Selegiline Percutaneous Absorption in Various Species and Metabolism by Human Skin

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Purpose. A Selegiline Transdermal System (STS) is under development for indications which may not be optimally or safely treated with oral selegiline. Studies were conducted to evaluate the *in vitro* penetration and skin metabolism of selegiline in order to better understand the toxicological findings and the observed plasma levels of selegiline and its metabolites in animals and man.

Methods. *In vitro* penetration studies were conducted in four different species (male hairless mice, male and female rats, female dog and male Micropig®) and compared to human skin. In another study, viable human skin was used to estimate the extent of metabolism of selegiline by human skin using Franz diffusion cells.

Results. Results indicated that female dog and male Micropig® skin were reasonable animal models for 24 hour *in vitro* selegiline penetration through human skin. Penetration of selegiline through rat skin and hairless mouse skin was 2-fold and 3-fold higher than through human skin, respectively. Metabolism was negligible in human skin. Selegiline metabolites (L-methamphetamine and N-desmethylselegiline but not L-amphetamine) were detected at all time points but the extent of selegiline metabolism was negligible. The cumulative 24 hour *in vitro* selegiline permeation from a 1.83 mg/cm^2 STS through human skin was 5.0 mg. This was similar to the *in vivo* permeation in humans as assessed by residual patch analysis.

Conclusions. The similarity of dog and human skin permeation results support the use of the dog as a species for evaluating the toxicology of transdermally-administered selegiline. Selegiline is not metabolized cutaneously and hence the metabolic profile following STS administration is likely due to hepatic metabolism only.

KEY WORDS: skin permeation; metabolism; selegiline; transdermal.

INTRODUCTION

Selegiline (SEL) is a selective inhibitor of MAO-B which is administered orally in the treatment of Parkinson's disease as an adjunct to L-DOPA. SEL undergoes extensive first-pass metabolism after oral administration which can be bypassed by transdermal delivery (1). The levels of selegiline after oral administration are highly variable (2). The recommended oral dose regimen of 5 mg bid has been defined on the basis of likely dose limiting toxicities, namely the potential for hypertensive crisis or 'cheese effect' and insomnia due to the selegiline's amphetamine metabolites. Non-MAO-B related pharmacology of selegiline suggests that the antagonistic actions of selegiline's amphetamine metabolites may be responsible for the lack of conclusive evidence for the neuroprotective effects of orally-administered selegiline HCl (3–7). Higher selegiline doses would be desirable in depressive states where the loss of central MAO-A selectivity (and expression of MAO-A inhibition) would be beneficial (3,8). These doses are currently precluded orally due to the potentiation of hemodynamic effects (also known as the 'cheese-effect'), which may be ultimately expressed clinically as hypertension, headache, palpitation, electrocardiographic abnormalities and arrhythmias (3,8). One rationale for the STS is that higher systemic selegiline levels may be attained at the expense of amphetamine metabolite formation without potentiation of tyramine sensitivity. A single administration of the STS has shown no increase in tyramine sensitivity at doses up to 200 mg of oral tyramine (9). The percutaneous absorption/penetration of most marketed transdermal systems (e.g. nitroglycerin and nicotine) has been established in animal and human skin. It is well known that skin has metabolic capabilities. Both phase I and phase II metabolism can take place in the skin (10). Phase I reactions such as oxidation (e.g. cortisol, norepinephrine), reduction (e.g. testosterone, progesterone), hydrolysis (e.g. glucocorticoids), and phase II reactions such as glucoronidation (e.g. benzopyrenes), sulfation (e.g. aminophenol), methylation (e.g. norepinepherine) and glutathione conjugation (e.g. styrene oxide) by the skin have been reported (10,11). Several enzymes such as NADPH-cytochrome c reductase and glutathione transferase are present in the skin. As shown in Figure 1, SEL is converted to N-desmethylselegiline (DES) and L-Methamphetamine (MET), and subsequently, both MET and DES are converted to L-amphetamine (AMP) (2,12). This conversion takes place in the gut as well as liver and leads to highly variable and low plasma levels of parent SEL after oral administration (2). These conversions are cytochrome P450-mediated (most likely CYP 2D6 and CYP 3A4) (2,13). Given the density of cytochrome P450 enzymes in human skin, it is possible that there is a dermal component to the metabolism of SEL after transdermal administration similar to topical testosterone (10).

The objectives of these studies were to evaluate the penetration of SEL through the skin of various species (i.e. hairless mice, rats, female dog, Micropig®) to determine the appropriate animal model for future pharmacokinetic and toxicokinetic studies with the STS, as well as to determine the extent of metabolism of SEL by viable human skin. The proposed duration of application of the STS is 24 hours, with the skin being the rate-limiting step for selegiline penetration since the patch contains no rate-controlling membrane or reservoir.

METHODS

Skin Penetration Studies

An *in vitro* study was conducted involving the application of the STS (1.83 mg/cm^2) to excised male hairless mouse, male and female rat, female dog, male Micropig® and human male abdominal skin preparations using Franz diffusion cells. Male

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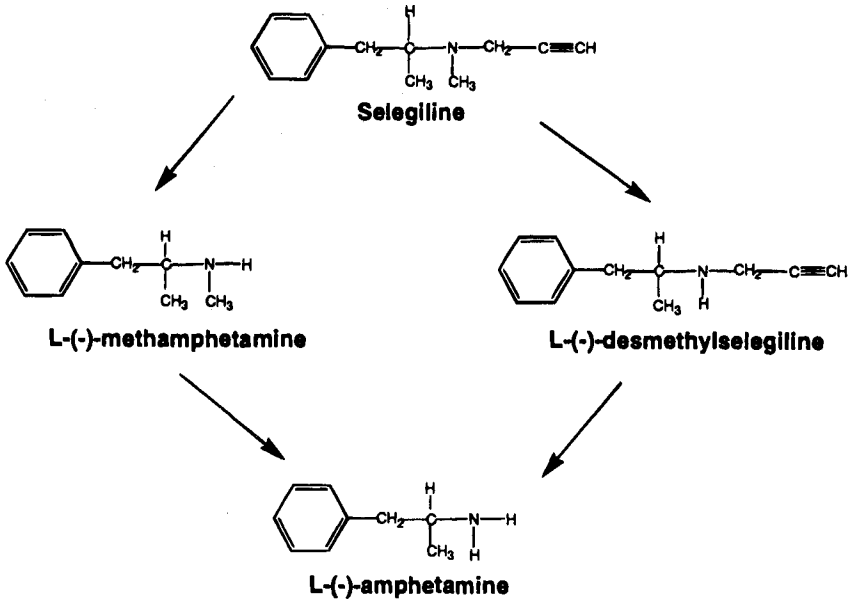

Fig. 1. Metabolic pathway of selegiline (2,12).

hairless HRS/J mice (7–8 week old) were obtained from Jackson Laboratory, Bar Harbor, ME. Male and female CRL CDBR VAF/+ rats (8–9 weeks) were obtained from Charles River Laboratories, Inc., Portage, MI. All animals were healthy and acclimatized before sacrifice to extract the skin. A sample of fresh skin from a 39 week old female beagle dog was obtained from Hazelton Wisconsin (Madison WI) and sample of fresh skin from a 17-week old male Yucatan Micropig® was obtained from Charles River Laboratories, Inc., Windham ME. Fresh samples of human abdominal skin were obtained from the International Institute for Advancement of Medicine (IIAM), Exton, PA. The dog (shaved skin), Micropig®, and human skin samples were shipped on wet ice and maintained in saline until initiation of the study.

Skin discs were prepared by modification of the method described by Kao *et al.* (14) for full-thickness excised skin preparations. The rats and mice were given an anesthetic overdose of carbon-dioxide, just prior to removal of skin. The fur of the rats were clipped from the dorsal trunk in the thoracic region. A piece of clipped skin was removed and placed on a petri dish. Skin samples from dog, Micropig® and man were processed in the same way. The fat and connective tissue were removed from skin pieces with a spatula. Discs (one inch in diameter) were cut from various skin samples. The thickness of the skin samples was also measured using a vernier caliper.

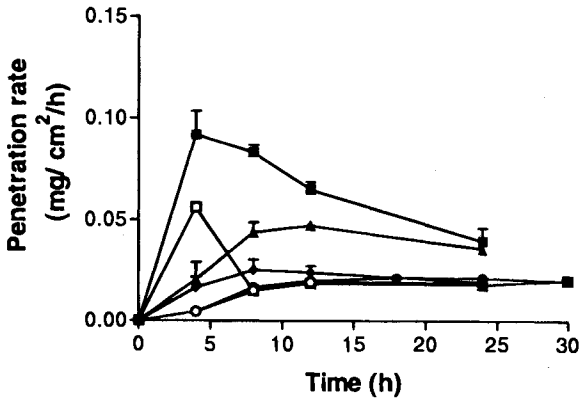

Fig. 2. Mean and SD rate of penetration of SEL through skins of male mouse (■), male rat (▲), female rat (♦), female dog (○), male micropig (●) and male human (□).

The Franz-type diffusion cells consisted of 10 mL flange diffusion cells mounted on a variable speed mounting console, which was set at 600 rpm. The cell itself was a water-jacketed, magnetically stirred chamber with a flat flange joint and cap for fastening the skin. The cell was filled with 0.9% sodium chloride solution (pH 7.0) and kept at 37°C. The excised skin was mounted such that the viscera was bathed in the fluid. In the case of human skin studies, the skin was chilled just before applying to the cell to improve the adhesion of the patch to the skin. In general, 3 skin samples of each type of species were used. The receptor medium was withdrawn at 4, 8, 12, 18 h for all the species except for Micropig® and human studies, in which 24 and 30 hour samples were also collected.

The amount of SEL that penetrated the skin was reported over the collection interval and described as cumulative amount/sq. area absorbed. The mean steady-state rate of penetration was calculated as the slope of the linear segment of the curve after plotting the cumulative amount/sq. area absorbed per interval versus time. The time to reach steady-state was variable but was usually about 8 h. The lag time between the application of dose and attainment of a steady-state rate of penetration was calculated by dividing the negative of y-intercept by the slope. With a zero (or positive) y-intercept, the lag-time is necessarily zero. Interspecies comparisons were performed using 2-tailed t-tests and a t-test adjusted for multiple comparison at a α-level of 0.05.

Penetration and Metabolism of Selegiline in Human Skin

Human skin samples were obtained within 24 h of surgery or death and stored at −70°C until initiation of study. The skin samples were from one caucasian male and two caucasian

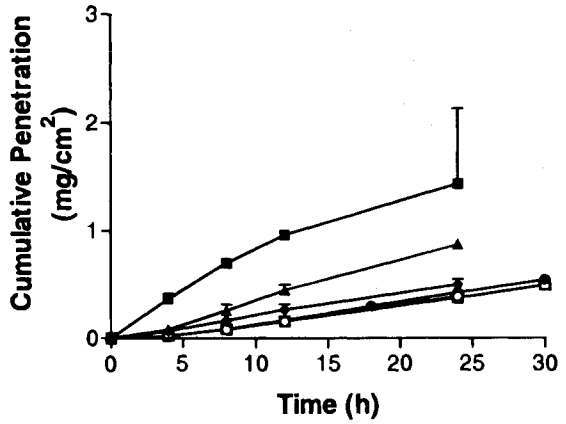

Fig. 3. Mean and SD cumulative SEL penetration vs. time plots for skins of male mouse (■), male rat (▲), female rat (♦), female dog (○), male micropig (●) and male human (□).

females between ages of 39–48 years and the thickness of the dermatomed skin was 0.2–0.6 mm. A total of six skin samples from each of the three individual donors were placed on the Franz diffusion cells with a 9 mm diameter circular skin exposure surface and approximately 4.8 mL receptor solution volume. The receptor (diffusate) solution consisted of HEPES-buffered Hank's balanced salt solution at pH 7.4 containing gentamicin sulfate (50 μg/mL). The apparatus was maintained at 37 ± 0.5°C by a constant temperature bath which circulated water through a jacket surrounding the receptor solution in the Franz diffusion chamber. The metabolic activity of the skin was tested prior and during the experiment by determination of lactate formed from glucose in the receptor solution (Sigma Diagnostics) (15). One of the samples from each donor did not receive the STS (1.0 mg/cm^2). The remaining 5 samples from each donor were exposed to the STS for the determination of SEL absorption and metabolism (four chambers in the Franz diffusion cell) and skin viability (one chamber). Four pieces of 8 mm in diameter were cut from each patch. Based on a patch concentration of 1.0 mg/cm^2 and diameter of 8 mm the loading dose was 0.5 mg. One of the pieces was placed on the skin and two were placed on a release liner at room temperature to account for any atmospheric losses. The fourth piece was designated as an assay control. The diffusate receptor solution was collected at 0.5, 1, 2, 4, 8, and 24 h after test article administration and replaced with fresh solution at each time point. At each time point, the concentration of SEL and metabolites were determined in single aliquots of receptor solution from the control and four chambers receiving the test article. Aliquots from the fifth chamber were exposed to assay lactate to determine the metabolic activity of the skin. After 24 hours, non-absorbed test articles were rinsed 3 times with approximately 1 mL of 0.9% saline from the skin surface and washings were stored for further analysis. After rinsing, the skin was cut into small pieces and homogenized in a buffered saline solution (0.9% NaCl with 50 mM Tris-HCl, pH 7.4). The homogenate was filtered through 0.45 micron nylon filter. The barrier integrity of each skin sample was confirmed by measuring the permeability of 1 μCi of tritiated water just prior to administration of the test article. Excess tritiated water was removed just before application of the patch and did not contaminate the receptor solution. Twenty-four hour cumulative amounts of SEL were calculated as the summation of incremental amounts of SEL over each time interval calculated by multiplying concentrations at different time points by the respective volumes of the receptor solution. The extent of metabolism was calculated as the percent of metabolite conversion relative to the amount of SEL in the receptor solution.

In Vitro Metabolism of Selegiline in Static Situation

Human skin samples were cut from the same pieces used in the percutaneous absorption/metabolism experiment and stored at −70°C until homogenization. Stock solutions of selegiline HCl were prepared in methanol: 20 mg/mL and 150 μg/mL. The latter solution served as a 500 × stock solution for the metabolism assay (final concentration in the reaction was 300 ng/mL). A stock solution (100 mM) of NADPH (Sigma) was prepared in water and the final concentration of NADPH in the assay tubes was 0.5 mM. Vials containing the skin pieces were transferred to an ice bucket, and 3.5 mL of Tris buffered saline (0.9% NaCl, 50 mM Tris-HCl, pH 7.4) was added to each vial. The skin pieces were cut and homogenized in this solution using a Polytron homogenizer and placed on wet ice. While on ice, the homogenates were supplemented with NADPH cofactor and SEL, swirled and transferred to room temperature (≈23°C). Immediately following the addition of SEL, aliquots of 1.0 mL were removed and 1 mL of methanol added to stop the reaction. The expected SEL concentration was 150 ng/mL. The same procedure was repeated at 30 and 120 minutes after selegiline addition. Tubes were kept at 0–4°C until analysis.

Bioanalytical

HPLC Assay for Penetration Studies

A Helwett-Packard HPLC and a variable detector (wavelength 257 nm), equipped with a Helwett-Packard 3395 Integrator and Phenomenex Bondclone 10 C-18, 10 μm (3.9 × 300 mm) column was used for analysis of diffusate fluid. The flow rate of mobile phase (60:40 of 50 nM sodium acetate buffer, pH 5: acetonitrile) was 1.0 mL/minute. An aliquot (2.5 mL) of each sample was transferred into the HPLC with 100 μL of each sample being injected onto the column for analysis. There was no interference from the matrix and selegiline yielded a single peak at approximately 8 minutes. The assay was linear from 0.5 to 250 μg/mL. The LLOQ for SEL was 0.5 μg/mL and the correlation coefficient for the regression of the calibration curve was 0.998 or better.

LC/MS/MS Assay for Metabolism Studies

An LC/MS/MS method was used to detect SEL, DES, MET and AMP in diffusate solution, skin extracts and washings. HPLC was performed with a Perkin-Elmer ISS-200 autosampler and LC-200 pump. The column was a Supelcosil LC-CN (4.6 × 33 mm, 3 μm particle size). The mobile phase consisted of 86:12:2 (V:V:V) methanol : 0.1% trifluoroacetic acid : 2.5 mM ammonium acetate. The flow rate was 1.2 mL/min. Injections volumes were either 3 or 10 μl depending on sensitivity requirements. A PE-Sciex API III^{plus} was used for detection. Standards were prepared in control receptor solution in order to neutralize

Table 1. Summary of Mean Selegiline Characteristics in Various Species (n = 3)

Species	Sex	Skin thickness (mm)	Mean steady state rate of penetration (mg/cm²/h)	Mean lag time (h)	Cumulative 24h amount penetrated (mg/cm²)
hairless mouse	male	0.64–0.67	0.0874	0.0	1.43 ± 0.07
rat	male	0.83–0.89	0.0405	2.33	0.87 ± 0.04
rat	female	0.76–1.04	0.0247	1.29	0.49 ± 0.05
dog	female	1.27	0.0189	3.87	0.38 ± 0.01
Micropig®	male	2.39	0.0204	3.61	0.42 ± 0.02
human	male	1.72	0.0184	3.58	0.37 ± 0.04

matrix effects resulting from the components of the solution. The limit of detection for SEL and metabolites was 1 ng/mL.

RESULTS

Skin Penetration Studies

The summary of selegiline skin penetration characteristics across various species are reported in Table 1. The highest mean steady state penetration rate was noted in hairless mice, which also possessed the thinnest skin of all species tested. The second highest mean steady state rate of penetration was noted with rats, which had the second thinnest skin. The human, dog and Micropig® skin ranged from 0.6–2.4 mm, and did not follow any trend with regards to SEL penetration. Table 1 also reports the summary of steady state rates of penetration, cumulative 24 hour penetration values and lag-times (time to achieve steady state rates of penetration). Male hairless mouse had the shortest lag-time, achieving steady-state rates almost immediately after application of the patch. Female rat skin had a time lag of 1.3 h, followed by male rat skin of 2.3 h. Lag times of the remaining species were higher and similar, ranging from 3.6–3.9 h. There were significant differences between male rat and female dog skin, between male rat and male Micropig® skin, male and female rat skin and between male rat and female dog skin. Based on cumulative 24 h penetration data, the female dog and male Micropig® skin were both reasonable models for *in vitro* penetration of selegiline through human male abdominal skin.

Penetration and Metabolism of Selegiline in Human Skin

Table 2 provides the amount of SEL and metabolites in the diffusate fluid as a function of time. The cumulative 24 h amount of selegiline permeated, scaled to the full size 20 cm² STS, varied from 1.5 to 2.0 mg. MET was the major metabolite and no AMP was detected. DES was 0.04% and MET was 0.2% of the parent drug. The total amount of metabolites was less than 1% of SEL suggesting that skin metabolism of SEL was negligible. Negligible quantities of SEL (less than 10% of those found in the diffusate solution) were found in the skin washings and skin homogenates as well.

In Vitro Validation of Metabolism of Selegiline in Static Situation

Selegiline was detected at all time points. SEL concentrations of approximately 130 ng/mL were observed for all time points, which was slightly less than the expected value of 150 ng/mL. No detectable metabolites were observed in two sets of skin samples for any time point. In one skin sample, less than 2% of MET was detected.

DISCUSSION

Selegiline is a weak base with a pK_a of 7.5, a low molecular weight of 187.3 and calculated partition coefficient (octanol/water) of 3.4 (16). These physicochemical characteristics along with the low loading dose make it an excellent candidate for transdermal delivery. After STS administration higher systemic selegiline levels are attained at expense of the amphetamine metabolite formation. This may offer an opportunity to study the neuroprotective effects of parent selegiline. While plasma levels following STS administration have been safe and well-tolerated in both healthy volunteers and patients, additional studies are required to substantiate the lack of tyramine sensitivity observed in the single dose study (3).

Table 2. Mean ± SD Selegiline and Metabolite Diffusate Concentration for Each Time Point

Time (h)	Analyte Concentration (ng/mL)			
	SEL	DES	MET	AMP
0.5	70.1 ± 66.4	0.0	0.0	0.0
1	155.3 ± 110.4	0.0	0.0	0.0
2	468.3 ± 302.3	0.0	0.5 ± 1.2	0.0
4	834.0 ± 449.0	0.0	1.8 ± 1.8	0.0
8	1845.0 ± 672.8	0.0	4.6 ± 1.7	0.0
24	5676.5 ± 1784.1	3.1 ± 0.7	14.1 ± 4.2	0.0
Cumulative 24 h amount (ng)	43374.1 ± 14710.5	15.0 ± 3.6	100.4 ± 38.7	0.0

While oral selegiline hydrochloride enjoys excellent safety profile, the STS has been evaluated in 3 month toxicity trial in dog (17). Based on toxicity comparisons, of margin of safety and comparative exposure of STS, to the historical oral safety data base, STS clinical trials have been initiated (17,18). Local toxicities have been evaluated in several dermal irritation studies (17).

The present studies were conducted to confirm the skin permeation of selegiline in various species. This would facilitate the evaluation of animal models for screening new formulations. Further, the of skin permeation of selegiline from toxicology studies in dogs and phase I trials in man could be compared to STS residual analysis. Both female dog and male Micropig® skin were reasonable models for *in vitro* 24-hour SEL penetration through skin. Penetration of SEL through rat skin and hairless mouse skin were 2-fold and 3-fold higher than through human skin, respectively. This was consistent with earlier observations that rodents overestimate permeation relative to humans (19–23). This may be partly associated with the effects of hydration, which reduces the barrier properties of rodent skin. The primary difference between rodent and human skin is the lipid composition and organization in stratum corneum (19–23). The high parent selegiline plasma concentrations achieved in rodents relative to man could be attributed partially to the greater transdermal penetration, but may also be due to species differences in metabolism. As selegiline metabolism has been shown to be cytochrome P-450 mediated, gender differences in the rat may be due to CYP 3A2 expression which has been implicated as an isozyme which metabolizes selegiline and is known to be expressed differently in male and female rats. Yoshida et al have previously documented these gender differences in the rat after oral administration of selegiline HCl (12). While, gender-related differences in penetration through rat skin may have a metabolic contribution, the metabolic viability and presence of metabolites were not investigated in these experiments making it difficult to speculate on other factors based on this data.

The calculated partition coefficient for selegiline is 3.4 (16). Given the lipophilicity of the selegiline base and lack of charge effects, the permeability of SEL through various skin types is likely to be driven by tortuousity of pathway through the skin and any potential skin metabolism. Differences in the lag times may also be due to formulation effects as well as species differences in biochemical properties, structural differences, epidermal and dermal characteristics, as well as differences in cutaneous appendages (i.e. hair follicles). While there are extensive and potentially diverse differences in each species' skin type, comparison of cumulative selegiline penetration has determined the suitable species to mimic human skin. These data have further permitted the correlation of relevant animal penetration data to human results, substantiating toxicokinetic findings with the STS.

The salient feature of this study was that the metabolism of the SEL was determined in a dynamic situation (i.e. while the drug was penetrating through the intact skin). The sensitivity provided by the LC/MS/MS method facilitated the estimation of metabolites without the use of radioactive material. Metabolism was negligible (i.e. less than 1%) through intact viable human skin. The lack of metabolism by human skin reduces another source of variability and may prove advantageous over oral administration, which suffers from a large first pass effect as stated earlier. Given the reduction in first-pass metabolism with both nitoglycerine and selegiline after transdermal delivery and the fact that both molecules are metabolized by cytochrome P450 isozymes, the lack of metabolism of SEL by human skin is in contrast to the high degree of cutaneous metabolism of nitroglycerine (49% by hairless mouse skin) (23). Hence, the variability in selegiline plasma levels following transdermal administration can not be explained by cutaneous metabolism unlike nitroglycerine.

The *in vivo* permeation in humans has been assessed in an earlier pharmacokinetic study in which a single STS was administered to six healthy male volunteers for 5 days with replacement every 24 hours (17). In addition *in vivo* permeation in rats and dogs was determined from two separate toxicokinetic studies in rats and dogs where single patches were administered for two and 5 days, with replacement every 24 h (17). The cumulative *in vitro* 24 hour permeation of SEL through human skin was 5.0 mg. This value was consistent with the *in vivo* permeation in humans, rats, and dogs of 7.0, 7.5 and 6.5 mg, respectively as determined by residual patch analysis (17,18). The slightly higher *in vivo* permeation was probably due to the higher clearance of drug available *in vivo* (19–23). The steady state SEL plasma levels achieved were 3.4, 124, and 3.9 ng/mL in healthy young humans, rats, and dogs respectively. *In vivo* rat plasma concentrations were higher and plasma concentrations in dogs were similar to that in man, suggesting the appropriateness of the dog as an animal model. Thus, *in vitro* penetration experiments were reasonable predictors of *in vivo* results and may help in optimizing the dose and selection of the final STS formulation.

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