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Penetration of ASM 981 in canine skin: a comparative study

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SUMMARY

ASM 981 has been developed for topical treatment of inflammatory skin diseases. It specifically inhibits the production and release of pro-inflammatory cytokines. We measured the skin penetration of ASM 981 in canine skin and compared penetration in living and frozen skin.

To make penetration of ASM 981 visible in dog skin, tritium labelled ASM 981 was applied to a living dog and to defrosted skin of the same dog. Using qualitative autoradiography the radioactive molecules were detected in the lumen of the hair follicles until the infundibulum, around the superficial parts of the hair follicles and into a depth of the dermis of 200 to 500 μm. Activity could not be found in deeper parts of the hair follicles, the dermis or in the sebaceous glands.

Penetration of ASM 981 is low in canine skin and is only equally spread in the upper third of the dermis 24 hours after application. Penetration in frozen skin takes even longer than in living canine skin but shows the same distribution.

INTRODUCTION

Atopic dermatitis (AD) in dogs is a commonly occurring skin disease with many similarities to atopic dermatitis in man (1, 2). Although the prevalence is unknown, it is judged to be the second most common cause of canine pruritus, after flea allergy dermatitis (3). Topical glucocorticosteroids are often used as sole treatment or in addition to systemic therapy with glucocorticosteroids, cyclosporine or hyposensitization (4).

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However, in dogs systemic absorption and local skin atrophy may develop following topical steroid therapy (4, 5).

ASM 981 (pimecrolimus) has been developed for topical treatment of inflammatory skin diseases (6). In many reports on adults and children with AD it has been described that pimecrolimus significantly reduced flare ups of AD symptoms and induced improvement in life quality (7).

Several studies in human and animal models have shown, that pimecrolimus specifically inhibits the production and release of pro-inflammatory cytokines after antigen-specific or non-specific stimulation in T cells and mast cells (8-11). Topical applications of drugs are often preferred as application is easy and frequently the unwanted effects of systemic administration are reduced while the concentration of the drug at the disease site is maximized. In contrast to

topical steroids, ASM 981 does not induce the common significant potential skin atrophy (5).

ASM 981 has a high lipophility allowing it to have a high affinity to the skin (12). Studies in human and animal models showed that ASM 981 effects a low blood level concentration of ASM 981, even over long-term treatment periods (13). Therefore, ASM 981 has a low potential for affecting the systemic immune response when topically applied. Also the adverse effects, observed after application of ASM 981 in humans were highly favourable, compared with glucocorticoids (8, 14).

Thus an ideal topical drug for treatment of inflammatory skin diseases should be able to penetrate into the relevant layers of the skin with limited permeation.

Skin penetration studies are usually conducted in vitro using full thickness excised frozen and thawed skin, relying on the fact that the barrier function of skin resides in the outer "dead" layer, the stratum corneum, thus justifying extrapolation to in vivo conditions. However, for canine skin there are no data available.

For ethical reasons it is important to establish a model using frozen canine skin for skin penetration studies.

The aim of this study was firstly to measure skin penetration of ASM 981 in canine skin and secondly to investigate if drug penetration in frozen canine skin is comparable to skin penetration in vivo.

MATERIAL AND METHODS

Animal

A 7 year–old-male healthy beagle dog, from the kennel of Novartis Centre de Recherche, St-Aubin, Switzerland was used. An individual collar number and a subcutaneous tag identified him. During the trial the dog was single housed in a stainless steel cage and fed with a certified pelleted standard diet and with free access to tap water. This study was approved by the ethical committee of the canton of Fribourg, Switzerland.

Compound

Tritium labelled ASM 981 with a specific activity of 310 MBq and a purity of >98% was synthesized by the Isotope Lab of Novartis Pharma, Basel, Switzerland.

The final test article used was produced by Formulation of Novartis Animal Health by mixing the labelled ASM (0.05% w/w) with cold ASM 981 (0.95% w/w) and a Carbopolgel-formulation to get a 1% gel with a specific activity of 0.3 MBq/mg. The created formulation showed particle sizes in the range of 5-30 microns and a needle-like shape.

The hair was clipped in the dog's neck. At 24h, 4h, and 20 minutes prior to euthanasia 100 mg of the compound was applied to marked sites of 3 cm in diameter on the clipped neck. To mimic normal application of the compound by a dog owner the test sites were not protected with a bandage. The dog was further observed but was not seen licking, scratching or rubbing at the test sites.

The dog was sedated with medetomidine (60 μg/ i.m.) prior to euthanasia with intravenous injection of barbiturates (100 mg/kg).

Skin sampling

Immediately after euthanasia, the three treated skin sites were harvested without whipping away surplus cream and was glued with tissue taker (Richard–Allan Scientific Neg-50 Frozen Section Medium, Microm international GmbH, Walldorf, Germany) with the epidermal surface perpendicular to the supporters of the cryostat (Microm international GmbH, Walldorf, Germany). The tissue was indirectly frozen by a metallic connection in isopenthane (Merck, Darmstadt, Germany) cooled in liquid nitrogen (Carbagas, Bern-Liebefeld, Switzerland). The specimens were then put in small plastic bags and kept at -80°C until sectioning.

After euthanasia the hair of the dog's back was clipped, and the skin was cut into two pieces of 20x10 cm, rolled with the epidermal surface in side after removal of the subcutis and frozen at -80°C for 24 hours. After defrosting the skin, a piece of 4 cm in diameter was cut to fit into the incubation chamber (diameter: 3 cm), where 200 mg (approximately 60 MBq) of the test compound were weighed into. After 4 and 24 hours the incubation was stopped by processing the skin in the same manner as described above before euthanasia.

Pieces of frozen, untreated skin served as a negative control and were prepared in the same way as the compound-treated skin. Measurements of the compound applied while the dog was alive are further referred to as the in vivo situation and those on thawed skin as the in vitro test situation.

Sections of 8-12 μm thickness were cut with a cryomicrotome perpendicular to the epidermal surface and thaw mounted on microscope slides. After a drying period of 24 hours the slides were put in boxes with silica gel and frozen at -20°C. No fixation was done to prevent displacement of the diffusible ASM 981.

Eight to ten sequential sections of all time points of the in vivo and in vitro situation were assessed.

Autoradiography

Autoradiography was performed according to the method described by Stumpf (15). Briefly, slides were left overnight at room temperature to prevent formation of condensed water. In a dark room they were dipped individually in a K.5 Ilford Emulsion (Ilford imaging group, Marly, Switzerland) for 2-3 seconds and then dried overnight in a box with silica gel at room temperature. Slides were put in lightproof boxes with silica gel, sealed, and incubated at 4°C. After 4 days of exposure the slides were developed with Kodak D-19 (Eastman Kodak Company, Rochester, NY, USA), fixed with Kodak fixer (Eastman Kodak Company, Rochester, NY, USA), stained with methyl green pyronin (MGP) (Sigma, Steinheim, Germany), rinsed with tap water, dried at room temperature and covers were applied.

Image analysis

The localization of the radioactivity within cutaneous structures was demonstrated in a microscopical environment (magnifications: 100-400X) with an Olympus Bx61 microscope (Olympus GmbH, Hamburg, Germany). Colour micrographs were taken with a JVC KY-F70 digital camera (JVC, Wayne, USA) and the analySIS software.

To evaluate the depth of penetration we measured the distance perpendicular to the epidermis from the stratum corneum until the silver grain density was not more than background level.

RESULTS

The results and figures represent the penetration measured in at least eight sequential sections of skin of each time point of the in vivo and in vitro situation.

Control samples

The cutaneous layers were well preserved after cryosectioning of the control (untreated) skin in both in vivo and in vitro samples. Minimal non-specific labelling was present as an evenly distributed, background activity (Figure 1a).

In vivo treatment (living skin)

20 minutes

Skin penetration of ASM 981 after 20 minutes was only examined in vivo. The radioactivity, visible by the black silver grains, was confined to the surface of the treated skin. Radioactivity was not seen in the dermis. Scanning all the samples, we found some sites in several slides where there might be some activity in the stratum corneum. We did not observe any accumulation of silver grains below the stratum corneum, in the hair follicle lumina, dermis or in the sebaceous glands.

4 hours

After 4 hours, a penetration of ASM 981 from the stratum corneum into the dermis was seen. However, the density of silver grains was not evenly distributed over the epidermal layers.

A higher density was observed on the surface of the epidermis, i.e. where the test article has been applied. We observed silver grains in the infundibular lumen of the hair follicles and in the surrounding dermis. Silver grains in the dermis were visible up to 200 µm skin depth.

Deeper parts of the hair follicles did not exhibit radioactivity above background level.

24 hours

After a 24 h treatment period a penetration into the dermis up to a depth of 500 µm was observed. A high activity was seen on top of the epidermis, where the test article had been applied. In the infundibular lumen of the hair follicles and the surrounding dermis a higher grain density was found (Figure 1b).

No accumulation was seen in the stratum corneum. Again deeper parts of the dermis and hair follicles and sebaceous glands did not contain silver grains above background level.

In vitro treatment (frozen skin)

4 hours

After 4 hours treatment in vitro, ASM 981 did not penetrate deeper than into the epidermis, just above the stratum basale.

24 hours

After 24 hours incubation, in frozen skin the results were very similar to those described in the living skin. We observed an accumulation of silver grains on the surface of the epidermis, where the labelled ASM 981 had been applied (Figure 1c).

In all skin sections a penetration to a depth of approximately 200 µm into the dermis was observed. Silver grains were evenly distributed in the dermis over the total horizontal length of the skin sections. Furthermore radioactivity was seen in and around the infundibulum of the hair follicles.

Radioactivity was absent in deeper parts of the dermis and adnexal structures as earlier described.

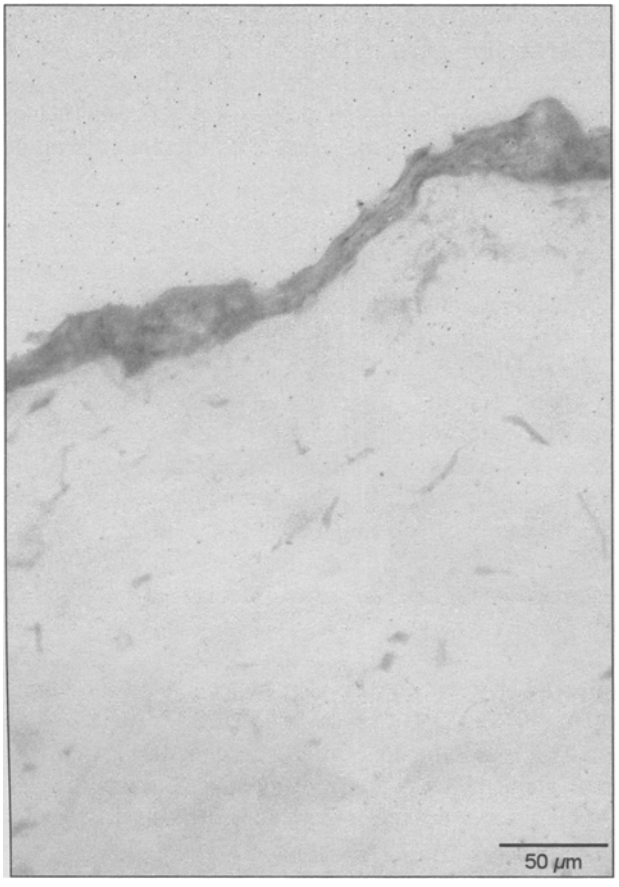

Fig. 1a: Negative control slide of untreated defrosted canine skin. Section was dipped in photo emulsion, developed after 4 days of exposure and stained with MGP. There is no evidence of radioactive labelling, except slight non-specific background labelling.

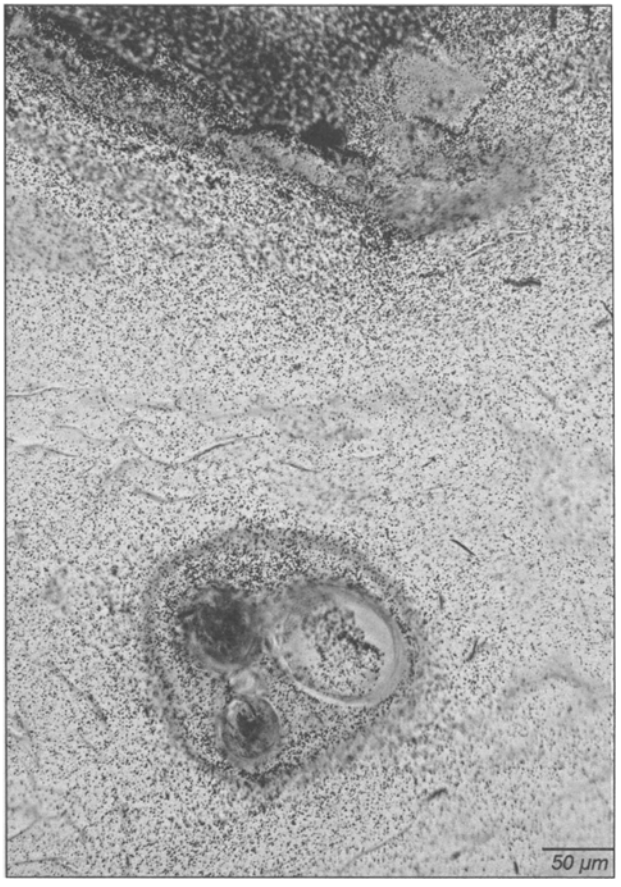

Fig. 1b: Surface area of an in vivo skin sample, incubated for 24 hours. Silver grains are present on the surface, in the epidermis, in and around the infundibulum of the hair follicle, surrounding dermis and superficial dermis.

DISCUSSION

Penetration of tritium-labelled ASM 981 was qualitatively studied following topical application onto multiple skin sites of a beagle dog after different incubation times.

Using this autoradiographic method we were able to show the localization of the molecules of the compound in the skin. In addition, this technique proved to be a technique with only little background noise due to a very low level of non-specific labelling.

The results showed that in canine skin the permeation of ASM 981 is very limited in the used formulation, i.e. without skin penetration enhancers. We found high activity on the external surface of the stratum corneum without accumulation in the stratum corneum or in the lower epidermal layers. Radioactivity was primarily visible in and around the infundibulum of the hair follicles. Although, ASM 981 is very lipophilic (7), we did not find accumulation of the compound in the sebaceous glands. An explanation for this finding may be that the ASM 981 did not penetrate deep enough into the hair follicle. In vitro skin penetration studies with ASM 981 have been performed in human, porcine and rat skin (16). These studies demonstrated that pimecrolimus in propylene glycol did not penetrate at al (16). However, applied with propylene glycol containing 10% oleyl alcohol, the concentration in the skin was within the same range as for steroids (16). Our canine data are comparable to the findings in humans. Canine skin however, differs from human skin because of much more hairs/cm^2 and differences in surface lipids (3). Hair follicles increase the skin surface area and may increase transappendageal drug transport (17). Additionally, the different composition of stratum corneum lipids could have influenced the permeation of ASM 981 in canine skin compared to human skin. Addition of penetration enhancers, accelerants or promotors may increase penetration of ASM 981 in canine skin.

We found a similar distribution pattern of penetration of the compound applied to "living" skin and "dead" skin.

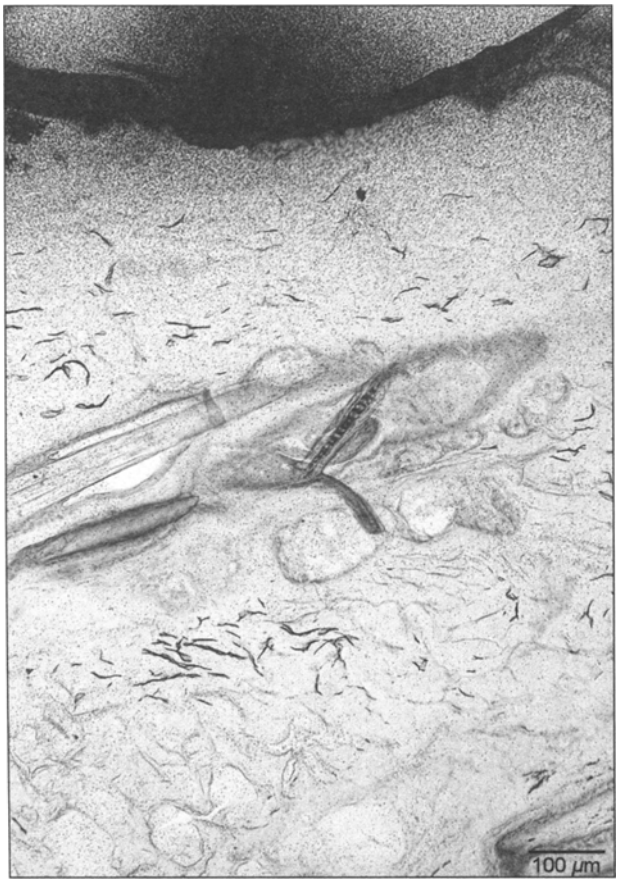

Fig. 1b: Surface area of an in vivo skin sample, incubated for 24 hours. Silver grains are present on the surface, in the epidermis, in and around the infundibulum of the hair follicle, surrounding dermis and superficial dermis.

Even though the results are not the same, as living skin allows a better penetration than the defrosted skin. These findings are comparable to reports in humans describing comparable drug permeation in frozen and fresh skin (18). Penetration in the living dog skin was slightly higher than in the defrosted one and after 24 hours approximately 10% of the applied test article was found at a depth of 250-300 µm.

One difference between the living skin and the defrosted skin is the temperature. The defrosted skin is not kept at canine body temperature. This may influence the permeation through the lipid rich stratum corneum and the lumen of the hair follicles.

Another difference between the frozen and the living skin is the missing blood stream. However, the measured activity is mainly in and around the hair follicles and the subepidermal layer of the dermis and not diffuse in the dermis. Therefore it is likely that blood stream has no influence on penetration. Additionally, blood flow may not have any influence because of the size and lipophilicity of the molecule.

Hence, frozen skin can be used for future skin penetration experiments in dogs to investigate potential penetration ability of a compound. These findings are important, as to our knowledge the comparison of in vivo to in vitro experiments have not been reported in dogs. For ethical reasons it is important to have a model validated using dead material. In addition, it may reduce the use of test animals.

In conclusion, the results show that ASM 981 has a low skin penetration in canine skin and demonstrate that "dead" skin can be used instead of "living" skin in penetration studies with ASM 981 in dogs. Furthermore, the best time to evaluate penetration is 24 hours after application.

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