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Skin permeation and metabolism of a new antipsoriatic vitamin D_3 analogue of structure 16-en-22-oxa-24-carboalkoxide with low calcemic effect

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Abstract

A new vitamin D_3 analogue, SMD-429, is an antipsoriatic candidate of structure 16-en-22-oxa-24-carboalkoxide exhibiting fewer side effects than known vitamin D_3 analogues. In this study, the permeation of SMD-429 through excised rat skin and three-dimensional cultured human skin model (LSE-high) was evaluated. The cumulative amount of SMD-429 permeated through the skin membranes was lower than that of either maxacalcitol or calcipotriol, whereas the amount of SMD-429 in the skin was the same. It was found from in vitro rat skin permeation experiment using [^{3}H]SMD-429 that SMD-429 was permeated through skin mainly in its metabolized form. The skin permeation profiles of vitamin D_3 analogues obtained were analyzed based on a one-layer diffusion model to estimate permeation parameters. The apparent diffusion coefficient of SMD-429 was 1.30×10^{-5} cm^2/h, which was 10-fold lower than that of maxacalcitol. The apparent metabolic rate constant of SMD-429 in skin was 1.01×10^{-1} h^{-1}, the same as maxacalcitol. Low apparent diffusivity of SMD-429 in skin might cause an increase in the probability of bioconversion. The same amount of SMD-429 in skin as known vitamin D_3 analogues would achieve sufficient therapeutic efficacy in skin. Such low skin permeability and high metabolic conversion in skin of SMD-429 would contribute to a reduction in the systemic side effects.

Keywords: Vitamin D_3 analogue; Skin permeation; Skin metabolism; Diffusion model; Lipophilicity

1. Introduction

1α,25-Dihydroxyvitamin D_3 analogues such as tacalcitol (Kato et al., 1986), maxacalcitol (Barker et al., 1999) and calcipotriol (Kragballe, 1995) are used for topical psoriasis treatment. The efficacy of these drugs is considered to be due to the induction of cell differentiation and the inhibition of keratinocyte proliferation (Takahashi et al., 2003). The skin is the target organ of vitamin D_3 analogues in psoriasis treatment. Therefore, maintaining a high drug concentration in skin is especially important for therapeutic efficacy. However, vitamin D_3 analogues permeated through skin into the systemic circulation would cause an undesirable side effect, hypercalcemia (Brown et al., 1989; Mortensen et al., 1996).

In order to separate the efficacy from the side effects of vitamin D_3, Shimizu et al. (2006) have designed and evaluated new vitamin D_3 analogues having 16-en-22-oxa-24-carboalkoxide structures, which show greater anti-proliferation potency and lower calcemic effect after percutaneous administration than maxacalcitol. These analogues have shown a greater metabolic clearance in liver microsomes and a greater clearance in rats as compared with maxacalcitol. The high clearance from the systemic circulation is likely one of the reasons for the reduction in side effects. Decrease in skin permeability may also effect a reduction in side effects because vitamin D_3 analogues permeated through skin into the systemic circulation would cause the calcemic action; however, skin permeation profiles of these analogues have not been clarified and the contribution of skin permeability to the reduction in side effects is not clear.

In the present study, in vitro skin permeation and metabolism of a new vitamin D_3 analogue, SMD-429, designed by Shimizu et al. (2006) was evaluated and compared with known vitamin D_3 analogues, maxacalcitol and calcipotriol, using excised rat skin and a three-dimensional cultured human skin model. Skin permeation profiles of these vitamin D_3 analogues were analyzed based on a diffusion model and the characteristics of each

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compound were clarified. Moreover, the contribution of skin permeability in the reduction of the calcemic effect of the new vitamin D_3 analogue, SMD-429, was discussed.

2. Materials and methods

2.1. Materials

A new vitamin D_3 analogue, SMD-429 [(*S*)-1-{(*S*)-4-[2-[(3*S*,5*R*)-3,5-dihydroxy-2-methylene-cyclohex-(*Z*)-ylidene]-eth-(*E*)-ylidene]-7*a*-methyl-3*a*,4,5,6,7,7*a*-hexahydro-3*H*-inden-1-yl}-ethoxy-acetic acid 1-ethyl-1-methyl-propyl ester], maxacalcitol and calcipotriol were synthesized by Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan). M1 [(*S*)-1-{(*S*)-4-[2-[(3*S*,5*R*)-3,5-dihydroxy-2-methylene-cyclohex-(*Z*)-ylidene]-eth-(*E*)-ylidene]-7*a*-methyl-3*a*,4,5,6,7,7*a*-hexahydro-3*H*-inden-1-yl}-ethoxy-acetic acid] and M2 [1α,3β-dihydroxy-20(*S*)-hydroxy-9,10-secopregna-5,7,10(19),16-tetraene] were also synthesized by Chugai Pharmaceutical. [^{3}H]SMD-429 (specific radioactivity: 629 GBq/mmol) was purchased from GE Healthcare Bio-Sciences Co. (NJ, USA). Chemical structures of these compounds are shown in Fig. 1. Fetal bovine serum was purchased from MP Biomedicals (Irvine, CA, USA). Medium chain triglyceride (MCT) was purchased from the Nisshin OilliO Group, Ltd. (Tokyo, Japan). White petrolatum was purchased from Kozakai Pharmaceutical Co., Ltd. (Tokyo, Japan). A three-dimensional cultured human skin model (LSE-high) was purchased from TOYOBO Co., Ltd. (Osaka, Japan).

2.2. Ointment preparation

An ointment containing 0.02% or 0.05% (w/w) drug (maxacalcitol, calcipotriol or SMD-429) and 3% (w/w) MCT was prepared using the following procedure. To a tube containing 60 mg of MCT was added 400 or 1000 μg of a test drug as an ethanol solution and the ethanol was evaporated under a nitrogen stream. To the MCT solution of drug, 1940 mg of white petrolatum was added. The tube was heated to 60 °C to melt the petrolatum, mixed to form an ointment, and stored at room temperature before use. The ointment containing 0.02% (w/w) drug was used for the LSE-high permeation experiments and the ointment containing 0.05% (w/w) drug was used for the rat skin permeation experiments. [^{3}H]SMD-429 ointment containing 0.05% (w/w) SMD-429 and 3% (w/w) MCT was also prepared according to the method described above.

2.3. Rat skin membrane preparation

The care of the animals and the present protocols complied with the "General Consideration for Animal Experiments" and were approved by the Ethics Committee for Treatment of Laboratory Animals at Chugai Pharmaceuticals. Male rats (Sprague–Dawley strain, 7–8 weeks old; CLEA Japan Inc., Tokyo, Japan) were used for the experiments. Rats were killed under ether anesthesia, the dorsal region of the skin was carefully shaved and excised, and the subcutaneous tissue removed using scissors.

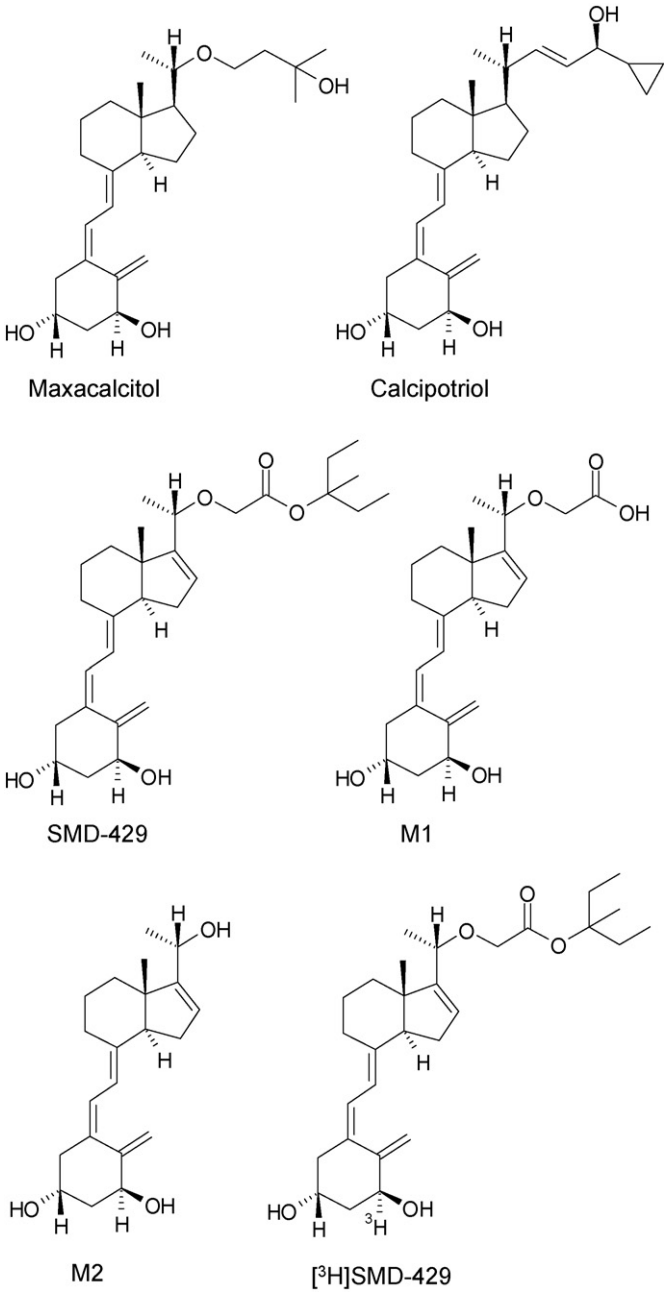

Fig. 1. Chemical structures of vitamin D_3 analogues. M1 and M2 are metabolites of SMD-429.

2.4. In vitro skin permeation experiment

2.4.1. Rat skin permeation experiment

We previously reported on in vitro rat skin permeation of maxacalcitol. In the present study, in vitro rat skin permeation experiments of calcipotriol and SMD-429 were carried out using the same method as reported (Yamaguchi et al., 2006). Franz-type diffusion cells having an effective diffusion area of 4.9 cm^2 and a receptor cell volume of 15 ml were used. The receptor cell, facing the dermis side, was filled with phosphate-buffered

saline (pH 7.4) containing 20% (v/v) fetal bovine serum. During the experiment, the receptor fluid was maintained at 37 °C and 24 mg (volume: 0.028 cm^3) of the ointment containing 0.05% (w/w) drug was applied to the skin surface from the donor side after 1 h pre-incubation of the skin in a receptor fluid. At 1, 4, 8, 20 or 24 h after application, the ointment was wiped off with cotton swabs, and the receptor fluid and the rat skin were collected. The ointment samples were stored at 4 °C; the receptor fluid and skin samples were stored in a freezer at −80 °C until sample preparation. Each experiment was carried out in triplicate ($n = 3$).

In vitro rat skin permeation experiments for [^{3}H]SMD-429 were carried out in order to evaluate the skin metabolism. The experimental conditions were the same as described above.

2.4.2. LSE-high permeation experiment

The LSE-high permeation experiments for maxacalcitol, calcipotriol and SMD-429 were carried out using the following method. Netwell™ six-well plates (diameter: 24 mm, mesh size: 500 μm; Corning, MA, USA) were used for the skin permeation experiments (van de Sandt et al., 2000). Each well was filled with 1.5 ml of phosphate-buffered saline (pH 7.4) containing 20% (v/v) fetal bovine serum as the receptor fluid. The LSE-high was set on a Netwell™ inserter in contact with the dermis side and the receptor fluid, and a silicone ring (inner area: 0.785 cm^2) was glued to the LSE-high surface. The Netwell™ plate was then moved to a CO_2 incubator (37 °C). After 1 h incubation, 20 mg (volume: 0.024 cm^3) of the ointment containing 0.02% (w/w) drug was applied to the LSE-high surface inside the ring. The receptor fluid was collected at a volume of 0.5 ml at 0.5, 1, 3, 5, 7, 20 and 24 h after application; 0.5 ml of fresh receptor fluid was added after each collection to keep the receptor volume constant. Each experiment was carried out in triplicate ($n = 3$). The receptor fluid samples were stored in a freezer at −20 °C until sample preparation.

2.5. Measurement of vitamin D_3 analogue in permeation experiment samples

2.5.1. Ointment

The ointment sample was mixed with 15 ml of *n*-hexane, then the vitamin D_3 analogues were extracted with 15 ml of acetonitrile. The acetonitrile layer was evaporated under a nitrogen stream. The extract was dissolved in 250 μl of *n*-hexane/*iso*-propanol/methanol (135:8:2, v/v/v) as the sample for HPLC. The HPLC apparatus consisted of a pump (LC-10AD_{VP}; Shimadzu Co., Kyoto, Japan), a UV detector (SPD-10A_{VP}; Shimadzu Co.) and an analytical column (YMC-Pack SIL A-004, 4.6 mm × 300 mm; YMC Co., Ltd., Kyoto, Japan). The mobile phase was *n*-hexane/*iso*-propanol/methanol (135:8:2, v/v/v) at a flow rate of 2.0 ml/min. The wavelength of the UV detector was set at 265 nm. HPLC samples were injected at a volume of 20 μl.

2.5.2. Skin

The skin sample was homogenized in 70 ml of 70% (v/v) methanol. The supernatant of the homogenate was recovered, the extraction procedure was done once again and the total volume of the skin extract was measured. As an internal standard, 20 μl of 400 ng/ml calcitriol was added to part of the skin extract and evaporated under a nitrogen stream; 0.5 ml of water and 3 ml of diethyl ether were added to the residue and the sample was mixed. The organic phase was evaporated under a nitrogen stream. The extract was reconstituted with 50 μl of methanol/10 mM ammonium acetate (85:15, v/v) to obtain the sample for LC–MS/MS. LC–MS/MS analysis was carried out by coupling an HP1090 liquid chromatograph system (Hewlett Packard, Palo Alto, CA, USA) to an API300 mass spectrometer (Applied Biosystems/MDS SCIEX, Concord, Ont., Canada). A CAPCELL Pack C_{18} column (UG120, 2.0 mm × 150 mm; Shiseido Co., Ltd., Tokyo, Japan) was used as the analytical column. The mobile phase was methanol/10 mM ammonium acetate (85:15, v/v) at a flow rate of 0.2 ml/min. The HPLC eluent was introduced into the source using a TurboIonSpray® interface and the mass spectrometer was operated in positive ion mode. Selected ions were *m/z* 377.3 (daughter ion of 430.3) for calcipotriol, *m/z* 313.3 (daughter ion of 490.3) for SMD-429 and *m/z* 363.3 (daughter ion of 434.3) for calcitriol.

2.5.3. Receptor fluid

The receptor fluid sample (50–400 μl) was mixed with 3 ml of acetonitrile and 20 μl of 400 ng/ml calcitriol, then the mixture was centrifuged (3000 rpm, 10 min, 4 °C). The supernatant was collected and evaporated. Water (0.5 ml) and diethyl ether (3 ml) were added to the residue and then mixed. The organic phase was recovered and evaporated, and the extract was dissolved in 50 μl of methanol/10 mM ammonium acetate (85:15, v/v) to obtain the samples for LC–MS/MS. The drug concentration in the receptor fluid was measured using the same LC–MS/MS system as described above. Selected ions were *m/z* 297.3 (daughter ion of 436.3) for maxacalcitol, *m/z* 377.3 (daughter ion of 430.3) for calcipotriol, *m/z* 313.3 (daughter ion of 490.3) for SMD-429, and *m/z* 363.3 (daughter ion of 434.3) for calcitriol.

2.5.4. Receptor fluid in the experiment using [^{3}H]SMD-429 ointment

The receptor fluid sample (2 ml) was mixed with 4 ml of acetonitrile. The mixture was centrifuged (3000 rpm, 5 min, 4 °C) and the collected supernatant was evaporated under a nitrogen stream. The residue was dissolved in acetonitrile/10 mM ammonium acetate adjusted to pH 3.5 with acetic acid (35:65, v/v) and measured by HPLC. The HPLC apparatus consisted of a pump (LC-10AD_{VP}; Shimadzu Co.), a UV detector (SPD-10A_{VP}; Shimadzu Co.), a flow-through scintillation counter (FLO-ONE; Packard Instrument Co., Meriden, CT, USA) and an analytical column (CAPCELL PAK C_{18}, UG120, 4.6 mm × 250 mm, 5 μm; Shiseido Co., Ltd.). A binary gradient was used; mobile phase A was acetonitrile/10 mM ammonium acetate adjusted to pH 3.5 with acetic acid (35:65, v/v) and mobile phase B was acetonitrile/10 mM ammonium acetate adjusted to pH 3.5 with acetic acid (80:20, v/v). The gradient at a flow rate of 1.0 ml was as follows: 0–12 min, isocratic at 100% A; 12–25 min, linear from 0 to 100% B; 25–40 min, isocratic at 100% B. Identification of SMD-429 and the metabolites was based on the comparison of retention times with authentic standards.

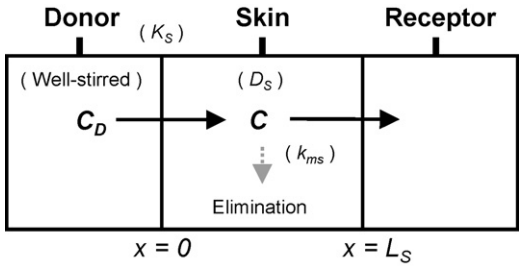

Fig. 2. Schematic representation of the diffusion model for rat skin permeation of a drug. K_S, D_S and k_{ms} are, respectively, the partition coefficient from donor to skin, the diffusion coefficient in skin and the metabolic rate constant in skin. L_S is the skin thickness. The positions at $x=0$ and $x=L_S$ are the interface of donor/skin and skin/receptor fluid, respectively.

2.6. Analysis of skin permeation of vitamin D_3 analogues based on a diffusion model

Fig. 2 shows the schematic representation of a diffusion model expressing drug permeation through skin. Assuming that (1) the behavior of a drug in skin obeys Fick's law of diffusion, (2) a drug is metabolized in skin at a rate of the first order kinetics and (3) a sink condition in the receptor fluid, the following partial differential equation is derived:

$$\frac{\partial C(x,t)}{\partial t} = D_S \frac{\partial^2 C(x,t)}{\partial x^2} - k_{ms} C(x,t) \quad (1)$$

where $C(x, t)$ (μg/cm^3) is the drug concentration in skin at time t at position x, D_S (cm^2/h) is the diffusion coefficient in skin and k_{ms} (h^{-1}) is the first order metabolic rate constant in skin. The initial conditions are expressed as the following equations.

$$C_D = \frac{\text{Dose}}{V_D}$$

$$C(x,0) = 0 \quad (0 \le x \le L_S)$$

where Dose (μg) is the drug amount in vehicle, V_D (cm^3) is the vehicle volume, C_D (μg/cm^3) is the drug concentration in vehicle, L_S (cm) is the thickness of skin and positions $x=0$ and $x=L_S$ correspond to the interface of donor/skin and skin/receptor fluid, respectively. The boundary conditions are expressed as the following equations.

$$C(x,t) = K_S C_D \quad (x=0) \quad (2)$$

$$V_D \frac{\partial C_D}{\partial t} = D_S \text{Area} \frac{\partial C(x,t)}{\partial x} \quad (x=0) \quad (3)$$

$$C(x,t) = 0 \quad (x = L_S)$$

where K_S is the skin/donor partition coefficient and Area (cm^2) is the effective diffusion area. The following boundary condition is obtained by combining Eqs. (2) and (3).

$$\frac{\partial C(x,t)}{\partial t} = \frac{K_S D_S \text{Area}}{V_D} \frac{\partial C(x,t)}{\partial x} \quad (x=0) \quad (4)$$

To obtain dimensionless equations, $y = x/L_S$ is used, then Eqs. (1) and (4) are transformed to the following equations.

$$\frac{\partial C'(y,t)}{\partial t} = \frac{K'_S D'_S \text{Area}}{V_D} \frac{\partial C'(y,t)}{\partial y} \quad (y=0)$$

$$\frac{\partial C'(y,t)}{\partial t} = D'_S \frac{\partial^2 C'(y,t)}{\partial y^2} - k_{ms} C'(y,t) \quad (0 < y < 1)$$

where $C'(y, t)$, K'_S and D'_S are equal to $C(x, t)L_S$, $K_S L_S$ and D_S/L_S^2, respectively. These partial differential equations are transformed to time-dependent ordinary differential equations and solved by the implicit EULER rule. The unchanged drug amount in donor ($Q_D(t)$) is estimated by the following equation.

$$Q_D(t) = \frac{V_D}{K'_S} C'(0,t) \quad (5)$$

The unchanged drug amount in skin ($Q_S(t)$) was estimated using the following equation.

$$Q_S(t) = \text{Area} \int_0^1 C'(y,t)\,dy \quad (6)$$

The cumulative amount of unchanged drug permeated through skin ($Q_R(t)$) was estimated using the following equation.

$$Q_R(t) = -D'_S \text{Area} \int_0^t \frac{\partial C'(y,t)}{\partial y}\,dt \Big|_{y=1} \quad (7)$$

Drug amount in donor (Q_D) was obtained from HPLC analysis. Drug amount in skin (Q_S) was calculated by the following equation: Q_S = (drug concentration in skin extract) × (skin extract volume). For the rat skin experiments using Franz-type diffusion cells, the cumulative drug permeated (Q_R) was calculated by the following equation: Q_R = (drug concentration in receptor fluid) × (receptor volume). For the LSE-high experiments using Netwell™ plates, the cumulative drug permeated (Q_R) was calculated by the following equation.

$$Q_R = C_i V + \sum_{k=1}^{i-1} C_k v \quad (Q_R = C_i V \text{ when } i = 1)$$

where i is the number of samplings, C_i is the drug concentration in receptor fluid at the ith sampling, v and V are the sampling volume and total volume of receptor fluid, respectively.

Mean values of Q_D, Q_S and Q_R at each sampling time of three experiments were calculated. The permeation profiles of the drugs through skin were fitted to Eqs. (5)–(7) using MULTI (Yamaoka et al., 1981) to obtain the parameters K'_S, D'_S and k_{ms}.

Simple regression analyses between the permeation parameters and lipophilicity were performed using the least squares method. Statistical analyses were performed using Microsoft Office Excel 2003 (Microsoft Co., Redmond, WA, USA).

3. Results and discussion

3.1. In vitro permeation of vitamin D_3 analogues

Using excised rat skin and a three-dimensional cultured human skin model (LSE-high) (Sugibayashi et al., 2004; Kano

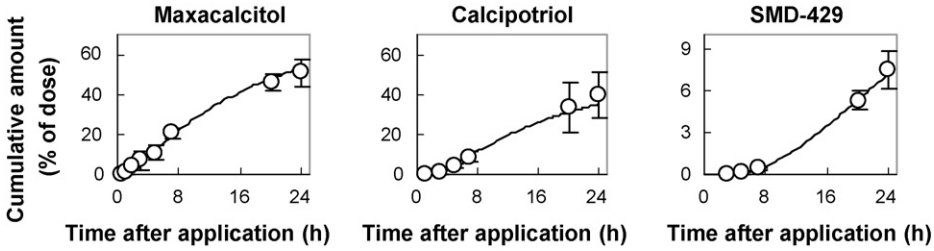

Fig. 3. Permeation profiles of vitamin D_3 analogues through LSE-high. After applications of maxacalcitol, calcipotriol and SMD-429 ointment to LSE-high, the cumulative amount of drug permeated was determined. (Open circles) Mean value of observed data from three experiments. (Error bars) S.D. of corresponding observed data.

and Sugibayashi, 2006), in vitro permeation experiments of the vitamin D_3 analogues were carried out. Fig. 3 shows the permeation profiles of the vitamin D_3 analogues through LSE-high; cumulative SMD-429 permeated over 24 h after application of ointment was 7.5% of dose, which was lower than maxacalcitol and calcipotriol. In this study, we used LSE-high as an alternative to human skin; the low level of cumulative permeated observed in the model suggests that the skin permeability of SMD-429 would also be low in human.

Fig. 4 shows the in vitro rat skin permeation profiles of SMD-429 and calcipotriol obtained from the present study and of maxacalcitol from a previous report (Yamaguchi et al., 2006). The unchanged SMD-429 remaining in the ointment at 24 h after application was 52.4% of dose; the transport of SMD-429 from ointment into skin was slow compared with that of maxacalcitol (7.1%) and calcipotriol (19.9%). The unchanged SMD-429 in skin was 33.6% of dose at 1 h after application and decreased to 16.1% at 24 h, which were almost the same as the rates for

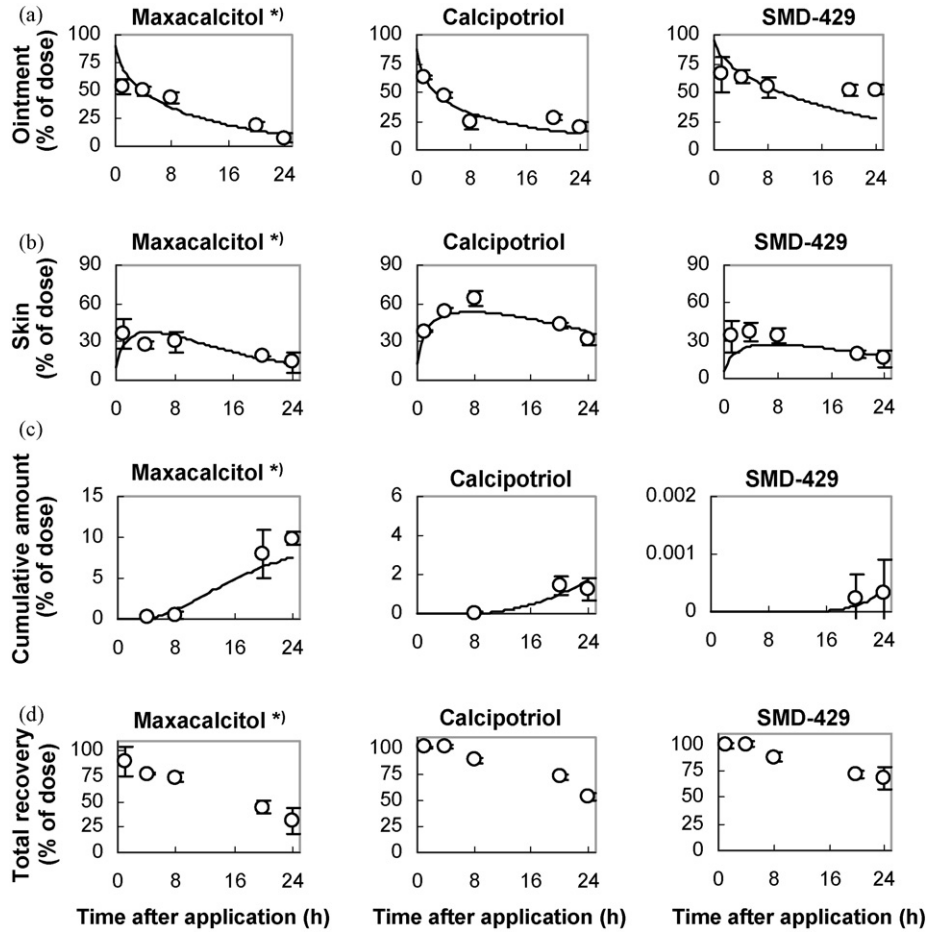

Fig. 4. Permeation profiles of vitamin D_3 analogues through excised rat skin. After applications of maxacalcitol, calcipotriol and SMD-429 ointment to rat skin, drug amount remaining in ointment (a), drug amount in skin (b) and cumulative drug amount permeated (c), were determined. Total recovery (d) was the sum of unchanged drug in ointment, skin and cumulative permeated. (Open circles) Mean value of observed data from three experiments. (Error bars) S.D. (*) Observed data of maxacalcitol was taken from a previous report (Yamaguchi et al., 2006).

maxacalcitol and approximately half the rates for calcipotriol. The cumulative unchanged SMD-429 permeated over 24 h was 0.0003% of dose, which was 4 orders of magnitude lower than either maxacalcitol or calcipotriol.

With both rat skin and LSE-high, the cumulative SMD-429 permeated was less than permeated maxacalcitol or calcipotriol. On the other hand, the amount of SMD-429 in the skin was almost the same as the others. These characteristics of SMD-429 seem to be preferable for psoriasis therapy because a greater amount of drug in skin would contribute to better therapeutic efficacy and low permeability would decrease hypercalcemia.

3.2. Metabolism of vitamin D_3 analogues in rat skin

Fig. 4d shows the time course of total recovery (the sum of drug in ointment, skin and cumulative amount permeated) of unchanged vitamin D_3 analogues. The total recovery for each compound was more than 90% at 1 h after application and time-dependently decreased. We had preliminarily evaluated the stability of these compounds in the receptor fluid and confirmed that 80% of maxacalcitol, 100% of calcipotriol and 73% of SMD-429 were recovered from a glass tube after 24 h incubation at 37 °C. Maxacalcitol and calcipotriol have been found to be metabolized in rat skin (Yamaguchi et al., 2006; Tomida et al., 1996) and cultured keratinocyte (Masuda et al., 1994, 1996). The present results showed that SMD-429 was also metabolized (Fig. 5). These findings suggest that the time-dependent decrease in the total recovery of unchanged drug was due to metabolic elimination in skin.

Fig. 5 shows the typical radio-HPLC chromatogram of [^{3}H]SMD-429 in receptor fluid at 24 h after application. The radioactivity in the receptor fluid was 9.2% of dose (mean value of two experiments). UNK (retention time: 3 min) and M1 (retention time: 11 min) were the main metabolites in the receptor fluid, and unchanged SMD-429 (retention time: 32 min) was not detected. M1 is a hydrolyzed form of SMD-429 and has no calcemic effect (Shimizu et al., 2006). We carried out a preliminary stability experiment with SMD-429 in receptor fluid and found that 73% was recovered from the receptor fluid after a 24 h incubation at 37 °C. We also performed a preliminary stability experiment with SMD-429 in the ointment at room temperature and found that 97% was recovered from the ointment 4 days after preparation. These results suggest that the hydrolysis of SMD-429 mainly took place in the skin and not in the receptor fluid or the ointment. No information was obtained on the structure of UNK; however, a comparison of elution time under reverse phase HPLC analysis suggests that UNK has a higher polarity than M2 (retention time: 13 min), which has no side chain and no calcemic activity (Shimizu et al., 2006). Thus, the bioconversion of SMD-429 in skin will contribute to a reduction in side effects from percutaneous administration of SMD-429.

Shimizu et al. (2006) reported that SMD-429 showed a high metabolic clearance in rat liver microsomes and that the clearance of SMD-429 in rats was greater than that of maxacalcitol. This finding suggests that the SMD-429 that penetrates into the systemic circulation would be immediately eliminated by metabolism. In this study, skin permeation of SMD-429 was found to be quite low; SMD-429 permeated through skin mainly in its metabolized form. In addition to the rapid elimination from the systemic circulation, low skin permeability and high metabolic conversion in skin would contribute to fewer systemic side effects from SMD-429. As shown in Fig. 3, low permeability of SMD-429 was observed with human skin model (LSE-high), expecting that SMD-429 would possibly have fewer calcemic effects in human.

3.3. Analysis of vitamin D_3 analogues permeation based on a diffusion model

The in vitro rat skin permeation profiles of SMD-429 and calcipotriol were presently analyzed based on a one-layer diffusion model including metabolic process (see Section 2). The in vitro rat skin permeation profile of maxacalcitol previously reported (Yamaguchi et al., 2006) was also analyzed using the present method. The simulation curves well correlated with the corresponding observed data (Fig. 4), suggesting that the present diffusion model was suitable to express the permeation of vitamin D_3 analogues through rat skin. Table 1 summarizes the permeation parameters of the vitamin D_3 analogues used in this study. The apparent partition coefficients (K_S) for maxacalcitol, calcipotriol and SMD-429 were 1.68×10^{-1}, 4.03×10^{-1} and 2.81×10^{-1}, respectively. The apparent diffusion coefficients (D_S) for maxacalcitol, calcipotriol and SMD-429 were 1.34×10^{-4}, 4.41×10^{-5} and

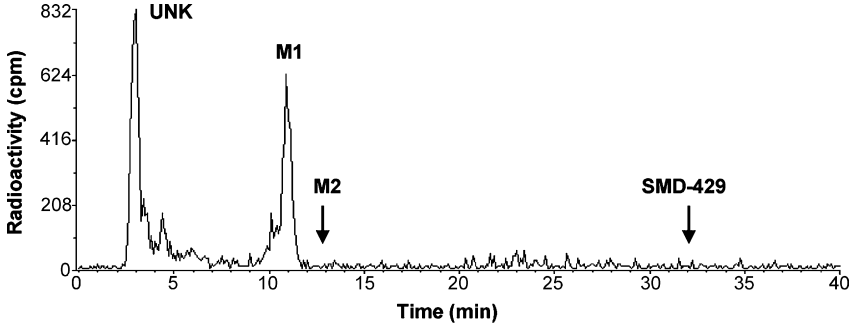

Fig. 5. Typical radio-HPLC chromatogram of a receptor fluid at 24 h after application of [^{3}H]SMD-429 ointment to excised rat skin. UNK, Unknown metabolite; M1, hydrolyzed SMD-429. M2, A predicted metabolite of SMD-429 (retention time: 13 min) and unchanged SMD-429 (retention time: 32 min) were not detected in the receptor fluid.

Table 1
In vitro permeation parameters of vitamin D_3 analogues through rat skin

Compound	Parameters					
	K'_S (cm)	D'_S (h^{-1})	k_{ms} (h^{-1})	P_S (cm/h)	K_S	D_S (cm^2/h)
Maxacalcitol	1.68×10^{-2} (0.27×10^{-2})	1.34×10^{-2} (0.23×10^{-2})	1.11×10^{-1} (0.18×10^{-1})	2.25×10^{-4}	1.68×10^{-1}	1.34×10^{-4}
Calcipotriol	4.03×10^{-2} (0.63×10^{-2})	4.41×10^{-3} (0.91×10^{-3})	4.11×10^{-2} (0.76×10^{-2})	1.78×10^{-4}	4.03×10^{-1}	4.41×10^{-5}
SMD-429	2.81×10^{-2} (0.82×10^{-2})	1.30×10^{-3} (0.09×10^{-3})	1.01×10^{-1} (0.29×10^{-1})	3.69×10^{-5}	2.81×10^{-1}	1.30×10^{-5}

Permeation parameters were estimated by analyzing permeation profiles of maxacalcitol, calcipotriol and SMD-429 based on a one-layer diffusion model. K'_S and D'_S are equal to $K_S L_S$ and D_S/L_S^2, respectively. The partition coefficient, K_S, and the diffusion coefficient, D_S, were estimated using a skin thickness (L_S) of 1000 μm. The permeability coefficient, P_S, is equal to $K'_S D'_S$. k_{ms} is the metabolic rate constant. Values in parentheses are S.D. of estimated parameters.

1.30×10^{-5} cm^2/h, respectively; the D_S value for SMD-429 was 10-fold lower than that for maxacalcitol. The apparent metabolic rate constants (k_{ms}) for maxacalcitol, calcipotriol and SMD-429 were 1.11×10^{-1}, 4.11×10^{-2} and 1.01×10^{-1} h^{-1}, respectively; the k_{ms} value for SMD-429 was the same as for maxacalcitol. The apparent permeability coefficients (P_S) for maxacalcitol, calcipotriol and SMD-429 were 2.25×10^{-4}, 1.78×10^{-4} and 3.69×10^{-5} cm/h, respectively. The P_S value for SMD-429 was lower than that of the other compounds. That might be due to the low apparent diffusivity of SMD-429 in skin.

The lipophilicity of the vitamin D_3 analogues used in this study was high; prolog $D_{7.4}$ values calculated by Pallas 2.0 (CompuDrug, Sedona, AZ, USA) were 4.57 for maxacalcitol, 5.34 for calcipotriol and 6.08 for SMD-429. The relationship between lipophilicity and apparent diffusion coefficient was evaluated, so that an inverse correlation ($r = -0.999$) was found (Fig. 6). It has been reported that an increase in the lipophilicity of alcohols (Cross et al., 2003) and steroids (Magnusson et al., 2006) caused a decrease in the diffusivity in full-thickness skin and dermis. These findings were similar to the present results. It is not clear why this interesting, but not common, relationship occurred. For clarification, further investigations are needed. The epidermal membrane is known to be a permeable layer for lipophilic compounds (Roberts et al., 1977; Potts and Guy, 1992), and the dermis acts as a depot for them (Moody and Nadeau, 1993; van de Sandt et al., 2000; Cnubben et al., 2002). Little is known about the barrier properties of the dermis; however, the hydrophilic layer (dermis) possibly functions as a permeation barrier for lipophilic compounds as does the lipophilic layer (*stratum corneum*) for hydrophilic compounds. The vitamin D_3 analogues used in the present study are highly lipophilic, thus, their behavior in skin might be affected by the dermis. The present inverse relationship of apparent diffusivity against lipophilicity might result from both the high lipophilicity of the vitamin D_3 analogues and the dermis barrier against lipophilic compounds. It is considered that the dermis might control the permeability of the vitamin D_3 analogues and the high lipophilicity of SMD-429 might result in low apparent diffusivity in skin and low permeation through skin.

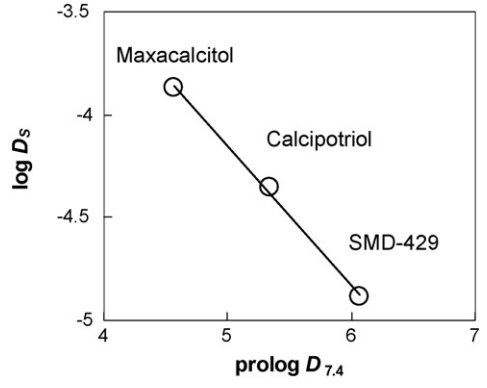

Fig. 6. Relationship between log D_S and prolog $D_{7.4}$ for vitamin D_3 analogues. The diffusion coefficients, D_S, were estimated by analyzing in vitro vitamin D_3 analogues permeation through rat skin based on a one-layer diffusion model.

Skin is a heterogeneous organ and thus the stratum corneum and the lower layers may exhibit different drug diffusivity, drug solubility and the enzymatic activity. In order to clarify the skin permeation process of a drug, a complex model such as a two-layer diffusion model (Bando et al., 1996; Yamaguchi et al., 2006) would be more useful than a one-layer diffusion model. We had analyzed the skin permeation profile of SMD-429 based on a two-layer diffusion model (data not shown), however, although the reason is not clear, the two-layer diffusion model previously reported (Yamaguchi et al., 2006) was not suitable to express the skin permeation profile of SMD-429. We successfully analyzed the permeation of vitamin D_3 analogues through full-thickness skin based on a one-layer diffusion model. A one-layer diffusion model is simple but has useful merits: if any relationships are found between the physico-chemical properties and the permeation parameters using a simple model composed of fewer parameters, those information would easily be applicable to the development of new drugs designed to control skin permeation.

We previously reported that unchanged maxacalcitol in a receptor fluid was 25.2% of total radioactivity permeated over 24 h after application of [^{3}H]maxacalcitol ointment to excised rat skin (Yamaguchi et al., 2006). In the present permeation experiments using excised rat skin, the metabolites of SMD-429 and unchanged SMD-429 in the receptor fluid over 24 h after application of [^{3}H]SMD-429 or SMD-429 ointment were found to be 9.1 and 0.0003% of dose, respectively. These results suggest that the metabolic conversion of SMD-429 in skin is higher than that of maxacalcitol, even though the apparent metabolic rate constant in skin (k_{ms}) was the same. Among the permeation parameters, the apparent diffusion coefficient in skin of SMD-429 and maxacalcitol showed the greatest difference in value. Low diffusivity in skin increases the mean transit time in skin (Hashida et al., 1988) and causes an increase in bioconversion

probability (Yamaguchi et al., 2006), and therefore might be one of the reasons for the high metabolic conversion of SMD-429 in skin. Boderke et al. (2000) simulated drug permeation through metabolizing tissue and found that a 10-fold decrease of diffusivity in skin caused a greater decrease in flux and a slight decrease in skin concentration. Their findings support our results on SMD-429 permeation: SMD-429 having low apparent diffusivity showed greatly decreased permeability and almost the same or a slightly lower amount in skin as compared with maxacalcitol and calcipotriol.

4. Conclusions

In this study, in vitro skin permeation of a new vitamin D_3 analogue with low calcemic effects, SMD-429, was evaluated and compared with two other vitamin D_3 analogues. The amount of SMD-429 in skin was the same as maxacalcitol and calcipotriol; however, the cumulative SMD-429 permeated was lower. These characteristics of SMD-429 would be an improvement in therapeutic efficacy in skin and result in fewer systemic side effects. In addition to high systemic clearance, the low skin permeability and high metabolic conversion of SMD-429 in skin have the potential to reduce the side effects for topical application in rats. The high metabolic conversion of SMD-429 might be due to its low apparent diffusivity in skin. An inverse correlation between apparent diffusivity in skin and lipophilicity was observed in the present study using high lipophilic vitamin D_3 analogues (prolog $D_{7.4}$ values ranging from 4.57 to 6.08). A topical drug having high lipophilicity would help decrease skin permeability and increase metabolic conversion in skin, resulting in a decrease of systemic side effects without an adverse effect on therapeutic efficacy.

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