovery in pig ears and human volunteers for both NPs, an excellent linear in vitro in vivo-correlation ($r^2=0.975$) was demonstrated.

In conclusion this study confirms pig ear skin as suitable skin model for quantifying follicular uptake of NPs. The uptake efficiency of NPs into hair follicles is relative low and seemed not to be influenced by the surface charge of the NPs.

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Wissenschaftliche Posterausstellung: Poster 2

Impact of Emulsifiers on the Performance of Sunscreen-loaded Nanosuspensions from Beeswax and Jojoba Oil

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Organic and natural ingredients have become a major trend in cosmetics due to the consumers' wish for ingredients friendly to both skin and environment. This is especially true for sunscreens in natural care cosmetics used for photo protection and thus skin cancer prevention.

A nanosuspension composed of titanium dioxide as inorganic sunscreen within a matrix of carnauba wax and decyl oleate in a 2:1 ratio and stabilized by the surfactant polysorbate 80 (polyoxyethylene (20) sorbitan monooleate) has previously been reported to yield a high sun protection factor (SPF) of about 60 (in vitro) [1]. The replacement of carnauba wax and decyl oleate by beeswax and jojoba oil also proved to result in high SPF values and small particle sizes in the nanometer range [2]. With regard to natural care cosmetics polysorbate 80 should be replaced by eudermic surfactants such as sugar esters and polyglyceryl esters. In this study, the impact of different surfactants on SPF and particle size distribution was investigated. Information about the morphology of the wax particles was obtained from transmission electron microscopy (TEM) and scanning electron microscopy (SEM), respectively.

Methods: Nanosuspensions were manufactured by dispersing a molten lipid phase into an aqueous phase by using high-pressure homogenization. Particle size distribution was determined by laser light diffraction and PIDS. SPFs were taken in vitro with an SPF analyzer. The visualization of the nanoscale particles was made using SEM and TEM after negative staining with a 2 % (w/w) solution of uranyl acetate, respectively.

Results: Sucrose laurate as sugar ester and polyglyceryl-4-laurate/succinate proved to be appropriate candidates for nanosuspensions with eudermic surfactants. From macroscopical evaluation nanosuspensions containing sucrose laurate seemed to be more viscous compared with corresponding formulations containing polyglyceryl-4-laurate/succinate. In terms of SPF, nanosuspensions with 6 % of titanium dioxide and 5 % of surfactant (both (w/w)) showed SPFs higher than 50 (polyglyceryl-4-laurate/succinate: 58.9, sucrose laurate: 51.6), whereas the plain formulations without titanium dioxide showed SPFs around 1.1 (polyglyceryl-4-laurate/succinate) and 1.5 (sucrose laurate), respectively.

In the case of polyglyceryl-4-laurate/succinate, increasing surfactant concentrations from 1 % up to 5 % led to a rise in SPFs from 13.2 up to 58.9. In contrast, nanosuspensions containing sucrose laurate showed lower SPFs with increasing surfactant concentration. While formulations containing 1 % of sucrose laurate yielded SPFs of about 70, those with 5 % sucrose laurate showed an SPF of about 50. This coincided with a reduction in particle size distribution upon



increasing surfactant concentration.

Furthermore, for the nanosuspensions containing polyglyceryl-4-laurate/succinate, no variation in particle size distribution occurred during a three-month storage at 20 °C. By using sucrose laurate a quick increase in particle size up to the micrometer range was observed after one week. Plain formulations without titanium dioxide showed a narrower particle size distribution than the corresponding nanosuspensions with titanium dioxide. Particularly with polyglyceryl-4-laurate/succinate a clear difference in particle sizes with regard to plain and titanium dioxide-loaded nanosuspensions could be observed.

Concerning morphology a close contact between beeswax and titanium dioxide crystals was confirmed. Nanosuspensions containing polyglyceryl-4-laurate/succinate showed plate-like particles of ellipsoidal to spherical shape with smooth edges. Particles of 400 nm and above showed furled or bent edges as well as an agglomeration of titanium dioxide in the middle of the particle. In the case of sucrose laurate as surfactant, the particles also seemed to have a plate-like shape with a central agglomeration of titanium dioxide, but showed rather irregular anisometric particles.

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Wissenschaftliche Posterausstellung: Poster 3

Permeation behavior of the NSAID ibuprofen from different poloxamer 407-based formulations through isolated human stratum corneum

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The non steroidal anti inflammatory drug (NSAID) ibuprofen is one of the most frequently used drugs in analgesic and antirheumatic therapy. Since dermal application of NSAIDs may reduce their typical gastro intestinal side effects, topical treatment is an important and effective alternative to a systemic therapy [1].

Quick and efficient pain relief requires an adequate drug permeation across the skin from appropriate vehicles which may influence the flux (= amount of drug permeated per area and time). Poloxamer-based systems have shown high in vitro permeation rates for different active pharmaceutical ingredients (API) [2-3]. Therefore, in the present study ibuprofen (IBU) was chosen as API. Several formulations containing poloxamer 407 (POX), medium chain triglycerides (MCT), isopropanol (IPA), dimethyl isosorbide (DMIS), and water were analyzed with regard to the influence of their quantitative composition on the ibuprofen flux. The results were compared to a commercially available poloxamer-based gel formulation, doc® Ibuprofen Schmerzgel.

Methods: Manufacture of the formulations was performed with a Cito Unguator® 2000 (Konietzko GmbH, D-Bamberg), doc® Ibuprofen Schmerzgel (Hermes Arzneimittel GmbH, D Großhesselohe/Munich) was purchased at a local pharmacy. In vitro permeation studies were carried out in modified Franz cells (37 °C, receiver solution: phosphate buffered saline pH 7.4) with isolated human stratum corneum. The skin samples originated from plastic surgery of healthy female abdomen and were prepared by trypsination [4]. Twelve samples were taken over a period of 32 hours. Quantification of the permeated drug amount was conducted with high performance liquid chromatography (Waters, D-Eschborn) by using a column of Hypersil® ODS 5 µm, 125 x 4 mm (Grom, D-Herrenberg-Kayh) with a mobile phase consisting of acetonitrile/water/acetic acid (55:45:1), a flow rate of 1.7 ml/min, and UV-detection at 246 nm.

Results: The variation of the quantitative composition produced significant differences in IBU permeation behavior [5]. An increased POX/MCT concentration led to a decreased IBU flux due to an increasing amount of poloxamer micelles which enabled partitioning of the API within these micelles and thus a slow API release [6]. In addition, an increase in consistency was observed and contributed to this phenomenon.

An increase in IPA/DMIS concentration produced higher ibuprofen fluxes. It has already been shown that IPA can act as permeation enhancer by fluidization of the lipid bilayer structure of the



stratum corneum [7]. However, high IPA concentrations may damage the stratum corneum so that skin tolerance of the formulations must be considered in the first place.

A doubling of the IBU concentration improved the permeation so that the flux of doc[®] Ibuprofen Schmerzgel was exceeded, but also changed the macroscopical appearance, mainly in terms of liquefaction, so that these systems may be used as spray formulations.

The present results clearly demonstrate the great influence of the vehicle composition on the in vitro permeation of a drug. Consequently, this queries the common practice of substitution of creams, ointments and gels in terms of the “aut idem” rule.

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Wissenschaftliche Posterausstellung: Poster 4

Caffeine Nanocrystals - Novel Concept for Improved Dermal Delivery & Production Method

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Since 2000 nanocrystals are pharmaceutically applied to increase the oral bioavailability (BA) of poorly soluble drugs of the Biopharmaceutical Classification System (BCS) class II (e.g. product Rapamune®/sirolimus). The rate limiting step of absorption of class II drugs is the low solubility and related low dissolution velocity. Transfer to the nanodimension changes the physico-chemical properties of materials, in case of poorly soluble drugs it increases the saturation solubility C_s , and related dissolution velocity dc/dt . The increased concentration gradient leads to increased membrane permeation and consequently BA.

However, completely forgotten was to apply this successful principle to improve dermal BA of cosmetic ingredients and of drugs. In 2007 we introduced the first cosmetic product on the market based on the poorly soluble antioxidant rutin (Juvena), in 2009 hesperidin nanocrystals (La Prairie). In a human study rutin nanocrystals showed a 1,000 fold higher antioxidant activity in the skin [1]. A novel approach is to apply this principle to well soluble actives such as caffeine (e.g. used in cellulite products). At the first glance, nanocrystals from soluble actives seem to make no sense, because the active is soluble anyway!

However, it was found that the penetration of caffeine increases with its concentration in the product, thus cellulite products compete with increasing caffeine concentrations. To avoid reduction of the caffeine concentration in the applied dermal formulation due to skin penetration, it makes sense to add additionally caffeine crystals as depot. Favourably are nanocrystals compared to μm -sized crystals, because they increase C_s and consequently the concentration gradient and related flux into the skin. In addition, crystals of optimal size (around 700 nm) can accumulate in the hair follicles and penetrate from there into the surrounding cutaneous tissue. Massage during application – performed anyway with some consumer care products – can further enhance follicular uptake.

However, when producing nanocrystals in a traditional high energy wet milling process of e.g. high pressure homogenization, supersaturation effects lead to pronounced crystal growth (Ostwald ripening effects), even larger than the starting material (fibre formation). A special process was developed based on low energy milling (bead mill) in combination



with dispersion media with low dielectric constant D (e.g. water-ethanol, water-glycerol mixtures) to yield nanocrystals of varying sizes, e.g. 900 nm to 660 nm, 250 nm, and by controlled separation 90 nm. In the next step, the nanocrystals will be tested in human to verify if their performance is superior to a caffeine solution. If yes, this principle could be applied to other soluble actives and introduced as novel dermal delivery concept.

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Wissenschaftliche Posterausstellung: Poster 5

Preparation Method for Ultra Small Gelatin Nanoparticles for Dermal Delivery of Peptides & Enzymes

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Cosmetic industry is increasingly interested to use peptides or enzymes in cosmetic products. Examples are the peptides by the Spanish company lipotec (e.g. the very successful Argireline) or enzymes like superoxide dismutase by American company Estee Lauder. As water soluble molecules they can be simply dissolved in the water phase of a cream/gel or of liposomes. However, to chemically stabilize these molecules and/or to achieve a controlled prolonged release, incorporation in a solid matrix particle is desirable. As preferable over hydrophobic matrices of polymeric nanoparticles appear nanoparticles from hydrophilic gelatin. Of special interest are ultra small gelatin nanoparticles (GNPs), that means < 100 nm, and especially < 50 nm. The smaller the particles, the more adhesive they are to the skin. In addition it was shown that core-multishell (CMS) nanoparticles by Haag et al of a size of about 40 nm were able to deliver more efficiently fluorescent marker into epidermis (1). However, most of the GNPs production methods yield nanoparticles above 250 nm. Very important is that incorporated enzymes are again released and retain their enzymatic activity! Therefore the aim was to produce GNPs below 100 nm, and to prove remaining enzymatic activity by using lysozyme as model.

A two-step desolvation method for the preparation of GNPs was developed by modification of a method described by Coester (2). Briefly the gelatin was dissolved in water and first desolvated by acetone to separate high molecular weight fractions. Glutaraldehyde was added as crosslinker after a second time desolvation and formation of particles. GNPs possessing a mean PCS size of about 60 nm were obtained with optimized production parameters, like a starting gelatin concentration of 2.5%, a pH of 2.5 and a precipitation time of 5 minutes. In the next step, GNPs were loaded with lysozyme, which changed the size only slightly to 78 nm. A drug loading efficiency of 89% was obtained. The activity of the released lysozyme was checked by HPLC, and proved to be 93%. Storage studies were performed over 6 months at 3 temperatures, apart from 40°C the GNPs remained physically stable.

Conclusion: GNPs distinctly below 100 nm could be produced, activity of the model enzyme remained. Further modification of the method should allow to produce GNPs ≤ 40 nm, having similar skin delivery potential to CMS nanoparticles. However, the GNPs have the



advantage that – in contrast to the dendrimer CMS – gelatin is a regulatorily accepted excipient and can be used in dermal formulations.

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Wissenschaftliche Posterausstellung: Poster 6

Film forming emulsions for sustained dermal release of nonivamide

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Nonivamide (NVA) is used in the therapy of chronic pruritus accompanied with diseases such as atopic dermatitis or psoriasis. Currently available formulations containing NVA need to be applied 4 to 6 times a day. As multiple applications a day frequently lead to poor compliance of patients, a formulation permitting sustained release of NVA is needed to reduce the number of applications per day. The aim of our study was to develop a film forming o/w emulsion containing NVA in the inner oil-phase, making it easy and convenient to treat larger areas of affected skin over a long time.

Simple oil-in-water emulsions were prepared from medium chain triglycerides and an aqueous solution of polyvinylalcohol. By addition of an aqueous dispersion of poly(ethylacrylate methylmethacrylate trimethylammonioethyl methacrylate chloride) (quaternary PMMA) and/or an aqueous dispersion of poly(ethylacrylate methylmethacrylate) (neutral PMMA) a water insoluble sustained release matrix was built around the droplets upon water evaporation. We investigated the ability of quaternary PMMA and neutral PMMA and their mixtures to entrap the oil droplets of an o/w emulsion in the dry state, and their ability to extend the contact time of drugs dissolved in the dispersed lipid phase to the skin as well as drug permeation from these dried emulsion films.

In-vitro permeation of NVA from film forming emulsions containing 1 % NVA was studied in comparison to the immediate release formulation "Hydrophilic Nonivamide Cream" containing 1 % or 0.1 % NVA (HNC 1 % or HNC 0.1 %). We found that film forming emulsions were able to reduce flux of the api by ten fold compared to HNC 1 %. This flux was found to be similar to that from HNC 0.1 %. Finite dose experiments revealed that NVA was released from film forming emulsions with constant flux over a period of 24 hours.

Therefore film forming emulsions may reduce application frequency and thereby enhance patient convenience and compliance. Thus the long term treatment of diseases such as chronic pruritus or pain may be improved.



Wissenschaftliche Posterausstellung: Poster 7

The use of concentrated heat improves burning, itching, swelling and quality of life during recurrence episodes of herpes labialis– results of a pharmacy based prospective, double-arm, observational cohort study with either acyclovir ointment or Herpothem[®] treatment under real life conditions

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Background

Recurrent herpes labialis, primarily caused by HSV-1 is a common skin-infection with occurrence of prodromes and crusts.¹ The prodrome phase is associated with itching, burning and pain prior to the appearance of erythema and papule formation.² All available antiviral drugs aim to block viral replication in order to shorten the duration of symptoms and accelerate the healing process. The inactivation of herpes simplex type 1 and 2 with heat has already been described.⁴ Until now, there is no real life data for topical herpes labialis treatments considering patient reported outcomes. We therefore performed a pharmacy based prospective, double-arm, observational cohort study with acyclovir ointment and Herpothem[®].

Methods

This study was performed in collaboration with 11 pharmacies in Germany with 103 volunteers. The study was approved by the Ethics Committee of the University of Greifswald (study protocol/ CRF).

The questionnaire used in this observational study contained the following items: age, sex, any prodrome visible, number of prodromes during former recurrences, the burden and duration of disease during former recurrences and willingness-to-pay for a treatment, which could prevent any herpes labialis outbreak.

Topical acyclovir ointments are widely used, even if they are advised to be applied numerous times a day for up to 5 days and their clinical benefit is regarded as small by only reducing the duration



of symptoms.³ Herpotherm® produces a microchip controlled concentrated topical thermal impulse of an average temperature range of 51-53 °C for 4 seconds.

Results

Both, the use of an acyclovir ointment and concentrated heat (Herpotherm®) led to a reduction of burning, itching, swelling and thus led to an improvement in the quality of life over a 7 day observation period. The Herpotherm® cohort showed a significant difference to acyclovir cohort in improvement in all items already after two days of treatment ($p < 0.04$) and at each following day of observation. The mean impairment of quality of life was reduced to 50 % of start value within 3 days of treatment in the Herpotherm® cohort and not before 5 days of treatment in the acyclovir cohort. Concentrated heat prevented the outbreak of a herpes labialis in 25 % of patients of the Herpotherm® cohort (7 out of 28 patients) and in 14 % of the acyclovir cohort (3 out of 21 patients), for patients without any prodromes before treatment. Furthermore there was a statistically significant lower development of crusts in the Herpotherm® cohort, than in the acyclovir cohort ($p < 0.01$). The burden and duration of disease was lower and shorter in the Herpotherm® cohort than in the acyclovir cohort.

Discussion

In this first observational cohort study the use of Herpotherm® resulted in a measureable benefit as far as patient outcome is concerned. In contrast to acyclovir, concentrated heat showed a higher prevention rate of herpes labialis outbreak. The Herpotherm® cohort showed a reduction of impairing factors as burning, itching and swelling (which are correlating with quality of life) initially after treatment. Larger randomized, controlled studies are still necessary to verify these results.

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Wissenschaftliche Posterausstellung: Poster 8

Thermo-sensitive hydrogels as carrier-systems for insect-based proteins in chronic wound management

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The aim of this study was the development of a thermo-sensitive hydrogel containing insect-based enzymes for treatment of chronic wound infections. The designed formulation based on gelling agent Poloxamer (a triblock copolymer with central polyoxy-propylene and two lateral polyoxy-ethylenes). For an ideal application, the wound-dressing should be liquid at ambient temperature and form a gel instantly at skin temperature in order to achieve sustained release of the drug. The gelling temperature represents the point of sol-gel transition and is reflected by the inflection point of viscous and elastic modulus (G'' and G'). The influence of several concentrations of different additives such as additional thickeners, moisturizing factors or preservatives on viscoelastic properties of the hydrogel were investigated by oscillatory measurements. The results indicated that generally with an increasing concentration of additives, the viscosity of the system and the gelling temperature were influenced. Based on experimental data two possible final-formulations were designed. The first one contained 16% (w/w) Poloxamer 407, 3% (w/w) glycerin, 0.2% (w/w) potassium sorbate, 0.1% (w/w) citric acid in water and a second formulation contained additionally 15% (w/w) Poloxamer 188.

Recently, chronic wound infections are a major challenge in health care. The insect-based enzyme IMPI (Inducible Metalloprotease Inhibitor) represents an innovative promising drug candidate in that field. It inhibits M4 Metalloproteases, which are built by several bacteria and are responsible for necrosis.

The API (active pharmaceutical ingredient), IMPI was produced as GST fusion protein by fermentation of E.coli and entrapped within these cold hydrogels in concentrations between 0.2 and 0.3 mg/ml. It could be demonstrated that IMPI as API, similar to the additives, influenced the rheological characteristics of the hydrogel. An increase of viscosity and a decrease of gelling temperature were observed. The effect might be caused by the hydrolysis of protein, which decreased the amount of free water molecules. However, the formulation fulfills the requirements and is approved by stability testing.

The quantitative analysis of proteins in the formulation was performed by analytic methods like HPLC, Bradford- and GST-Assay. HPLC analytics revealed a high limit of quantification. The



other methods exhibited immense standard deviation and are partially influenced by hydrogel compounds. Qualified detection of bioactivity of the enzyme was reached with casein fluorescence quenching assay. The results showed that the activity of IMPI in hydrogel after preparation was comparable to its activity in water solution. Furthermore, the bioactivity was not altered during storage.

It could be concluded, that the prepared thermo-sensitive hydrogels are suitable formulations for treatment of chronic wound infection with IMPI. In further studies the time-release profile of IMPI from the hydrogel will be further elucidated.



Wissenschaftliche Posterausstellung: Poster 9

Black cumin as a traditional vulnerary – Little seeds with great effects?

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The history of medicinal plants is probably as old as humanity itself. The plants were chosen for the medical therapy according to empiric observations of their effects and handed down first by oral, later by written tradition from generation to generation. Often, indications and applications for medicinal plants can be observed over centuries. These traditional applications – explained by respecting and correctly understanding the contemporary concepts of illness and therapy – can be indications of a real effect. Our research project is dedicated to medicinal plants from the medieval Arabic medicine. First, the medicinal plants mentioned and described in selected medieval sources are identified under the aspects of modern botany. The next step is the analysis of the tradition of selected medicinal plants in the European pharmacy and medicine. Finally, medicinal plants with defined long-term use will be studied concerning the present knowledge about their constituents and efficacy and after the evaluation recommended as potential phytotherapeutics with the analysed indications or as resources for active substances.

Black cumin (*Nigella sativa* L.) is a medicinal plant with a long Arabic and European tradition in treating wounds and skin diseases. Recent research on animal models showed very promising enhancing effects of black cumin oil on wound healing. It seems to be essential to trace back its medical-pharmaceutical tradition and to investigate in how far it can explain and support present applications and suggest future potential of the plant.



Wissenschaftliche Posterausstellung: Poster 10

An innovative vehicle based on lyotropic formulations containing up to 20 % diclofenac for combating local peripheral pain

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The problem

Combating local peripheral pain by a systemic therapy is often contra-indicated or even impossible, because of serious comorbidities of the patients. In addition systemic pain therapy often fails, because the local level of effectiveness cannot be reached because the therapeutic quotient of the pain drug employed is too low.

But in many cases also the local therapy fails, because of too a low skin permeation of the applied formulation, so that the local effective concentration of the drug cannot be achieved.

The new vehicle

Three main components constitute the new vehicle.

First, a lyotropic fluid based on nonionic tensides. These fluids have two very interesting properties: they are very good solvents for all kinds of substances (drugs): hydrophilic, lipophilic, amphiphilic and they render possible a good permeation through the horny layer of the skin.

Second, the new vehicle contains capsaicin, which opens the cutaneous vessels to the maximum. This increases the skin perfusion and so the drug transport to the inner tissues. Also it empties the substance P stores, combating the second pain of capsaicin very effectively, but capsaicin generates a strong first pain.

Third, to cut off this first pain, the vehicle contains also lidocain.

The formulations

In order to combat local pain with the new vehicle, we have chosen diclofenac as pain drug. Two special formulations were developed: DoloCur for the hairless skin of men and HippoCur for the haired skin, especially for horses. The first formulation contains 20% diclofenac, the second 15%. In hairless skin mainly the intercellular permeation is working, whereas in haired skin additionally there is working the transfollicular penetration mechanism.

The formulations are colourless clear gelic fluids and at least stable for 2 years.

The application

The standard application procedure comprises 7 applications daily in the first week and 7 applications every second day in the following fortnight. In case of hairless skin (DoloCur) 10 drops are rubbed into 100 cm² skin, taking 2 fingers protected with a cot. 1 drop taken from an injection needle weighs 25mg and contains 5mg diclofenac. In case of haired skin 1g of HippoCur (i.e. 150mg diclofenac) are sprayed onto the skin parallel to the hair around the carpal joint of a horse.

Results

1) Dynamic light scattering (DLS) investigation:



The results of both formulations are different: HippoCur has 2 maxima at 45 and 340 nm, DoloCur at 15 and 89 nm. Both preparations show polydispersity and low signals.

2) Drug levels in the carpal joints of horses:

Five horses were treated with HippoCur as described above. After treatment synovial fluid was taken from the joints and analysed for diclofenac, at the same time the blood plasma of the animals was analysed (the analysis was done with HPLC combined with mass spectrometry for detection). The average value for diclofenac level in the synovial fluids was 210 ± 5 ng/ml (mean \pm SD, n= 5). Diclofenac could not be detected and measured in blood plasma, the content was under the limit of proof, which is 3 ng/ml.

3) Clinical case series in horses with HippoCur:

In 94 horses with different orthopedic indications concerning the limbs of the animals HippoCur was applied as described. The quote of clinical success (abolishing laming) was found to be 90%.

4) Clinical case series in men with DoloCur:

In men (more than 100 cases) with different indications like heel spur or CRPS (complex regional pain syndrome) or tendinosis the average quote of clinical success (abolishing pain) was 75%. No case of skin irritation was observed.

Discussion

The results of the light scattering investigations indicate the existence of mesophases within the formulations.

It is clear from the description of the procedure that the treatment is the more successful the nearer the locus of pain lies to the skin surface. We estimate the deepness of effectivity to be about 2cm, because it was possible to treat successfully also a man's wrist.

The effective tissue level of diclofenac is discussed thoroughly by Chlud and Wagner [1]. The authors regarding synovial fluid come to the effective range of 100-500ng/ml for diclofenac. It is seen that the effective diclofenac levels, achieved in the investigations reported here, lies well within this range.

Kriwet und Müller-Goymann have shown, that diclofenac itself is mesogenic in diethylamine water system [2].

The new vehicle may also be used for formulations with other drugs, because the vehicle is universal.

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Wissenschaftliche Posterausstellung: Poster 11

Stabilisation of a W/O/W Multiple Emulsion using a Natural Polymers

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Introduction

Multiple emulsions are of major interest as potential skin delivery systems and are also known as “emulsions of emulsions”. They show high potential for the transport of hydrophilic drugs across the skin and the internal droplets can serve as an entrapping reservoir for hydrophilic drugs. However, multiple emulsions are thermodynamically unstable due to a high tendency of the internal droplets to coalesce, aggregate or rupture. The aim of the present study was to prepare multiple emulsions by a simple one-step emulsification using a natural polymer as a thickener of the external water phase to improve long-term stability and increase the release under shear. Furthermore 5-fluorouracil was incorporated as a model drug to investigate skin penetration properties.

Experimental methods

Formulations

Multiple emulsions using Span 80 as a lipophilic and Tween 80 as a hydrophilic surfactant were prepared using a One-Step Emulsification Method [1]. The total amount of surfactant was kept at 4%. Both surfactants were solved in the oil phase. Oil and aqueous phase containing 1% 5-fluorouracil as a model drug were separately heated up to $50 \pm 5^\circ\text{C}$. Subsequently the water phase was slowly added into the oil phase under moderate stirring. Afterwards the natural polymer Solagum™ AX (Acacia Senegal Gum and Xanthan Gum) was incorporated and stirred for 20 min at 750 rpm.

Optical light microscopy

The characterisation of the multiple droplets was performed using a photo microscope (Zeiss Axis Observer.Z1 microscope system, Carl Zeiss, Oberkochen, Germany).

Stability assessment

Droplet size was determined using a laser diffraction particle size analyser (Mastersizer 3000, Malvern, United Kingdom). The particle size distribution was calculated according to the Mie theory.

In vitro skin penetration

Skin penetration of 5-fluorouracil from W/O/W multiple emulsions was determined by tape stripping experiments. Formulation was applied on full-thickness porcine ear skin for one hour. Subsequently the uppermost layers of the stratum corneum were removed with 20 tape strips. Corneocytes and 5-fluorouracil were quantified by NIR and HPLC, respectively. The entire horny



layer thickness was determined by removing the whole stratum corneum until the limit of detection of the NIR densitometer was reached.

Results

It was possible to create stable W/O/W multiple emulsions using Solagum™ AX as a natural thickener. The use of a thickener led to a decrease of droplet size from approximately 36 μm to 17 μm and an improvement of droplet-stability. Furthermore the number of inner water droplets entrapped in the oil droplets was increased. After 10 weeks of storage there were rarely any entrapped water droplets left in the multiple emulsion without a thickener, whereas the oil droplets of the multiple emulsion with a polymer contained still a lot of inner water droplets. The mean droplet sizes of the thickened multiple emulsion remained largely constant over the whole observation period. The skin penetration of the model drug 5-fluorouracil proved to be excellent from the obtained W/O/W multiple emulsion with a thickener. 5-Fluorouracil could be detected up to 20 strips, which approximates 82% of the entire stratum corneum thickness.

Acknowledgements

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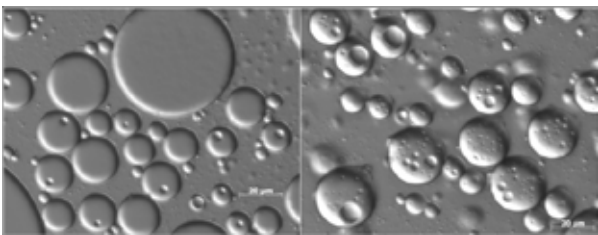


Figure 1: Differential interference contrast microscopic (DIC) images of a W/O/W multiple emulsion. Left: Without a thickener, Right: With a natural polymer as a thickener

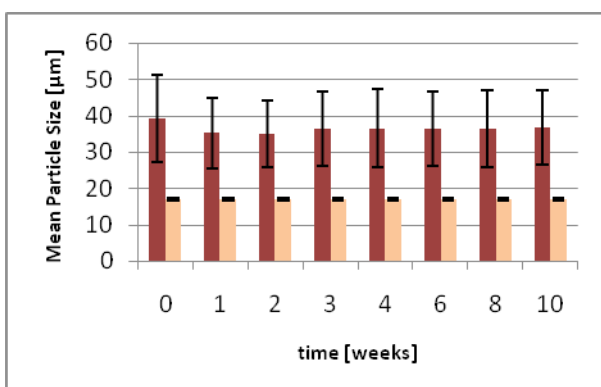


Figure 2: Droplet sizes of the multiple W/O/W emulsions presented as the mean diameter based on the volume distribution $D(v,0.5)$. Red bars: without thickener, orange bars: with thickener

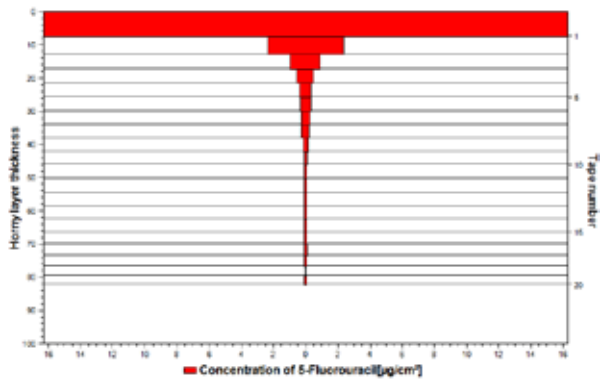


Figure 3: Skin penetration profile of 5-fluorouracil from a W/O/W multiple emulsion using Solagum™ AX as a thickener (n=6)

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Wissenschaftliche Posterausstellung: Poster 12

Effects of INLB321-CD on VEGFA Gene Up-Regulation in Immortalized Human Keratinocytes and on a Model of Wounded Skin

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Internalin B (InlB) is an invasion protein of *Listeria* which facilitates its uptake into host cells by activating the receptor tyrosine kinase MET. It was proposed that activation via receptor dimerization is mediated by an InlB dimer. The dimerized fragment of Internalin B, InlB321-CD¹ (crystal dimer), was designed to stabilize the InlB dimer in solution. In binding studies and in in vitro scatter assays¹, InlB321-CD revealed to be a stronger agonist than monomeric InlB321 and Internalin B.

In human skin, mainly epithelial cells express the MET receptor whose activation leads to proliferation, migration and vascularization. Its endogenous agonist hepatocyte growth factor (HGF/SF), which is secreted by e.g. dermal fibroblasts, plays an important role in the regeneration of the epidermis of the skin. That is why in preliminary studies, InlB321-CD was investigated with focus on its mitogenic and motogenic properties^{2,3} on human epidermal immortalized cellline (HaCaT).

The present study aims at InlB321-CD's influence on the up-regulation of the vascular endothelial growth factor (VEGFa) at RNA level, because HGF/SF stimulates vascularization via secretion of VEGF4. In vivo, particularly in chronic wounds, HGF/SF is degraded by proteases causing retarded vascularization.

Furthermore, InlB321-CD's effect on a mechanically wounded and differentiated epidermis model was analyzed in terms of its wound healing properties.

Methods:

A confluent HaCaT monolayer was serum-starved (24 h), then incubation with serum-free medium, 0.5 nM HGF, 0.5 nM InlB321-CD and 1 nM InlB321 took place for 6 h. Total RNA was extracted with Trizol[®] according to the manufacturer's guidelines. RNA concentration was quantified with an UV spectrometer. Prior to performing PCR with a pair of gene specific primers for VEGFa, first strand DNA synthesis was carried out. The PCR products were separated with an agarose gel electrophoresis stained with ethidium bromide and detected under UV light (260 nm).



HaCaT cells were cultivated on a polycarbonate membrane (3µm pore size) which was set on a dermis consisting of living fibroblasts incorporated in a collagen matrix⁵. After 3 weeks of co-culture, 3 days of serum-starvation was conducted. Then the membrane with the differentiated epidermis was removed. Subsequent to perforating the epidermis with a punch, cultivation on a dermis with either dead or living fibroblasts was conducted for further 5 days in serum-free medium with or without 0.5 nM InlB321-CD supplementation. An MTT assay was used to test for viability of the epidermis.

Results:

The VEGF gene expression at RNA level after incubation with 0.5 nM InlB321-CD was slightly higher compared with medium control, and comparable to 0.5 nM HGF/SF, which served as positive control. However, the equimolar dose of monomeric InlB321 did not increase VEGF gene on mRNA level.

Subsequent to incubation with 0.5 nM InlB321-CD, the epithelial model of wounded skin cultured on a dead dermis showed a higher relative cell viability compared to that treated with plain medium. In contrast to that, the differentiated keratinocytes co-cultured on a living dermis did not benefit the same way from 0.5 nM InlB321-CD supplementation versus medium. This might be due to fibroblasts' secretion of growth factors, i.e. HGF/SF that endogenously stimulates the mitogenic process in keratinocytes.

In conclusion, for proof of wound healing potential of InlB321-CD treatment of a wounded 3D skin model, the endogenous HGF/SF secretion of fibroblasts from the dermal layer underneath the epidermal layer has to be suppressed.

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Wissenschaftliche Posterausstellung: Poster 13

Toxicology and Pharmacokinetics of Novel Anti-Tumor Guanosine Phosphate Analogues - an In Silico Approach

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Keywords

human polymerase inhibitors, nucleoside analogs, non-melanoma skin cancer, predictive toxicology, alternative testing

Abstract

Drug candidates must prove both effectiveness and safety. A series of novel guanosine phosphate analogues for the treatment of non-melanoma skin cancer (NMSC) was recently identified by molecular modeling[1]. Efficacy in vitro has been proved in monolayer cultures of malignantly transformed human cells[2] and in organotypic NMSC models.

In this study we assess drug safety in silico by predicting key parameters such as acute and chronic toxicity, absorption and distribution[3,4]. We compared the entire series of novel guanosine phosphate analogues, especially the most promising drug candidates OxBu and OxHex, to a series of reference compounds: adefovir, tenofovir, aphidicolin, 5-fluoruracil, ingenol mebutate and δ aminolevulinic acid. Adefovir and tenofovir are close in structure to the test compounds, aphidicolin addresses the identical target. In NMSC therapy 5-fluoruracil is the gold standard, ingenol mebutate is the latest innovation and δ aminolevulinic acid is widely used.

Acute and chronic toxicity. OxBu and particularly OxHex are less hepatotoxic in man than 5-fluoruracil. Nephrotoxicity will probably occur and can be expected according to the effects of adefovir, tenofovir and high doses of aciclovir. OxBu, OxHex are predicted less toxic in the rat than adefovir, tenofovir and ingenol mebutate. LD50, LC50 and chronic LOAEL values predicted for OxBu and OxHex are close to each other but higher than for therapeutically used reference compounds.



Absorption and distribution. Human skin permeability is predicted to be equal for OxBu and 5-fluoruracil and higher for OxHex following the application of aqueous solutions. Applied orally, human intestinal absorption is low. Blood brain barrier permeation of OxBu and OxHex is predicted to be lower than of 5-fluoruracil or of ingenol mebutate in mice.

In conclusion, the application of in silico technologies may contribute to effective and safe drugs by reducing animal experiments in pre-clinical drug assessment. Although prediction power of in silico methods depends on the investigated parameter, novel guanosine phosphate analogues are predicted to have a desirable benefit-to-risk-ratio.

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