Endocrine pharmacology

Calcium effects and systemic exposure of vitamin D₃ analogues after topical treatment of active vitamin D₃-containing ointments in rats

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ABSTRACT

Topical agents containing vitamin D₃ (VD₃) analogues such as calcipotriol, maxacalcitol and tacalcitol and the combination of calcipotriol/betamethasone dipropionate (betamethasone) are prescribed for patients with psoriasis. However, they are known to occasionally cause hypercalcemia, and the frequency of hypercalcemia is suggested to vary according to the VD₃ analogue used. In this study, to address the reason for these differences, the calcemic effects of maxacalcitol-, calcipotriol- and calcipotriol/betamethasone-containing ointments in rats were evaluated. The serum calcium levels in rats treated with ointments containing maxacalcitol, but not calcipotriol or calcipotriol/betamethasone, were significantly elevated, which is consistent with clinical observations. The serum concentration of VD₃ analogue in rats treated with ointments containing calcipotriol and calcipotriol/betamethasone was lower than that in rats treated with maxacalcitol-containing ointment. Thus, the calcemic effects appear to be associated with the systemic exposure of VD₃ analogues in rats. To understand the mechanism underlying the different systemic exposures of VD₃ analogues, skin permeation and metabolic stability of VD₃ analogues were evaluated. The cumulative amount of calcipotriol permeated through rat skin was significantly lower than that of maxacalcitol. On the other hand, the metabolic clearance of calcipotriol in rat hepatocytes was higher than that of maxacalcitol. Similar results were obtained using human skin and human hepatocytes. The current study demonstrates that the lower calcemic effects of calcipotriol- and calcipotriol/betamethasone-containing ointments are caused by the low systemic exposure of calcipotriol according to low skin permeability and rapid hepatic elimination after topical application.

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1. Introduction

Topical agents containing active vitamin D₃ (calcitriol, 1α, 25-dihydroxyvitamin D₃, VD₃) analogues such as tacalcitol, calcipotriol and maxacalcitol are widely used for psoriasis therapy. However, topical VD₃ agents are occasionally known to cause hypercalcemia in patients with psoriasis. The efficacy of these agents is considered to be due to the induction of cell differentiation, the inhibition of keratinocyte proliferation and the inhibition of cytokine production in T cells, dendritic cells and keratinocytes (Lovato et al., 2016) through vitamin D receptor, while the action via vitamin D receptor is likely to result in an elevation of the serum calcium level through modulating the expression levels of transient receptor potential vanilloid 6, calbindin, osteocalcin, and parathyroid hormone (Barthel et al., 2007; Jaaskelainen et al., 2003; Naveh-Many and Silver, 1993; Saito and Harada, 2014). Some reports have referred to the side effect of calcitriol analogues in humans. Yamamoto et al. (2012) suggested that calcipotriol is associated with lower serum calcium levels than tacalcitol and maxacalcitol. Moreover, animal studies have been conducted following oral and intraperitoneal administration to evaluate the calcemic potential of several VD₃ analogues and the calcemic mechanisms. However, there are few reports about the calcemic actions/mechanisms after the transdermal application of VD₃ analogues, even though they are typically applied to the skin for psoriasis treatment. Hence, the calcemic potencies after topical application of VD₃ ointments in clinical situations have not yet been adequately investigated.

One reason for the calcemic effect is presumably due to the high systemic exposure of VD₃ analogues through transdermal application (Baroni et al., 2008; Saito and Harada, 2014). In general, the plasma concentration of a transdermal agent is dependant on the balance between the skin permeation profile and the...
clearance of systemic circulating drugs (Nakamura et al., 2012). Therefore, in vitro studies using skin permeation with human skin and metabolic stability in human hepatocytes of transdermal agent are considered to be useful for predicting systemic exposure in humans.

The present study aimed to clarify the relationship between the systemic exposure of each VD₃ analogue and the following calcemic effect in rats and demonstrate differences in systemic exposure among VD₃ analogues according to the pharmacokinetic, hepatic metabolism and skin permeation properties.

2. Materials and methods

2.1. Chemicals, tissues and experimental animals

Calcipotriol was purchased from Tocris Bioscience (Bristol, UK). Maxacalcitol was purchased from ChemScene, LLC (Monmouth Junction, NJ, USA). Simvastatin was purchased from Wako Pure Chemical Industries (Osaka, Japan). Marketed ointment containing either 25 μg/g of maxacalcitol (maxacalcitol ointment), 50 μg/g of calcipotriol (calcipotriol ointment) or 50 μg/g of calcipotriol and 0.643 mg/g of betamethasone dipropionate (calcipotriol/betamethasone ointment) were used in this study. All other reagents were commercially available. Male Hairless Wistar Yagi (HWY)/Slc rats (5–6 weeks of age) were obtained from Japan SLC Inc. (Shizuoka, Japan). Male Sprague Dawley rats (7–9 weeks of age) were obtained from Charles River Laboratories Japan Inc. (Yokohama, Japan). The experimental protocols were reviewed and approved by the Kyowa Hakko Kirin Co., Ltd. Animal Care Committee in accordance with the “Company Policy on the Care and Use of Laboratory Animals”.

Rat (Lot. OJK) and human (Lot. HDI) cryopreserved hepatocytes were purchased from Celsis In Vitro Technologies (Baltimore, MD, USA). Excised human skin (female, Caucasian, 34–70 years of age, Lot. NO. TRA002001C014-C016, TRA002001C077-C079, TRA002001C083-C084) was obtained from Biopredic International (Saint-Gregoire, France) as frozen skin sets. The experimental protocols were maintained according to the “Company Policy on the Research Ethics Review Committee” by Kyowa Hakko Kirin Co., Ltd.

2.2. In vivo study

For the evaluation of the calcemic effects of VD₃ analogue-containing ointments after topical administration in rats, 200 mg of each ointment was applied on the abdominal skin of HWY/Slc rats anesthetized with isoflurane, followed by occlusion with Finn Chamber discs (18 mm diameter; Bio Diagnostics Ltd.), Tegaderm Transparent Dressing (3 M HealthCare) and bandages. In sham-treated rats, Finn Chamber discs without any ointment was applied on the skin, and then occluded with Tegaderm Transparent Dressing and bandages. Serum samples were collected from the abdominal vein of the rats under isoflurane anesthesia at 1, 2, 6, 10, 24 and 48 h after the application of each ointment and were stored at −20 °C until the measurement of calcipotriol, maxacalcitol and calcium. The serum calcium concentration was measured using the ortho-cresolphthalein complexone method with an automatic analyzer (7180, Hitachi High Technologies).

For the pharmacokinetic study in rats, calcipotriol and maxacalcitol were diluted in N, N-dimethylacetamide, and injected into the femoral vein at 1 mg/kg under isoflurane anesthesia. Plasma samples were obtained from the tail vein at 0.083, 0.25, 0.5, 1, 2, 4, 6 and 8 h after the dosing, and were stored at −20 °C.

The elimination half-life (\( t_{1/2} \)), area under the plasma concentration-time curve from 0 to infinity (AUC₁₋₋), and total plasma clearance (CL\(_\text{plasma} \)) were calculated using the Phoenix WinNonlin software program (version 6.1, Pharsight) based on a non-compartmental analysis.

2.3. In vitro metabolism

The metabolic stabilities of calcipotriol and maxacalcitol in rat and human cryopreserved hepatocytes were evaluated. The intrinsic clearance was determined using the substrate depletion approach. The study was performed using the methodologies described in a previous report (Sohlenius-Sternbeck et al., 2010). In brief, hepatocytes were diluted in William’s medium E, and these viabilities were determined using the Trypan blue exclusion method. Hepatocytes were seeded into a 24-well plate at a cell density of 1 × 10⁶ cells/ml. After pre-incubation for 15 min in an incubator at 5% CO₂, the metabolism was initiated by adding a substrate to each well. The hepatocytes were incubated with VD₃ analogues for 0, 15, 30, 60 and 90 min, and the reactions were stopped by the addition of ice-cold acetonitrile containing internal standard. The intrinsic metabolic clearance in hepatocytes (CL\(_\text{int,H} \)) was corrected by an unbound fraction of the test compound in the incubation medium (Kilford et al., 2008).

2.4. In vitro skin permeation

HWY/Slc rats were killed by CO₂ gas. The abdominal skin was carefully excised, and subcutaneous tissue was removed. Human skin was thawed at room temperature and cut into the appropriate size.

Franz-type diffusion cells with an effective diffusion area of 0.785 cm² (radius: 0.5 cm) and a receptor cell volume of 5 ml were used. Diffusion cells were filled with receptor fluid (phosphate-buffered saline containing 5 w/v% of bovine serum albumin). The skin was set dermis-side down on the diffusion cell, and the temperature of the receptor fluid was controlled to maintain the test system at 32 °C. Approximately 50 mg of each ointment was applied to the skin surface. Receptor fluid was sampled at 3, 18, 20, 22 and 24 h in HWY/Slc rat skin, and 3, 6, 18, 20, 22 and 24 h in human skin after application. The sampling volume was 2 ml, and 2 ml of fresh receptor fluid was added after each sampling.

2.5. Sample preparation for liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) measurement

The concentrations of calcipotriol and maxacalcitol in the samples were measured by LC-MS/MS. All samples were mixed with ice-cold acetonitrile containing internal standard. After centrifugation, the supernatant was collected and liquid-liquid extraction by ethyl acetate was performed. The extract was evaporated under nitrogen gas at 40 °C and reconstituted with methanol/water/5 mol/l ammonium acetate/1 mol/l ammonium formate (500/500/2/2, vol%) the same as the LC-MS/MS sample.

2.6. LC-MS/MS analysis

LC-MS/MS analyses were performed with the API5000 system (AB SCIEX) coupled with the Agilent 1200 series HPLC system (Agilent Technologies) and HTC PAL autosampler (CTC Analytics). The Analyst software program (version 1.6.1) was used for the calculation of the test compound concentrations in individual samples.

An XBridge C18 column (3.5 μm, 4.6 mm I.D. × 100 mm, Waters) with OPTI-GUARD (C18, Optimize Technologies) was used as the analytical column and the pre-column filter at room temperature, respectively. The mobile phases were A: methanol/water/5 mol/l ammonium acetate (200/800/2, v/v/v) and B:
methanol/water/5 mol/l ammonium acetate (980/20/2 or 1000/0/2, v/v/v) at a flow rate of 0.6 ml/min. Chromatography of both calcipotriol and maxacalcitol was performed under the following gradient condition: 0–0.2 min 85% B, 0.2–3.5 min 85–100% B, 3.5–5 min 100% B, and 5–7 min 85% B. The LC eluent was introduced into the source using an electrospray ionization interface in the positive ionization mode. Detected ions (Q1/Q3) of calcipotriol, maxacalcitol, and simvastatin were m/z 395.3/133.2, 436.3/297.3, and 419.3/285.1, respectively. Simvastatin was used as an internal standard based on the protocol described in a previous report (Li et al., 2013).

2.7. Statistical analysis

Bartlett’s test was performed to test equality of variance. Multiple comparisons among each group were assessed by one-way analysis of variance, followed by the Tukey test or Kruskal-Wallis test, followed by the Steel-Dwass test. Values of \( P < 0.05 \) were considered to be statistically significant. All statistical calculations were performed using the Statistical Analysis System software program (SAS; Release 9.2; SAS Institute, Cary, NC, USA).

3. Results

3.1. Calcemic effects and systemic exposure of VD₃ analogues after the topical administration of VD₃ ointments in rats

To evaluate the effects of VD₃ analogue-containing ointments on calcium homeostasis in rats, the serum concentration of calcium was measured at 24 h after the topical application of maxacalcitol ointment, calcipotriol ointment or calcipotriol/betamethasone ointment (Fig. 1). No significant change in the serum calcium level was observed in the rats treated with calcipotriol ointment or calcipotriol/betamethasone ointment compared to the sham-treated rats. On the other hand, the serum calcium level in the rats treated with maxacalcitol ointment was significantly higher than that in the sham, calcipotriol ointment and calcipotriol/betamethasone ointment-treated rats.

As shown in Fig. 2, in both the calcipotriol ointment and calcipotriol/betamethasone ointment-treated rats, the serum concentration of calcipotriol was below the lower limit of quantification (0.1 ng/ml) at all time points examined. On the other hand, maxacalcitol in the serum was detected at 1, 2, 6 and 10 h, but not at 24 and 48 h, after the topical application, and the maximum serum concentration (Cₘₐₓ) of maxacalcitol in rats treated with maxacalcitol ointment was 0.289 ± 0.142 ng/ml. Thus, the serum concentration of VD₃ analogue in rats treated with maxacalcitol ointment was higher than that in the rats treated with calcipotriol ointment or calcipotriol/betamethasone ointment.

3.2. A pharmacokinetic study after intravenous administration of calcipotriol and maxacalcitol in rats

The plasma pharmacokinetics of calcipotriol and maxacalcitol were evaluated after a single intravenous administration to the rats (Fig. 3(A) and (B)). After the intravenous administration of calcipotriol at a dose of 1 mg/kg, the plasma concentration of calcipotriol decreased monophasically. The \( t_{1/2A} \), \( \text{AUC}_{0-\infty} \), and \( \text{Cl}_{\text{plasma}} \) were 0.229 ± 0.012 h, 2590 ± 70.9 ng h/ml and 2.88 ± 0.07 l/h/kg, respectively. The plasma concentration of maxacalcitol also decreased over time. The \( t_{1/2A} \), \( \text{AUC}_{0-\infty} \), and \( \text{Cl}_{\text{plasma}} \) were 0.383 ± 0.012 h, 2590 ± 282 ng h/ml and 0.390 ± 0.042 l/h/kg, respectively. These pharmacokinetic parameters are described in Table 1.

3.3. In vitro metabolism

The metabolic stabilities of calcipotriol and maxacalcitol in the rat and human cryopreserved hepatocytes were evaluated using a substrate depletion approach (Fig. 4(A) and (B)). In rat hepatocytes, calcipotriol rapidly decreased compared with maxacalcitol, and the \( \text{Cl}_{\text{int,H}} \) of calcipotriol and maxacalcitol were 2.06 ± 0.09 and 0.0485 ± 0.0012 ml/min/10⁶ cells, respectively. In human hepatocytes, calcipotriol rapidly decreased as well, and the \( \text{Cl}_{\text{int,H}} \) of calcipotriol and maxacalcitol were 1.48 ± 0.07 and 0.0208 ± 0.0033 ml/min/10⁶ cells, respectively. The \( \text{Cl}_{\text{int,H}} \) is described in Table 1.

3.4. In vitro skin permeation

Skin permeation of VD₃ analogue-containing ointment through rat and human skins was evaluated using Franz-type diffusion
The cumulative amounts of permeated VD₃ analogues at 24 h after application through rat skin from maxacalcitol, calcipotriol and calcipotriol/betamethasone ointments were 413 ± 7140, 62.1 ± 730.4 and 203 ± 767 ng/cm², respectively. The cumulative amounts of permeated VD₃ analogues at 24 h after application through human skin from maxacalcitol, calcipotriol and calcipotriol/betamethasone ointments were 93.0 ± 49.5, 10.2 ± 8.4 and 15.3 ± 9.0 ng/cm², respectively. Thus, the skin permeability of maxacalcitol after the topical application of maxacalcitol ointment was significantly higher than that of calcipotriol after the topical application of calcipotriol or calcipotriol/betamethasone ointment in rat and human skins.

### 4. Discussion

The calcemic effects in psoriasis patients have been reported to differ among ointments containing these VD₃ analogues such as tacalcitol, calcipotriol and maxacalcitol (Yamamoto et al., 2012). However, the reason for such differences remains to be fully understood.
elucidated because there are few applicable experiments which mimic the clinical situation. Therefore, in this study, a potential reason for the difference was evaluated according to their pharmacokinetic profiles, including skin permeation and hepatocyte metabolism. Baroni et al. (2008) reported that rats are likely to be a relevant species to predict an acute calcemic response of a VD3 analogue in human. Therefore, the calcemic effects of VD3 analogue-containing ointments in rats were evaluated in this study. Our present study demonstrated that the calcemic effects of calcipotriol- and calcipotriol/betamethasone-containing ointments were significantly lower than that of maxacalcitol-containing ointment (Fig. 1), and that the Cmax of VD3 analogue in the rats treated with calcipotriol- and calcipotriol/betamethasone-containing ointments was lower than that in the rats treated with maxacalcitol-containing ointment (Fig. 2). Based on our findings and the findings that binding activities of metabolites of calcipotriol and maxacalcitol generated by keratinocytes to vitamin D receptor are approximately two orders of magnitude lower than their parent compounds (Masuda et al., 1994; Masuda et al., 1996), these results suggest that high systemic exposure of VD3 analogues therefore induces calcemic effects in rats. These findings were supported by previous reports which showed the serum calcium levels in rats after intraperitoneal or oral administration of VD3 analogues to increase with the AUC and/or Cmax of VD3 analogues (Baroni et al., 2008; Brown et al., 1993; Saito and Harada, 2014). Thus, high systemic exposure of VD3 analogues is thought to be a relevant factor of hypercalcemia in rats.

On the other hand, the concentration of VD3 analogues in the skin has been suggested to play an important role in their efficacy (Morimoto et al., 1986). Although the amounts of VD3 analogues in the skin was not measured in this study, it has been reported that the amounts of maxacalcitol and calcipotriol in the skin of rats treated with VD3 analogue-containing ointments were almost the same (Yamaguchi et al., 2008). Based on our findings and those of other previous reports which showed that tacalcitol, maxacalcitol and calcipotriol suppressed the proliferation of keratinocytes with a similar in vitro potency, the efficacy of maxacalcitol and calcipotriol in the skin was suggested to be comparable after transdermal dosing.

To understand the reason why the systemic exposure differed among VD3 analogues, a pharmacokinetic study after intravenous administration, hepatic metabolism studies, and skin permeation studies were conducted. As shown in Fig. 3, the CLplasma of calcipotriol in rats was much greater than that of maxacalcitol, and the in vitro CLint, H in rat cryopreserved hepatocytes provided supportive evidence of the in vivo clearance (Fig. 4(A)). Subsequently, in the skin permeation study, the cumulative amounts of calcipotriol permeated through rat skin applied with calcipotriol- and calcipotriol/betamethasone-containing ointments were significantly lower than that of maxacalcitol permeated through rat skin applied with maxacalcitol-containing ointments (Fig. 5(A)), which were consistent with the results of previous reports (Yamaguchi et al., 2008). These results suggested that the lower systemic exposure of calcipotriol in rats treated with calcipotriol- and calcipotriol/betamethasone-containing ointments resulted from the low skin permeability and the high metabolic clearance in liver associated with calcipotriol. Interestingly, similar results were observed in a study using human hepatocytes and human skin (Figs. 4(B) and 5(B)), implying that the systemic exposure of calcipotriol is probably low in humans as well as rats.

In general, the unbound fraction of the drug in plasma protein (fu, p), as well as CLint, H, is an important factor affecting the in vivo hepatic clearance (CLH) of drug because CLH is simply expressed by fu, p and CLint, H (CLH = fu, p × CLint, H) (Riley et al., 2005). As previously reported, plasma VD3 interaction with vitamin D binding protein (VDBP), which is involved in the disposition of plasma VD3 was demonstrated using VDBP knockout mice, suggesting that VDBP may be an important factor for the disposition of VD3 analogues (Brown et al., 2013; Safadi et al., 1999). Indeed, the relative affinity of maxacalcitol to VDBP has been reported to be about 5 times less than that of calcipotriol (Dusso et al., 1991). This finding could thus possibly explain the gap between CLint, H differences (CLH: 42-fold to fu, p × CLint, H: 8.2-fold) and CLplasma difference (7.4-fold) in the current study. Therefore, the hepatic metabolic rate appears to be more dominant in VD3 disappearance compared to VDBP interaction in the case of calcipotriol and maxacalcitol.
VD₃ analogue-containing ointments are often used in combination with ointments containing steroids for the treatment of psoriasis. In addition, a combination ointment of calcipotriol and betamethasone dipropionate has been developed and prescribed for patients with psoriasis. In this study, we showed that there was no difference in the calcemic effects between calcipotriol-containing ointment and calcipotriol/betamethasone-containing ointment in rats. In in vitro skin permeation studies, the skin permeability of calcipotriol in humans was comparable between these ointments, whereas the skin permeability of calcipotriol in rats was higher for calcipotriol/betamethasone-containing ointment. In general, VD₃ analogues such as calcipotriol and maxacalcitol have been shown to be metabolized in rat skin and human cultured keratinocytes (Yamaguchi et al., 2006, 2008). Betamethasone might therefore have a potential to affect the expression or activity of the enzyme which is responsible for the metabolism of calcipotriol in the rat skin. Further investigation is therefore needed to identify the mechanisms of the VD₃ analogue metabolism in the skin.

In conclusion, the current study indicated that the lower calcemic effect of calcipotriol- and calcipotriol/betamethasone-containing ointments is caused by the low systemic exposure of calcipotriol based on its low skin permeability and rapid hepatic elimination after topical application.

References


