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ORIGINAL ARTICLE

Human hepatic metabolism of the anti-osteoporosis drug eldecalcitol involves sterol C4-methyl oxidase

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Abstract

The metabolism of eldecalcitol (ED-71), a 2β-hydroxypropoxylated analog of the active form of vitamin D_3 was investigated by using *in vitro* systems. ED-71 was metabolized to 1α,2β,25-trihydroxyvitamin D_3 (1α,2β,25$(OH)_3D_3$) in human small intestine and liver microsomes. To identify the enzymes involved in this metabolism, we examined NADPH-dependent metabolism by recombinant P450 isoforms belonging to the CYP1, 2, and 3 families, and revealed that CYP3A4 had the activity. However, the CYP3A4 -specific inhibitor, ketoconazole, decreased the activity in human liver microsomes by only 36%, suggesting that other enzymes could be involved in ED-71 metabolism. Because metabolism was dramatically inhibited by cyanide, we assumed that sterol C4-methyl oxidase like gene product (SC4MOL) might contribute to the metabolism of ED-71. It is noted that SC4MOL is physiologically essential for cholesterol synthesis. Recombinant human SC4MOL expressed in COS7, *Saccharomyces cerevisiae*, or *Escherichia coli* cells converted ED-71 to 1α,2β,25$(OH)_3D_3$. Furthermore, we evaluated the metabolism of ED-71 by recombinant CYP24A1, which plays an important role in the metabolism of the active form of vitamin D_3 (1α,25$(OH)_2D_3$) and its analogs. The k_{cat}/K_m value for 24- or 23-hydroxylation of ED-71 was only 3% of that for 1α,25$(OH)_2D_3$, indicating that ED-71 was resistant to CYP24A1-dependent catabolism. Among the three enzymes catalyzing ED-71, SC4MOL appears to be most important in the metabolism of ED-71. To the best of our knowledge, this is the first study showing that SC4MOL can function as a drug-metabolizing enzyme. The yeast and *E. coli* expression systems for SC4MOL could be useful for structure-function analyses of SC4MOL.

Abbreviations

ADX, adrenodoxin; ADR, adrenodoxin reductase; BMD, bone mineral density; DBP, vitamin D-binding protein; DMSO, dimethylsulfoxide; ERG26, C4-position of 4,4′-dimethylzymosterol in concert with C-3 sterol dehydrogenase; 1α,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; ED-71, eldecalcitol or 2β-(3-hydroxypropoxy)-1α,25-dihydroxyvitamin D_3; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; O2C3, 2α-(3-hydroxypropoxy)-1α,25-dihydroxyvitamin D_3; P450, cytochrome P450; PCR, polymerase chain reaction; SC4MOL, sterol C4-methyl oxidase like; UGT, UDP-glucuronosyltransferase; VDR, vitamin D receptor.

Introduction

Eldecalcitol (ED-71), a 2β-hydroxylpropoxylated analog of the active form of vitamin D_3 (1α,25$(OH)_2D_3$; 1α,25-dihydroxyvitamin), has been developed by Chugai Pharmaceutical Co., Ltd. (Japan) and was approved as a new drug for the treatment of osteoporosis in Japan in 2011 (Miyamoto et al. 1993; Matsumoto et al. 2005, 2010; Matsumoto and Kubodera 2007; Matsumoto 2012). ED-71 showed stronger inhibitory effects on bone resorption and increase of bone mineral density (BMD) to prevent osteoporotic fractures than alfacalcidol (1α(OH)D_3) which has been used for the treatment of osteoporosis in Japan (Matsumoto et al. 2011), although its binding affinity for vitamin D receptor (VDR) is less than that of 1α,25$(OH)_2D_3$ (Tsugawa et al. 2000). The greater potency of ED-71 appears to be based on its longer circulating half-life than 1α,25$(OH)_2D_3$ (Okano et al. 1989). Recently, Brown et al.(2013) demonstrated that vitamin D-binding protein (DBP) has a major influence on circulating levels of vitamin D analogs. Thus, the higher affinity of ED-71 for DBP than 1α,25$(OH)_2D_3$ (Tsugawa et al. 2000) might result in its great efficacy. However, ED-71 also showed stronger effects than 1α,25$(OH)_2D_3$ in DBP knockout mice, suggesting that its efficacy does not involve in DBP (Brown et al. 2013). As reported by them, the resistance of ED-71 to CYP24A1-dependent catabolism in the target cells might be related to its greater efficacy (Ritter and Brown 2011). CYP24A1 plays a key role in the metabolism of 1α,25$(OH)_2D_3$ and its analogs in the target cells. When they bind to VDR as ligands to induce transcriptional activation of the *CYP24A1* gene (Ohyama et al. 1994), the overexpressed CYP24A1 enzyme could inactivate them via multiple oxidation steps. Therefore, the resistance to CYP24A1-dependent catabolism could be a key property in prolonging the biological effects of ED-71 in the target cells (Sakaki et al. 2013). We have successfully expressed rat or human CYP24A1 in *Escherichia coli* cells to reveal the metabolism of the active form of vitamin D and its derivatives (Sakaki et al. 1999a, 2000; Sawada et al. 2004; Abe et al. 2005; Urushino et al. 2007). In this study, we examined human CYP24A1-dependent metabolism of ED-71 by using the recombinant human CYP24A1 expressed in *E. coli* cells.

Our previous studies revealed that the C2-epimer of ED-71, 2α-hydroxypropoxylated-1α,25$(OH)_2D_3$ named O2C3 is a suitable substrate for CYP3A4 which is the most important drug-metabolizing P450 enzyme expressed in human liver and intestine (Yasuda et al. 2013). Recently, Ono (2014) demonstrated that the liver, not the kidney, plays an essential role in the metabolism of ED-71. Based on these findings, we have attempted to identify the hepatic enzymes responsible for the metabolism of ED-71 by using recombinant human drug-metabolizing P450s and human liver microsomes. In this report, we describe that CYP3A4 and sterol C4-methyl oxidase (SC4MOL) are involved in the metabolism of ED-71.

The *SC4MOL* (sterol C4 methylsterols oxidase-like) gene is considered to be a homolog of *Saccharomyces cerevisiae ERG25* (Li and Kaplan 1996). ERG25 had been isolated and functionally shown to catalyze sequential removal of the two methyl groups from the C4-position of 4,4′-dimethylzymosterol in concert with C-3 sterol dehydrogenase (ERG26) and 3-keto reductase (ERG27) (Bard et al. 1996; Gachotte et al. 1998, 1999) (Fig. 1). Erg25 has three histidine clusters common to nonheme iron binding and a KKXX Golgi-to-endoplasmic reticulum retrieval signal (Bard et al. 1996). Four decades ago, it was shown that the rat sterol C-4 demethylation enzyme is localized in microsomes and that its activity is inhibited by cyanides (Gaylor and Mason 1968); next, Miller's group (Miller and Gaylor 1970; Miller et al. 1971) revealed that sterol C-4 demethylation depends on O_2 and NAD(P)H. It should be noted that this enzyme is resistant to carbon monoxide, isocyanide, and nicotinamide, but is strongly inhibited by cyanide. Recent studies showed that mutation in the human SC4MOL gene causes serious diseases such as psoriasiform dermatitis, microcephaly, and developmental delay, because of the accumulation of 4,4′-dimethyl-5α-cholest-8-en-3β-ol, the substrate of this enzyme (He et al. 2011, 2013). Based on these findings, it is assumable that SC4MOL corresponds to the sterol C-4 demethylation enzyme. Thus, it is surprising that SC4MOL, which plays a crucial role in cholesterol synthesis, is involved in the metabolism of ED-71 in the liver.

To the best of our knowledge, this is the first report to identify the enzymes responsible for the metabolism of ED-71. One of the most remarkable findings is a novel function of SC4MOL as a drug-metabolizing enzyme. The heterologous expression systems for human SC4MOL established in this study will be useful to reveal the enzymatic properties of SC4MOL.

Materials and Methods

Materials

1α,25$(OH)_2D_3$ was purchased from Wako Pure Chemicals (Osaka, Japan). NADPH and NADH were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). A 50 donor human liver microsomes pool, human small intestinal microsomes pool, and microsomes prepared from recombinant baculoviruses-infected insect cells containing human CYP3A4, human NPDPH-P450 reductase, and human cytochrome b_5 were purchased from BD Biosciences (Woburn, MA). 2β-(3-Hydroxypropoxy)-

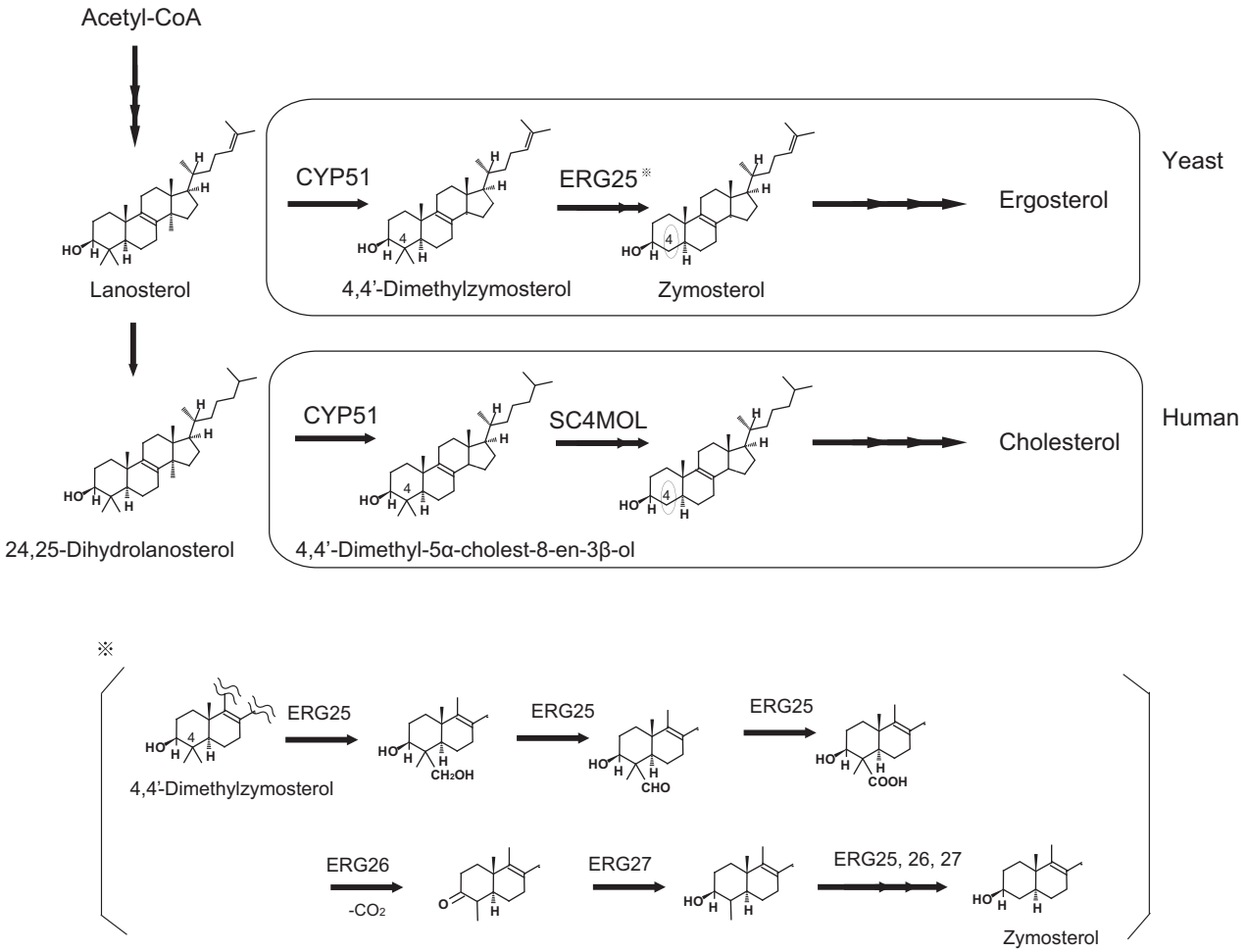

Figure 1. Biosynthesis pathways for ergosterol and cholesterol. As shown in the parenthesis, yeast Erg25 and human SC4MOL catalyze the three-step monooxygenation of two methyl groups at the C4 and C4′-positions of 4,4′-dimethylzymosterol and 4,4′-dimethyl-5α-cholest-8-en-3*β*-ol, respectively. ERG26 and ERG27 are also required to produce zymosterol.

1α,25$(OH)_2D_3$ (ED-71 or eldecalcitol), 24(*S*)OH-ED-71, 24(*R*)OH-ED-71, and 1α,2*β*,25$(OH)_3D_3$ (ED-138) were kindly provided by Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan) (Ono et al. 2006). 1α,2α,25$(OH)_3D_3$ was kindly provided by Dr. Atsushi. Kittaka of Teikyo University, Japan. Human CYP24A1, bovine adrenodoxin reductase (ADR) and adrenodoxin (ADX) were prepared from recombinant *E. coli* cells as described previously (Akiyoshi-Shibata et al. 1994; Sakaki et al. 1999b; Kusudo et al. 2004). The recombinant *S. cerevisiae* AH22 cells expressing each of 11 human P450s (CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, and 3A4) with the vector pGYR were kindly provided by Sumitomo Chemical Co., Ltd. (Osaka, Japan) (Yasuda et al., 2010). The vector pET17b was purchased from Novagen (Madison, WI). The vector pCR-Blunt II TOPO was purchased from Invitrogen (Carlsbad, CA). The vector pEBMulti-Neo was purchased from Wako Pure Chemicals. The GroEL/ES expression plasmid, pGro12, was kindly given by the HSP Research Laboratory (Kyoto, Japan). All other chemicals were purchased from standard commercial sources of the highest quality available.

Metabolism of ED-71 in human liver or small intestine microsomes

In P450-dependent oxidation of ED-71, the reaction mixtures containing 0.5 mg protein/ml of the liver or small intestinal microsomes, 1–50 *μ*mol/L ED-71, 1 mmol/L NADPH in 100 mmol/L potassium phosphate buffer (pH 7.4) was incubated for 10–30 min at 37°C, and the metabolites were analyzed as described in *high performance liquid chromatography (HPLC) Analysis of the Metabolites*. In UDP-glucuronosyltransferase (UGT)-dependent glucuronidation of ED-71, the reaction mixtures containing 0.5 mg protein/mL of the liver micro-

somes, 10 μmol/L ED-71, 2 mmol/L UDP-glucuronic acid, and 1 mmol/L $MgCl_2$ in 100 mmol/L potassium phosphate buffer (pH 7.4) was incubated for 60 min at 37°C, and the reaction was terminated by addition of equal volume of methanol. After centrifugation at 20,000*g* for 15 min, the supernatant was analyzed as described in *HPLC Analysis of the Metabolites*.

Inhibition of ED-71 *O*-depropoxylation in human liver microsomes by ketoconazole or cyanide

The reaction mixtures containing 0.5 mg protein/mL of the liver microsomes, 5 μmol/L ED-71, 1 mmol/L NADPH, and 1 μmol/L ketoconazole or 0.01–1 mmol/L potassium cyanide in 100 mmol/L potassium phosphate buffer (pH 7.4) was incubated for 15 min at 37°C, and the metabolite was analyzed as described in *HPLC Analysis of the Metabolites*.

Metabolism of ED-71 by whole cell fraction of recombinant yeast cells

The recombinant *S. cerevisiae* cells expressing each of human CYPs (CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, and 3A4) were cultivated in synthetic minimal medium containing 2% glucose, 0.67% yeast nitrogen base without amino acids, and 20 mg/l L-histidine. When the cell density (OD_{660}) of culture reached 0.5, 1 mmol/L ED-71 in ethanol was added to the culture at a final concentration of 10 μmol/L. At 24 h after addition of ED-71, the cultures were extracted with four volumes of chloroform/methanol (3:1. v/v). The organic phase was recovered and dried in a vacuum evaporator centrifuge (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). The residue was solubilized with methanol and analyzed as described in *HPLC Analysis of the Metabolites*.

Metabolism of ED-71 by recombinant CYP3A4

The reaction mixtures containing 40 pmol/mL of recombinant human CYP3A4 microsomes prepared from recombinamt baculoviruses-infected insect cells, 1 mmol/L NADPH, and 2.5–40 μmol/L ED-71 in 100 mmol/L potassium phosphate buffer (pH 7.4) was incubated for 20 min at 37°C, and the metabolite was analyzed as described in *HPLC Analysis of the Metabolites*.

cDNA cloning of human SC4MOL

Human SC4MOL cDNA (NP_006736) was obtained from human liver cDNA library HL1145y (Clontech Laboratories Inc., Palo Alto, California) with polymerase chain reaction (PCR) methods. PCR was performed in a 50 μL of mixture containing 10 ng cDNA, 0.2 mmol/L dNTP, 0.2 μmol/L two sets of primers (i) 5′-ATATAAGCTTAAAAAAATGG CAACAAATGAAAGTGT-3′ (ii) 5′-ATATAAGCTTTTATT CAGTCTTTTTCTC-3′, 1 mmol/L $MgSO_4$, 1U KOD-plus-DNA polymerase for 30 cycles at temperatures of 94°C for denaturation (15 sec), 52°C for annealing (30 sec), 68°C for extension (1 min). PCR fragment (0.9 kbp) was cloned to the pTA2-vector using Target clone-plus (Toyobo Co., Ltd., Osaka, Japan) according to the manufacturer's instructions to obtain pTA2-SC4MOL. All the nucleotides of human SC4MOL cDNA were confirmed by DNA sequencing.

Expression of human SC4MOL in COS7, S. cerevisiae and *E. coli* cells

The expression plasmid for human SC4MOL with the His-Tag at the C-terminus in the COS-7 cells was constructed as described below. The cDNA-encoding human SC4MOL with the His-tag (His x 6) at the C-terminus was amplified by PCR using pTA2-SC4MOL as a template with the two sets of primers (iii) 5′-ATATCTCGAGATG GCAACAAATGAAAGTGTCAGC-3′ and (iv) 5′-ATATGC GGCCGCTAGTGGTGATGGTGATGATGTTCAGTCTTTT TCTCAA ACTTCTTC-3′. The PCR fragment (0.9 kbp) was inserted into the pCR-Blunt II TOPO vector (Invitrogen), and then digested by *Xho*I and *Not*I. The resultant *Xho*I-*Not*I fragment (0.9 kbp) was inserted into the pEB-Multi-Neo-vector to obtain pEBMulti-Neo-SC4MOL.

The expression plasmid for human SC4MOL in *S. cerevisiae* was constructed by using the vector pGYR which is an episomal *S. cerevisiae*/*E. coli* shuttle vector containing a 2 μm DNA ori, *Leu2* gene as a marker, *S. cerevisiae* NADPH-P450 reductase gene, pUC ori, Amp^r, and a glyceraldehydes-3-phosphate dehydrogenase (GAPDH) promoter and terminator derived from *Zygosaccharomyces rouxii*. The SC4MOL cDNA fragment (0.9 kbp) was obtained by digestion of pTA2-SC4MOL with *Hin*dIII and sequentially inserted into the pGYR-vector to obtain pGYR-SC4MOL. The plasmid pGYR-SC4MOL was introduced into *S. cerevisiae* AH22 by lithium acetate method (Ito et al. 1983).

The expression plasmid for human SC4MOL with the His-tag (His x 6) at the C-terminus in *E. coli* was constructed. The cDNA encoding human SC4MOL with the His-tag (His x 6) was amplified by PCR using pTA2-SC4MOL as a template with the two sets of primers (V) 5′-ATATCA TATGGCAACAAATGAAAGTGTCAGCATCTTTA-3′ and (vi) 5′-ATATAAGCTTAATGATGATGATGATGATGTTC AGTCTTTTTCTCAAACT-3′. The PCR fragment (0.9 kbp) was cloned in the pTA2-vector, and then digested by *Hin*dIII and *Nde*I. The resultant *Nde*I-*Hin*dIII fragment (0.9 kbp)

was inserted into the pET17b-vector to construct pET17b-SC4MOL. The *E. coli* BL21 (DE3) harboring pGro12, which is a GroEL/ES expression plasmid was transformed with the expression plasmid pET17b-SC4MOL by the $CaCl_2$ procedure.

Western blot analysis of human SC4MOL protein expressed in COS7 cells

COS-7 cells were cultivated in Dulbecco's Modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C in an incubator with 5% CO_2. Cells were seeded in 6-well plates at a density of 2×10^5 cells/well, and transfected with pEBMulti-Neo-SC4MOL plasmids, using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's instruction. At 48 h after the transfection, cells were harvested and lysed using protein lysis buffer containing 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 0.1% SDS, 1% sodium cholate, 1% Triton X. Proteins separated by SDS-PAGE were electrically transferred onto a PVDF membrane (GE Healthcare UK Ltd, Buckinghamshire, England). The membranes were incubated with mouse anti-His-Tag antibody (1:1000) (MBL Co., Ltd., Nagoya, Japan) at 4°C overnight. The membranes were reacted with secondary rabbit anti-mouse IgG HRP-linked antibodies (1:2000) (Cell Signalling Technology, Inc., Danvers, Massachusetts, USA) and visualized using an ECL prime Western blotting reagent (GE Healthcare UK Ltd., Buckinghamshire, England). Chemiluminescence signals were detected using the Fujifilm LAS-1000 imager (Fuji Photo Film Company, Tokyo, Japan).

Metabolism of ED71 in the human SC4MOL-expressing COS-7 cells

ED-71 was added to the culture of COS-7 cells expressing human SC4MOL at a final concentration of 1 μmol/L. The cells and medium were separated after 24 h, and ED-71 and its metabolites were analyzed as described in *HPLC Analysis and liquid chromatography-mass spectrometry (LC-MS) Analysis of the Metabolites.*

Metabolism of ED71 by whole cell fraction of recombinant yeast cells expressing human SC4MOL

The recombinant *S. cerevisiae* cells expressing human SC4MOL were cultivated in synthetic minimal medium containing 2% glucose, 0.67% yeast nitrogen base without amino acids, and 20 mg/L L-histidine at 30°C. When the cell density (OD_{660}) of culture reached 0.5, ED-71 was added to the culture at a final concentration of 10 μmol/L. After 24 h incubation at 30°C, ED-71 and its metabolites were extracted and analyzed as described in *HPLC Analysis and LC-MS Analysis of the Metabolites.*

Metabolism of ED-71 or 1α,2β,25(OH)$_3$D$_3$ by recombinant human CYP24A1

The activity of CYP24A1 toward ED-71 and 1α,2β,25$(OH)_3D_3$ was measured in the reconstituted system containing the membrane fraction prepared from the recombinant *E. coli* cells expressing human CYP24A1 (Kusudo et al. 2004; Abe et al. 2005). The reaction mixture containing 2.0 μmol/L ADX, 0.2 μmol/L ADR, 20 nmol/L CYP24A1, 1-50 μmol/L ED-71 or 1α,2β,25$(OH)_3D_3$, 1 mmol/L NADPH, 100 mmol/L Tris-HCl (pH 7.4), and 1 mmol/L ethylenediaminetetraacetic acid (EDTA) was incubated for 10–30 min at 37°C, and the metabolites were analyzed as described in *HPLC Analysis and LC-MS Analysis of the Metabolites.*

Metabolism of ED71 by whole cell fraction of recombinant *E. coli* cells expressing human SC4MOL

The recombinant *E. coli* cells expressing human SC4MOL and GroEL/ES were cultivated in TB medium (pH 7.0) containing 50 μg/mL ampicillin and 25 μg/mL kanamycin at 37°C. When the cell density (OD_{660}) of culture reached 0.5, 1-thio-β-D-galactopyranoside (IPTG), arabinose, and ED-71 were added to the culture at final concentrations of 1 mmol/L, 4 mg/mL, and 10 μmol/L, respectively. The cultures were subsequently incubated at 27°C for 24 h and then IPTG and arabinose were added for transcriptional induction of SC4MOL and GroEL/ES, respectively. ED-71 and its metabolite were extracted and analyzed as described in *HPLC Analysis and LC-MS Analysis of the Metabolites.*

Measurement of binding affinity of the metabolite of ED-71 for VDR

The binding affinity of 1α,25$(OH)_2D_3$, ED-71, and its metabolite 1α,2β,25$(OH)_3D_3$ for VDR was examined using a nuclear receptor cofactor assay system (Enbio RCAS for VDR; EnBioTec Laboratories, Tokyo, Japan) according to the manufacturer's instructions as described previously (Yasuda et al. 2013).

HPLC analysis and LC-MS analysis of the metabolites of ED-71

The metabolites of ED-71 were extracted with chloroform/methanol (3:1), and analyzed by HPLC under the

following conditions: column, YMC-pack ODS-AM (4.6 × 300 mm) (YMC Co., Tokyo, Japan); UV detection, 265 nm; flow rate, 1.0 ml/min; column temperature, 40°C; liner gradients of 20–100% acetonitrile aqueous solution per 25 min. To separate 24*R*(OH)-ED-71 and 24*S*(OH)-ED-71, HPLC using a chiral cyclodextrin column was performed under the following conditions; SUMICHIRAL OA-7000 (4.6 × 250 mm) (Sumika Chemical Service, Ltd., Osaka, Japan); UV detection, 265 nm; flow rate, 0.7 mL/min; column temperature, 25°C; mobile phase, methanol/water (75:25, v/v) and LC-MS analysis was performed as described in our previous study (Yasuda et al. 2013). Here 1α,2α,25$(OH)_3D_3$ and 1α,2β,25$(OH)_3D_3$ were separated under the same HPLC conditions with the exception of 100% methanol as the mobile phase.

Periodate oxidation

Periodate oxidation was performed to identify the presence of vicinal diol or α-ketol group as described previously (Urushino et al. 2009).

Results

Metabolism of ED-71 in human liver or small intestine microsomes

Figure 2A shows the HPLC profile of the metabolite of ED-71 in human liver microsomes in the presence of NADPH. One major metabolite designated as M1 was detected at the retention time of 21.4 min. The mass spectrum of M1 showed the molecular ion at m/z 433 (M + H), 8%; and fragment ions at m/z 415 (M+H-H_2O), 74%; m/z 397 (M+H-2H_2O), 86%; and m/z 379 (M+H-3H_2O), 100%; m/z 361 (M+H-4H_2O), 48%; as shown in Figure 2B, which suggest that M1 would be 1α,2β,25$(OH)_3D_3$ or 1α,2α,25$(OH)_3D_3$ produced by removal of a 3-hydroxypropyl group of ED-71 at C2 position. HPLC analysis using a chiral cyclodextrin column showed the same retention time as M1 and 1α,2β,25$(OH)_3D_3$ (22.7 min) (Fig. 2C), suggesting that M1 would be 1α,2β,25$(OH)_3D_3$. Our previous studies revealed that O2C3, C2-epimer of ED-71, was converted into 1α,2α,25$(OH)_3D_3$ in human liver by CYP3A4 (Yasuda et al. 2013). Thus, we assumed that CYP3A4 or some other P450s could convert ED-71 into 1α,2β,25$(OH)_3D_3$.

M1 was also detected in human small intestine microsomes. Kinetic analysis revealed that V_{max}/K_m value of liver microsomes was nearly the same as that of small intestine microsomes (Table 1). To estimate the *in vitro* intrinsic clearance (CL_{int}) of the reaction, we used the following equation (eq. 1):

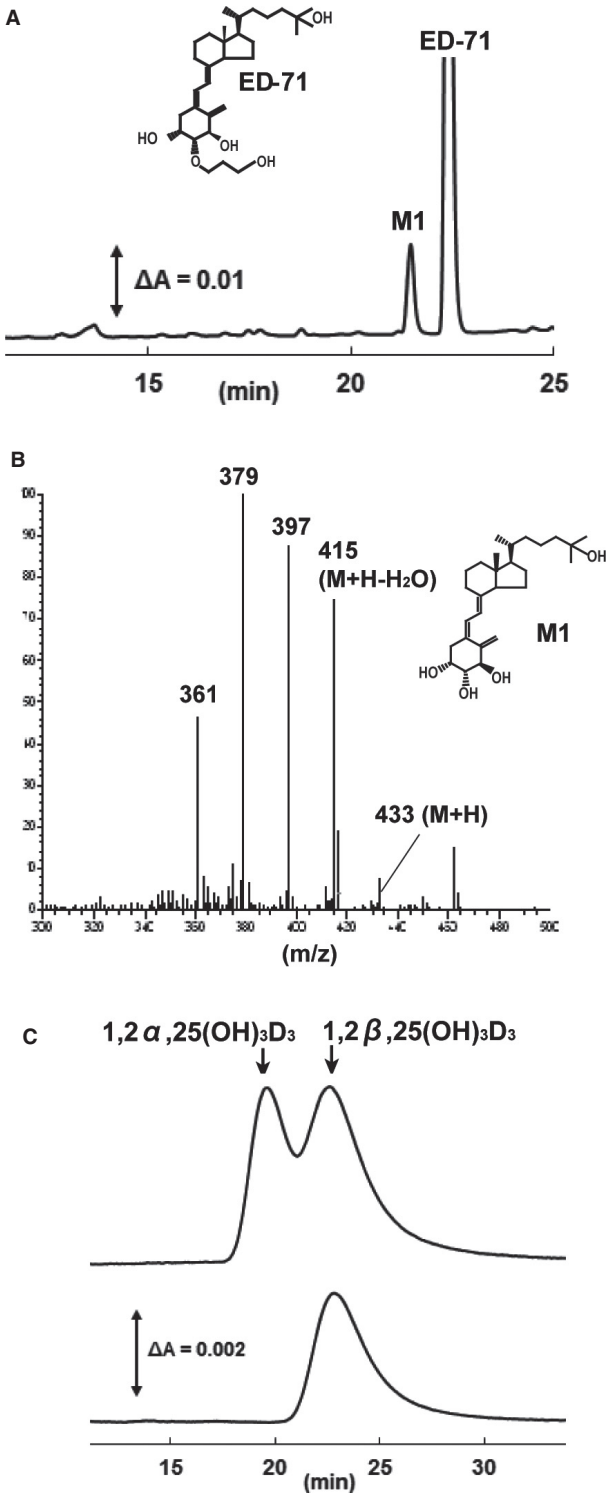

Figure 2. HPLC profile of ED-71 and its metabolite (M1) in human liver microsomes (A) and mass spectrum of M1 (B). The equal amounts of authentic standards of 1α.2α,25$(OH)_3D_3$ and 1α.2β,25$(OH)_3D_3$ were analyzed by HPLC using a chiral cyclodextrin column (C, upper). The retention time of M1 was identical with that of 1α.2β,25$(OH)_3D_3$ (C, lower).

Table 1. Kinetic parameters of human liver or small intestine microsomes.

Substrate		V_{max} (nmol/mg protein per minute)	K_m (μmol/L)	V_{max}/K_m	CL_{int} (mL/min per kg)
ED-71	Liver	0.45 ± 0.092	66 ± 22	0.0068	6.1
	Small intestine	0.21 ± 0.045	35 ± 15	0.0060	0.54

$$CL_{int} = \frac{V_{max}}{K_m} \times \frac{\text{milligram of microsomal protein}}{\text{gram of tissue}} \times \frac{\text{gram of tissue}}{\text{killogram of body weight}} \quad (1)$$

We calculated the CL_{int} values for the dehydroxypropylation of ED-71 from human liver microsomes (45 mg microsomal protein/g liver and 20 g liver/kg body weight) and the human small intestinal microsomes (3 mg microsomal protein/g intestine and 30 g intestine/kg body weight) (Soars et al. 2002). The CL_{int} values for ED-71-dehydroxypropylation in human liver and intestine were 6.1 and 0.54, respectively, (Table 1), suggesting the metabolism of ED-71 in the liver is more important than that in the intestine. No metabolites were detected in the presence of UDP-glucuronic acid in the liver microsomes, suggesting that ED-71 was hardly metabolized by UGTs.

Identification of P450-isoform responsible for ED-71 *O*-dehydroxypropylation in the Liver

To identify the CYP species responsible for the metabolism in humans, ED-71 was metabolized by recombinant human P450 enzymes expressed in *S. cerevisiae* cells. Among 11 isoforms (CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, and 3A4), the metabolite was detected only by CYP3A4, at the same retention time of M1 (21.4 min). The same metabolite was also detected in the recombinant human CYP3A4 microsomes prepared from the baculoviruses-infected insect cells (Fig. 3A). HPLC analysis using a chiral dextrin column and LC-MS analysis suggested that the metabolite was 1α,2β,25 $(OH)_3D_3$. These results indicate that CYP3A4 is the enzyme responsible for *O*-dehydroxypropylation of ED-71 in human liver. To confirm this assumption, we examined the inhibitory effect of the CYP3A4-specific inhibitor, ketoconazole, on ED-71 *O*-dehydroxypropylation activity in human liver microsomes. As shown in Figure 3B-a, ED-71 *O*-dehydroxypropylation activity in human liver microsomes was reduced to 64% in the presence of 1 μmol/L of ketoconazole, whereas recombinant human CYP3A4-dependent activity was reduced to 3% with

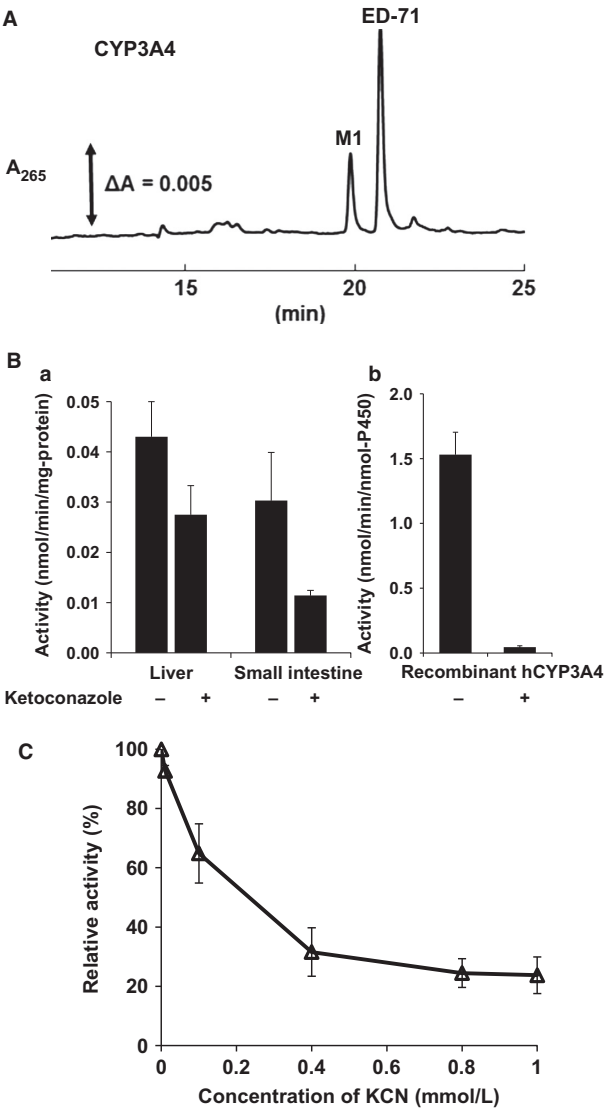

Figure 3. HPLC profile of ED-71 and its metabolite (M1) by recombinant human CYP3A4 (A). ED-71 metabolism in human liver and small intestine microsomes (a) and recombinant human CYP3A4-dependent metabolism of ED-71 (b) were inhibited by 1 μmol/L of ketoconazole (B). The relative ED-71 *O*-dehydroxypropylation activity in human liver microsomes in the presence of 0.01, 0.1, 0.4, 0.8, and 1.0 mmol/L of potassium cyanide (C).

1 μmol/L ketoconazole (Fig. 3B-b). These results suggest that the contribution of CYP3A4 is less than 40% of total ED-71 *O*-dehydroxypropylation activity in human liver. In contrast, ED-71 *O*-dehydroxypropylation activity in human small intestine microsomes was reduced to 37% with 1 μmol/L ketoconazole (Fig. 3B-a), suggesting that the degree of contribution of CYP3A4 in human small intestine is more than 60%.

To identify another enzyme to catalyze ED-71 *O*-dehydroxypropylation, we examined the coenzyme dependence of ED-71 *O*-dehydroxypropylation in human liver. The activity in human liver microsomes at 5 μmol/L ED-71 with 1 mmol/L NADH or NADPH was 0.030 ± 0.005 and 0.043 ± 0.007 (nmol/min/mg-protein), respectively. Furthermore, this reaction was markedly inhibited by potassium cyanide (Fig. 3C). The *O*-dehydroxypropylation activity was reduced to 22% in the presence of 1 mmol/L potassium cyanide, whereas the CYP3A4-specific testosterone 6-hydroxylation activity in human liver microsomes was reduced to 62%. These results strongly suggest that the enzyme responsible for ED-71 *O*-dehydroxypropylation in human liver uses both NADPH and NADH as cofactors. We found that these enzymatic properties correspond to those of SC4MOL that catalyzes C4-demethylation of C4-methylsterols

Expression of SC4MOL in COS7 cells and SC4MOL-dependent metabolism of ED-71

To confirm our assumption that SC4MOL is involved in ED-71 metabolism, we have cloned human SC4MOL cDNA, and expressed it in COS7cells. Western blot analysis of COS7 cells expressing SC4MOL with His-Tag at C-terminus using anti His-Tag antibody indicated a specific band at 36 kDa (Fig. 4). This value coincides with the calculated molecular weight of human SC4MOL containing the His-tag ($6 \times$ His). When ED-71 was added to the medium, a major metabolite was detected at the retention time of 21.4 min, which was the same as that of $1\alpha,2\beta,25(OH)_3D_3$ (Figs. 2 and 4) while the control COS7 cells showed no metabolite. These results strongly suggest that human SC4MOL expressed in the COS7 cells derived from Simian kidney cells had the activity to convert ED-71 to $1\alpha,2\beta,25(OH)_3D_3$.

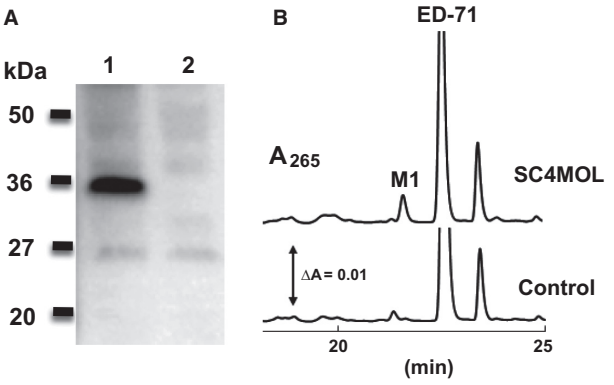

Figure 4. Western blot analysis of human SC4MOL protein expressed in COS-7 cells (A) (lane 1: COS-7 cells transfected with SC4MOL expression vector pEBMulti-Neo-SC4MOL, lane 2: control COS-7 cells, Marker: Pre-Stained Protein Markers [Broad Range] [Nacalai Tesque, Inc., Japan]). Anti-His-Tag IgG was used for detection as described in Materials and Methods. HPLC profiles of ED-71 and its metabolite (M1) in control COS-7 cells or COS-7 cells expressing human SC4MOL (B).

Metabolism of ED-71 by Whole-cell fraction of recombinant yeast and *E. coli* expressing SC4MOL

Human SC4MOL cDNA was also expressed in *S. cerevisiae* and *E. coli* cells to confirm the activity of SC4MOL. The whole-cell fraction of recombinant yeast or *E. coli* cells expressing SC4MOL was examined for metabolism of ED-71. On both expression systems, one major metabolite was detected at the retention time of 21.4 min, which was the same as that of $1\alpha,2\beta,25(OH)_3D_3$ (Figs. 5 and 6) (Li and Kaplan 1996).

We further examined the metabolism of ED-71 in microsomal fraction prepared from the recombinant yeast cells expressing SC4MOL in the presence of NADH or NADPH. As shown in Figure 4B, the metabolite designated as M1 was detected, whereas no metabolite was detected in the control microsomal fraction. The activity in the microsomal fraction of recombinant yeast expressing SC4MOL with 5 μmol/L ED-71 was 3.5 ± 0.4 and 2.9 ± 0.5 (pmol/min per mg protein) in the presence of NADH and NADPH, respectively. This metabolite was not detected in the presence of 1 mmol/L of cyanide. These results strongly suggested that SC4MOL catalyzed conversion of ED-71 into $1\alpha,2\beta,25(OH)_3D_3$. The V_{max} and K_m values in the presence of NADH were estimated to be 19 ± 1 (pmol/min/mg protein) and 19.5 ± 3.0 μmol/L, respectively (Table 2).

Metabolism of ED-71 and $1\alpha,2\beta,25(OH)_3D_3$ (M1) by human CYP24A1

Human CYP24A1-dependent metabolism of ED-71 was studied using recombinant human CYP24A1 expressed in *E. coli* cells. As shown in Figure 7A, two major metabolites were detected at the retention times of 17.9 and 18.2 min. Based on our previous studies on human CYP24A1-dependent metabolism of $1\alpha,25(OH)_2D_3$ and its derivatives, we assumed that they would be 23- and 24-hydroxylated metabolites of ED-71 (Akiyoshi-Shibata

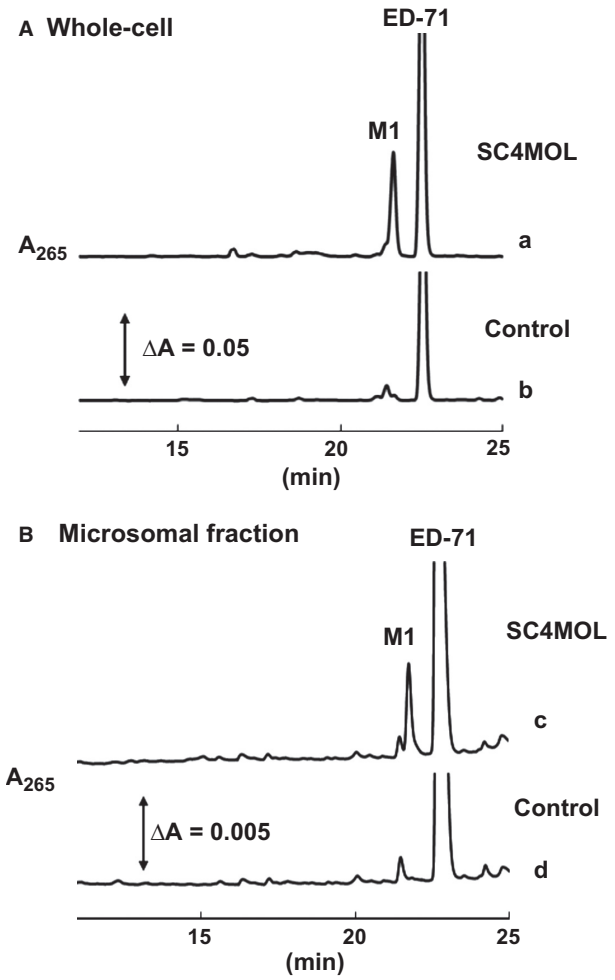

Figure 5. HPLC profiles of ED-71 and its metabolite (M1) in the whole-cell (A) and microsomal fractions (B) prepared from recombinant *Saccharomyces cerevisiae* cells expressing human SC4MOL.

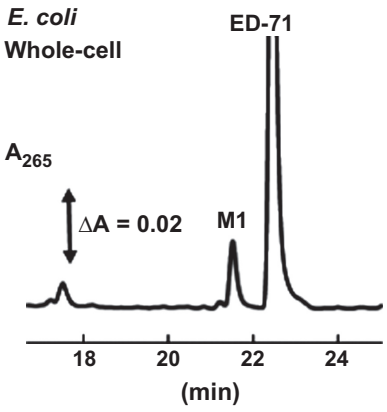

Figure 6. HPLC profile of ED-71 and its metabolite (M1) produced in the recombinant *Escherichia coli* cells expressing human SC4MOL.

Table 2. Kinetic parameters of each recombinant microsomes.

CYP3A4	
k_{cat} (min^{-1})	8.8 ± 1.2
K_m (μmol/L)	27.2 ± 7.3
k_{cat}/K_m	0.32
SC4MOL	
V_{max} (pmol/mg protein per minute)	19 ± 1
K_m (μmol/L)	19.5 ± 3.0
V_{max}/K_m	1.0

et al. 1994; Sakaki et al. 1999a, 2000; Kusudo et al. 2004; Sawada et al. 2004; Abe et al. 2005). The authentic standards of 24(*R*)OH-ED-71 and 24(*S*)OH-ED-71 showed the retention time of 18.2 min (data not shown), which was the same with that of M3. To confirm their chemical structures, we collected them separately in the effluents from HPLC, and subjected them to mass spectrometric analysis. As shown in Figure 7C and 7D, molecular ion and major ion fragments of both M2 and M3 were shown at m/z 507 (M+H), 489 (M+H-H_2O), and 471 (M+H-$2H_2O$). The fragment ion at m/z 413 (M+H-76-H_2O), 395 (M+H-76-$2H_2O$), and 377 (M+H-76-$3H_2O$) showing a loss of a 3-hydroxypropoxy group were also observed in mass spectrum of both M2 and M3 (Fig. 7C-D). In contrast, the fragment ions at m/z 339 (M+H-76-74-H_2O), 321 (M+H-76-74-$2H_2O$), 303 (M+H-76-74-$3H_2O$), which are generated by the cleavage between C-23 and C-24 and are characteristic of the 23-hydroxylated compounds as described previously (Kusudo et al. 2004), were only observed in M2 (Fig. 7C and D). These results strongly suggested that M2 and M3 were 23(OH)-ED-71 and 24(OH)-ED-71, respectively. To confirm this assumption, periodate oxidation of M2 and M3 was performed. As shown in Figure 8, M3 was converted into the lower polarity compound designated as M10 by periodate oxidation treatment, whereas M2 was not converted. The mass spectrum of M10 showed a molecular ion and fragment ions at m/z 447 (M+H), 429 (M+H-H_2O), 411 (M+H-$2H_2O$), 353 (M+H-76-H_2O), and 335 (M+H-76-$2H_2O$) (Fig. 8). Based on our previous study (Kusudo et al. 2004; Urushino et al. 2009), M10 appeared to be 24-oxo-25,26,27-trinor-ED-71, which is produced by cleaving C24-C25 bond by oxidation. This result agrees with our assumption that M3 is 24(OH)-ED-71. Furthermore, we analyzed M3 with authentic standards of 24(*S*)OH-ED-71 and 24(*R*)OH-ED-71 using chiral HPLC column. Retention time of M3 and authentic standards of 24(*S*)OH-ED-71 and 24(*R*)OH-ED-71 were 27.3, 25.3 and 27.3 min, respectively, suggesting that M3 was 24(*R*)OH-ED-71 (Fig. 7B). It should be noted that 23(OH)-ED-71 was also observed as a metabolite of ED-71 by human CYP24A1 while Ono et al. (2006) had not predicted it as a metabolite of ED-71. In the metabolism of ED-71 by

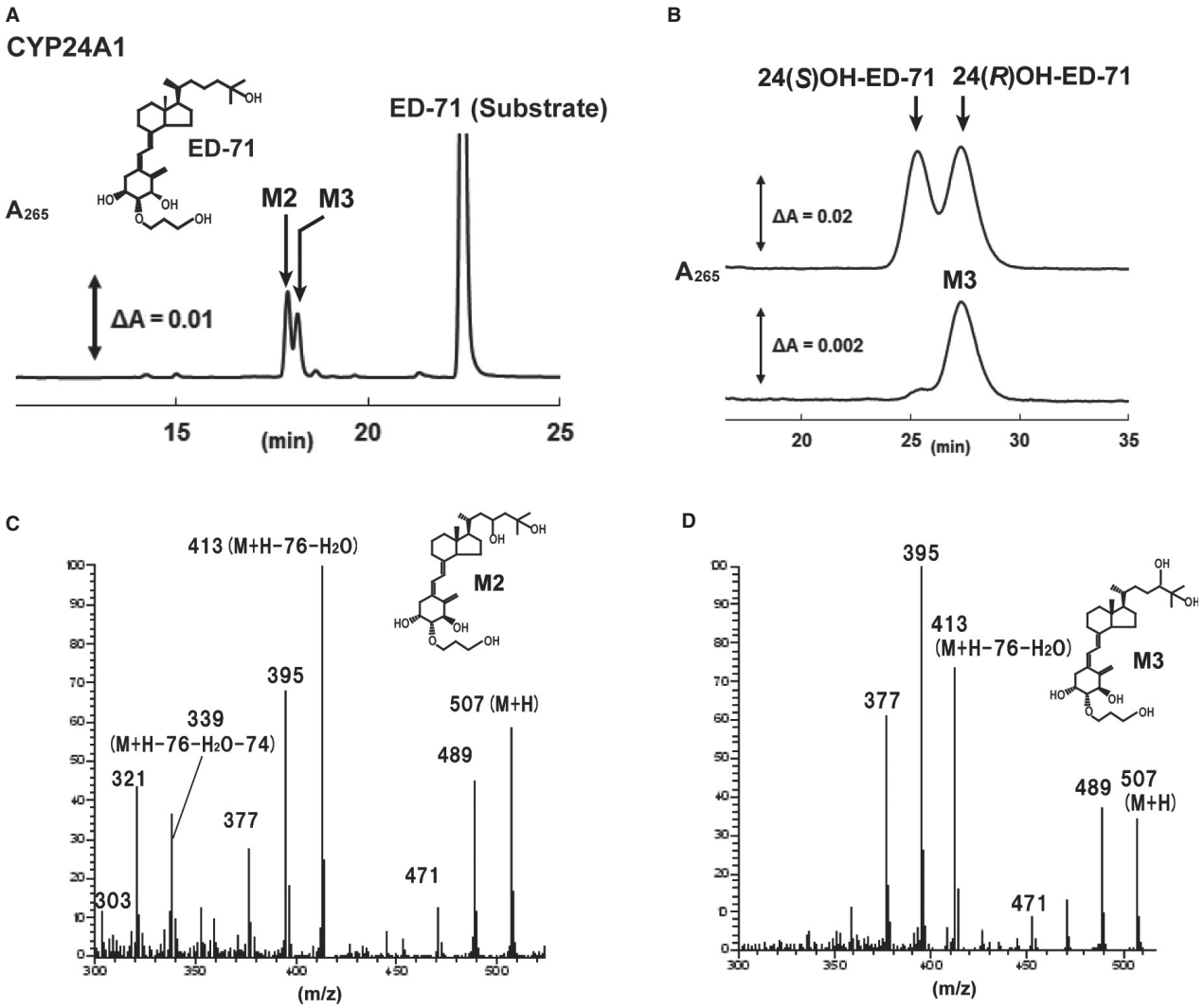

Figure 7. HPLC profiles of ED-71 and its metabolites (M2 and M3) by recombinant human CYP24A1 expressed in *Escherichia coli* cells (A and B) and mass spectra of M2 (C) and M3 (D). The equal amounts of authentic standards of 24(S)-hydroxy- and 24(R)-hydroxy-ED-71 were analyzed by HPLC using a chiral cyclodextrin column (B, upper). The retention time of the metabolite M3 was identical with that of 24(R)-hydroxy-ED-71 (B, lower).

rat CYP24A1, 24(OH) ED-71 was a major metabolite, and the ratio between 23(OH) ED-71 and 24(OH) ED-71 was 12: 88 (Fig. 9). Thus, it is possible that i*n vivo* studies using rats lack 23(OH) ED-71 as a metabolite of ED-71.

Next, we examined the metabolism of 1α,2β,25$(OH)_3D_3$ (M1) by CYP24A1. Figure 10A shows human CYP24A1-dependent metabolism of 1α,2β,25$(OH)_3D_3$. At least six metabolites were detected, whereas only two metabolites were detected when ED-71 was used as a substrate (Fig. 7A). HPLC profile of the metabolites was quite similar to that of the metabolites of 1α,25$(OH)_2D_3$ or its A-ring analogs as shown in our previous studies (Kusudo et al. 2004; Sawada et al. 2004). Based on our previous results, we could speculate their structures as follows; M4: 1α,2β,23,25,26$(OH)_5D_3$, M5: 24-oxo-1α,2β,23,25$(OH)_4D_3$, M6: 1α,2β,23,25$(OH)_4D_3$, M7: 1α,2β,24,25$(OH)_4D_3$, M8: 24-oxo-1α,2β,25$(OH)_3D_3$, M9: 25,26,27-trinor-24-ene-1α,2β$(OH)_2D_3$. Mass spectral analysis confirmed our assumption (Fig. 10). Figure 11 shows putative metabolic pathways of 1α,2β,25$(OH)_3D_3$ by human CYP24A1, which suggests that 1α,2β,25$(OH)_3D_3$ is metabolized in a manner similar to 1α,25$(OH)_2D_3$.

The kinetic parameters of CYP24A1 for ED-71 and 1α,2β,25$(OH)_3D_3$ were shown in the Table 3. The k_{cat}/K_m value for ED-71 was only 3% of that for 1α,25$(OH)_2D_3$, suggesting that ED-71 was remarkably resistant to CYP24A1-dependent metabolism, compared with 1α,25$(OH)_2D_3$. The K_m values for 1α,2β,25$(OH)_3D_3$ was 10.2 ± 2.9 μmol/L, which was nearly the same as that for

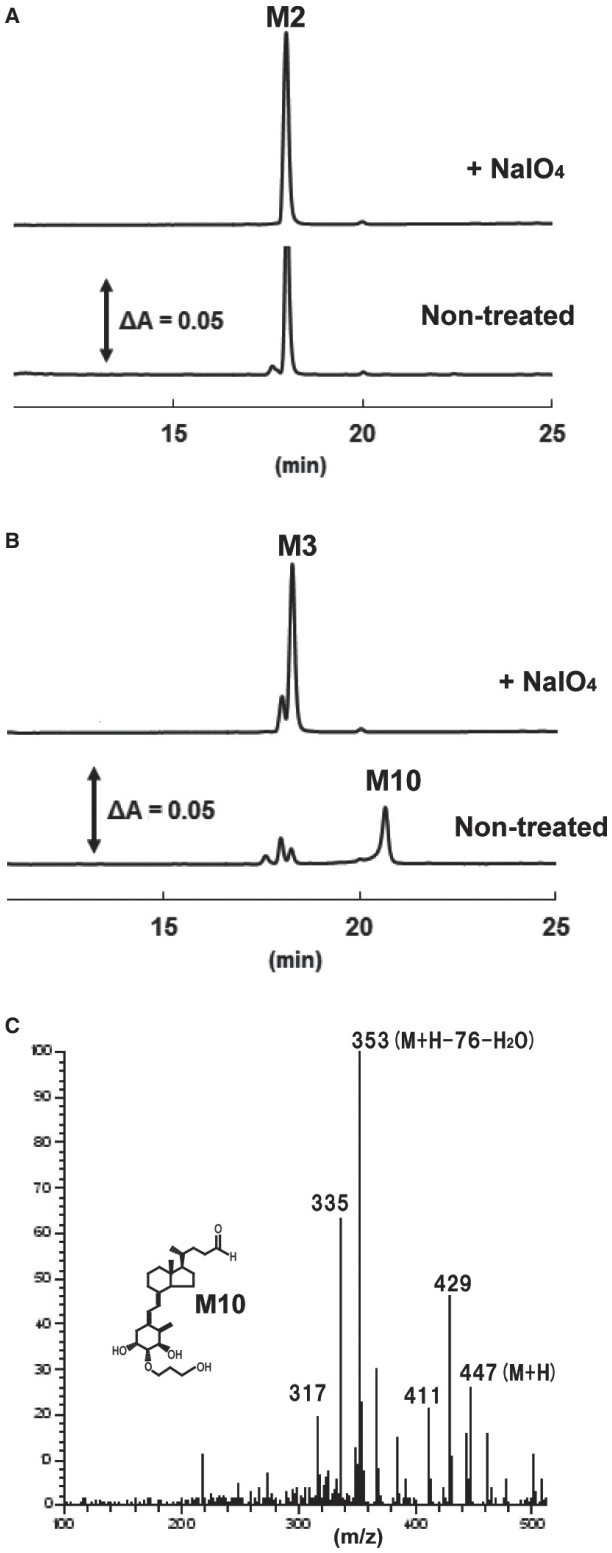

Figure 8. HPLC analysis of the metabolites M2 (A) and M3 (B) with or without periodate oxidation treatment. The mass spectrum of M10 produced from M3 by periodate oxidation treatment (C).

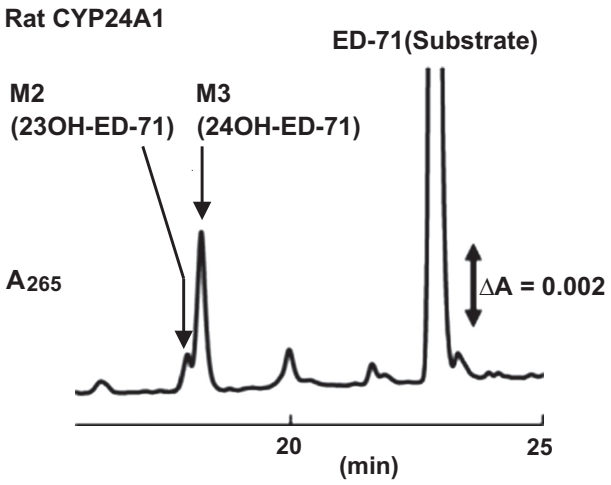

Figure 9. HPLC analysis of metabolites of ED-71 catalyzed by recombinant rat CYP24A1 expressed in *Escherichia coli* cells. The retention times of the metabolites were identical with those of M2 (23OH-ED-71) and M3 (24OH-ED-71) shown in Figure 7.

ED-71, but k_{cat} values for 1α,2β,25(OH)$_3$D$_3$ was 3.5-times higher than that for ED-71. Thus, the k_{cat}/K_m value for 1α,2β,25(OH)$_3$D$_3$ was 3.5-times higher than that for ED-71. These results indicate that the major metabolite of ED-71, 1α,2β,25(OH)$_3$D$_3$, is a better substrate of human CYP24A1 than ED-71 itself, although it is a significantly worse substrate of CYP24A1 than 1α,25(OH)$_2$D$_3$.

Comparison of VDR-affinity and metabolism between ED-71 and its major metabolite in the liver

To evaluate the biological effects of 1α,2β,25(OH)$_3$D$_3$, we examined its VDR-binding affinity. Figure 12 shows comparison of VDR-binding affinity among 1α,25(OH)$_2$D$_3$, ED-71, and 1α,2β,25(OH)$_3$D$_3$. The concentrations of 1α,25(OH)$_2$D$_3$, ED-71, and 1α,2β,25(OH)$_3$D$_3$ for 50% B/B$_{max}$ values were 3.6, 4.4, and 3.9 nmol/L, respectively. Thus the VDR affinity of ED-71 and 1α,2β,25(OH)$_3$D$_3$ was 82 and 92% as compared with that of 1α,25(OH)$_2$D$_3$. These results demonstrated that 1α,2β,25(OH)$_3$D$_3$ had almost the same affinity for VDR with 1α,25(OH)$_2$D$_3$.

Discussion

ED-71 is a second-generation vitamin D analog which has recently been approved as a very promising drug for the treatment of osteoporosis. Phase III clinical trials revealed that ED-71 has a strong inhibitory effect on bone resorption and causes a significant increase in BMD. In addition, the incidence of vertebral and wrist fractures was

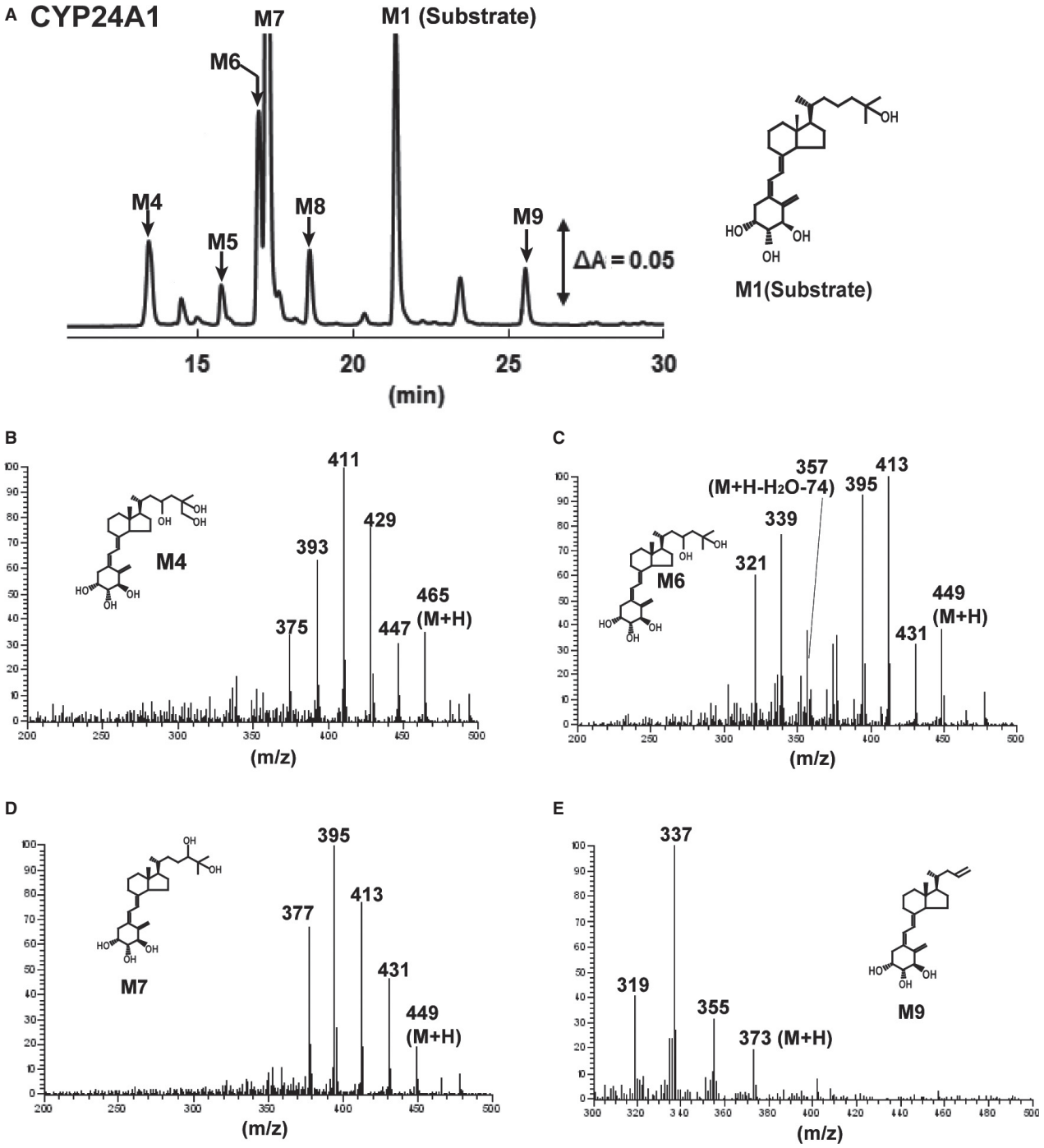

Figure 10. HPLC profiles of 1α,2β,25(OH)$_3$D$_3$ (M1) and its metabolite (M4-M9) by recombinant human CYP24A1 expressed in *Escherichia coli* cells (A). Mass spectra of M4 (B), M6 (C), M7 (D), and M8 (E), and their putative structures are shown.

significantly reduced in the group administrered ED-71 (0.75 μg/day) compared with the group administered alfacalcidol (1α-hydroxyvitamin D$_3$, which has been approved for the treatment of osteoporosis in Japan) (1.0 μg/day). Alfacalcidol is known to be converted into 1α,25(OH)$_2$D$_3$ in the liver by CYP27A1 to function as a VDR ligand. ED-71 showed slightly lower affinity for VDR and remarkably higher affinity for DBP than 1α,25(OH)$_2$D$_3$. However, the most remarkable feature of ED-71 appears to be resistance to CYP24A1-dependent metabolism. Recent studies have shown that 1α,25(OH)$_2$D$_3$ is completely degraded by CYP24A1-dependent

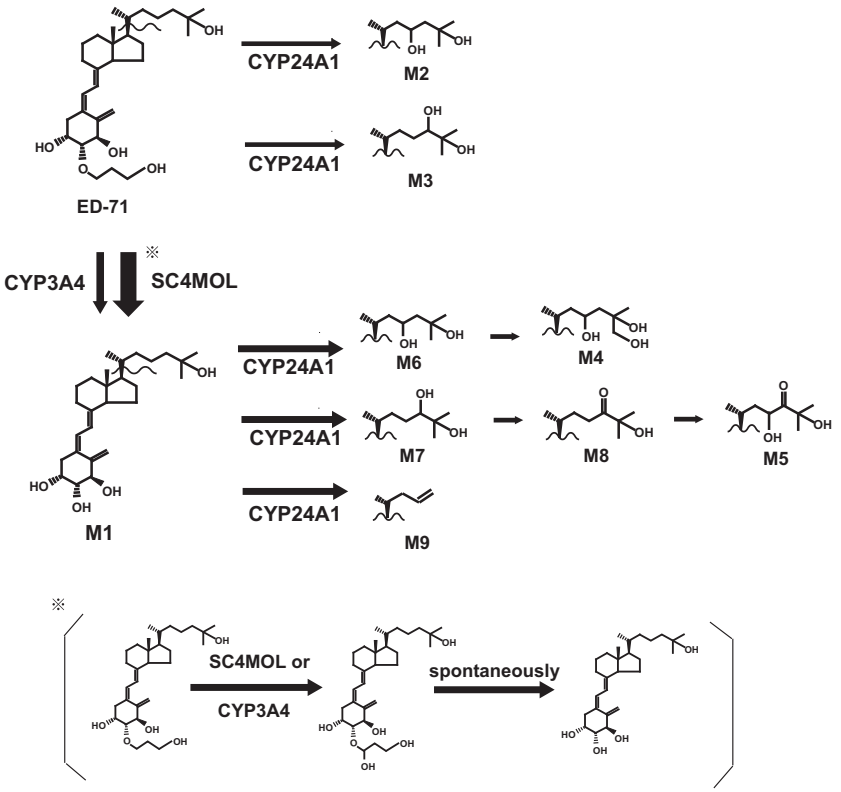

Figure 11. Putative metabolic pathways of ED-71 in humans by SC4MOL, CYP3A4 and CYP24A1. Microsomal SC4MOL and CYP3A4 are mainly localized in the liver, while mitochondrial CYP24A1 is mainly localized in the kidneys.

Table 3. Kinetic parameters of CYP24A1 for each substrate.

	k_{cat} (min^{-1})	K_m (μmol/L)	k_{cat}/K_m	Relative k_{cat}/K_m
ED-71	10.7 ± 2.3	9.9 ± 3.7	1.1	0.034
1α,2β,25$(OH)_3D_3$ (M1)	38.1 ± 5.7	10.2 ± 2.9	3.7	0.12
1α,25$(OH)_2D_3$	20.0 ± 1.5	0.64 ± 0.13	31	1

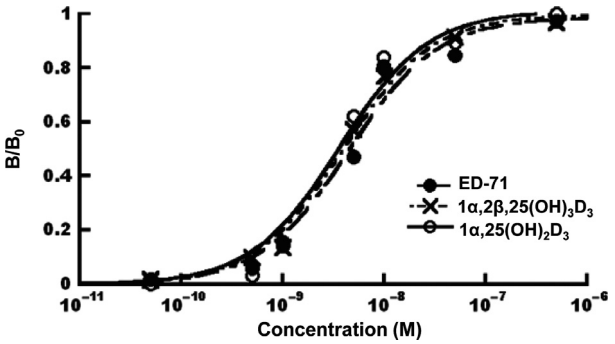

Figure 12. Comparison of VDR affinity among 1α,25$(OH)_2D_3$, ED-71, and 1α,2β,25$(OH)_3D_3$ (M1). The concentrations of 1α,25$(OH)_2D_3$, ED-71, and 1α,2β,25$(OH)_3D_3$ for 50% B/B_{max} values were 3.6, 4.4, and 3.9 nmol/L, respectively.

multi-step oxidation, but ED-71 was not metabolized in cells despite the transcriptional induction of *CYP24A1* gene by ED-71(Ritter and Brown 2011; Munetsuna et al. 2014). We have shown that several C2α-substituted vitamin D analogs are resistant to CYP24A1 catabolism, suggesting that they tend to retain their active forms in the target tissue longer than native 1α,25$(OH)_2D_3$ (Abe et al. 2005; Yasuda et al. 2013). For example, the k_{cat}/K_m value of CYP24A1 for O2C3 (a C2-epimer of ED-71) was only 3% of that for 1α,25$(OH)_2D_3$ (Abe et al. 2005). As shown in Figure 1, ED-71 has a hydroxypropoxy group at the C2β position of 1α,25$(OH)_2D_3$, and its k_{cat}/K_m value of CYP24A1 for ED-71 was 3% of that of 1α,25$(OH)_2D_3$, similarly to O2C3. Thus, the resistance to CYP24A1-dependent catabolism could be a key property of ED-71 that prolongs its effects in target cells (Sakaki et al. 2013).

Preclinical and clinical studies have demonstrated that 1α,2β,25$(OH)_3D_3$ is the major circulating metabolites of ED-71 (Ono 2013). Our previous study demonstrated that O2C3 was metabolized to 2-dehydroxypropylated metabolite, 1α,2α,25$(OH)_3D_3$, in human liver microsomes by CYP3A4. Thus, we expected that ED-71 could be metabolized to 1α,2β,25$(OH)_3D_3$ by CYP3A4. As expected, the recombinant CYP3A4 converted ED-71 into 1α,2β,25$(OH)_3D_3$, while the V_{max}/K_m value for ED-71 *O*-dehydroxypropylation was 28-fold smaller than that for O2C3 dehydroxypropylation. Based on these results, we hypothesized that CYP3A4 is a major enzyme for ED-71 metabolism in the human liver. However, a CYP3A4-specific inhibitor, ketoconazole, inhibited only 36% of ED-71 *O*-dehydroxypropylation activity in human liver microsomes, whereas the same concentration of ketoconazole almost completely inhibited O2C3 *O*-dehydroxypropylation (Yasuda et al. 2013). These results suggest that the degree of contribution of CYP3A4 to ED-71 metabolism in the liver is approximately 40%, and that other enzyme (s) located in liver microsomes are also involved in metabolism of ED-71.

We found that the conversion of ED-71 to 1α,2β,25$(OH)_3D_3$ in human liver or intestine microsomes was strongly inhibited by potassium cyanide as shown in Figure 3. Previous reports demonstrated that a sterol C-4 demethylation enzyme localized in rat liver microsomes was strongly inhibited by cyanide, but was resistant to carbon monoxide (CO), isocyanide, or nicotinamide (Gaylor and Mason 1968; Miller et al. 1971). Recent reports have suggested that human *SC4MOL* (sterol C4 methylsterols oxidase-like) gene product is a homolog of *S.cerevisiae ERG25* gene product (Li and Kaplan 1996). The ERG25 protein had been isolated, and functional studies have shown that it catalyzes sequential removal of the two methyl groups at the ERG26 and 3-keto reductase (ERG27) (Bard et al. 1996; Gachotte et al. 1998, 1999) (Fig. 1). Taken together, these findings indicate that cyanide-sensitive SC4MOL catalyzes multi-step monooxygenation of the 4,4′-dimethyl group of 4,4′-dimethyl-5α-cholest-8en-ol. In addition, we expected that SC4MOL would catalyze monooxygenation (hydroxylation) at the C1 position of the 2β-hydroxypropoxy group of ED-71 to produce 1α,2β,25$(OH)_3D_3$ (Fig. 11). To confirm this assumption, we performed cloning and heterologous expression of human SC4MOL cDNA. It should be noted that SC4MOL is localized on endoplasmic reticulumn (ER) membrane and requires the electron transfer system consisting of NADPH-P450 reductase, NADH b_5-reductase, and cyt b_5 for its activity (Fig. 13) (Kawata et al. 1986). First, we examined the expression of SC4MOL in COS7 cells. Because COS7 is derived from simian kidney cells expression level of endogenous SC4MOL appears low. As expected, control COS7 cells showed no detectable activity while SC4MOL-expressing COS7 cells showed conversion of ED-71 to 1α,2β,25$(OH)_3D_3$. For the kinetic analysis of SC4MOL-dependent ED-71 metabolism, we selected the yeast *S. cerevisiae* as a host, because it possesses endoplasmic reticulumn and the electron transfer system containing NADPH-P450 reductase, NADH- b_5-reductase, and cyt. b_5. Conversion of ED-71 to 1α,2β,25$(OH)_3D_3$ was observed in the whole-cell and microsomal fractions of recombinant yeast cells expressing SC4MOL. Furthermore, addition of human cyt b_5 or human NADPH-P450 reductase to the SC4MOL-containing microsomal fraction increased the SC4MOL activity, suggesting that they donated electrons to SC4MOL. Surprisingly, the same metabolite was also detected in the whole-cell fraction of recombinant *E. coli* cells expressing SC4MOL. Although *E. coli* cells contain no such electron

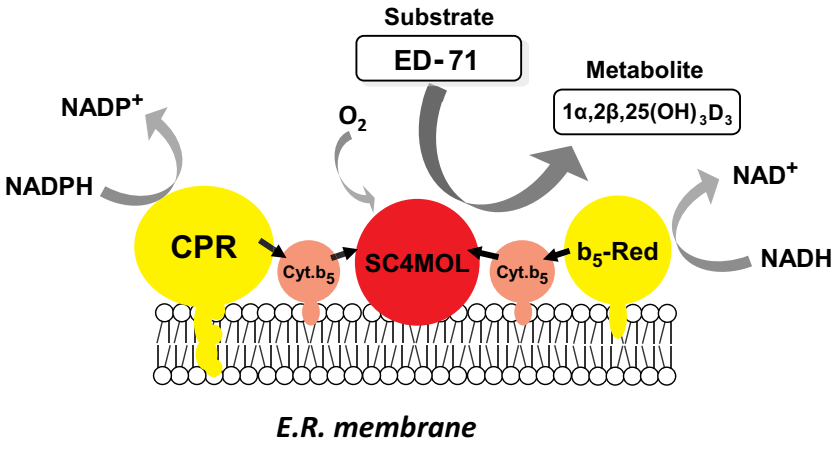

Figure 13. Putative electron transfer chains consisting of NADPH-P450 reductase (CPR), NADH-cyt.b_5 reductase, cyt.b_5, and SC4MOL in human liver. Electrons are transferred from NADPH or NADH to SC4MOL via CPR or NADH-cyt.b_5 reductase to cyt.b_5.

donors, unknown enzymes or ferredoxins have acted as electron donors. Although the expression level of SC4MOL in *E. coli* cells is low in this study, overexpression and crystallization of SC4MOL may make it possible to reveal binding of 4,4′-dimethylzymosterol or ED-71 to the active site of SC4MOL.

In this study, we have identified three enzymes responsible for the metabolism of ED-71. Because the SC4MOL- or CYP3A4-dependent metabolite 1α,2β,25$(OH)_3D_3$ was further metabolized by CYP24A1, many additional metabolites may be produced in the human body (Fig. 11). Of the three enzymes, SC4MOL appeared to be the most important, because some clinical studies have demonstrated that the metabolism of ED-71 is not kidney- but liver-dependent (Ono 2013), and our results demonstrated that SC4MOL made a greater contribution than CYP3A4 in the metabolism of ED-71 in the liver. Because SC4MOL is known to be crucial for cholesterol synthesis (He et al. 2011, 2013), the inhibitory effect of ED-71 on SC4MOL activity in cholesterol synthesis should be considered. However, inhibition of SC4MOL-dependent cholesterol synthesis by ED-71 may be negligible, because the daily intake of ED-71 is only 0.75 μg, which is much less than the required intake of most of prescription drugs. To the best of our knowledge, no reports have been published on the side-effects of ED-71 on cholesterol synthesis.

It remains unclear how many drugs are metabolized by SC4MOL. Recently, we have shown that several vitamin D analogs modified at the C-2 position are metabolized by SC4MOL (data not shown). If drug-metabolizing activity is observed in microsomes, where both NADH and NADPH are effective, and the activity is remarkably inhibited by cyanide but not inhibited by CO, SC4MOL could be responsible for the activity. In addition, if the activity is inhibited by ED-71, involvement of SC4MOL would be confirmed. Thus, ED-71 could be used as a probe drug for SC4MOL. Further studies are needed to evaluate SC4MOL as a drug-metabolizing enzyme. The heterologous expression systems for SC4MOL established in this study appear to be useful for revealing the enzymatic properties of SC4MOL.

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Authorship Contribution

Yasuda, Ikushiro, and Sakaki participated in the research design. Yasuda, Iwanaga, Ogawa, Mano, Ueno, Kimoto, Ohta, and Kamakura conducted the experiments. Yasuda, Ohta, and Sakaki performed the data analysis. Yasuda and Sakaki wrote or contributed to the writing of the manuscript.

Disclosures

None declared.

References

Abe D, Sakaki T, Kusudo T, Kittaka A, Saito N, Suhara Y, et al. (2005). Metabolism of 2 alpha-propoxy-1 alpha,25-dihydroxyvitamin D3 and 2 alpha-(3-hydroxypropoxy)-1 alpha,25-dihydroxyvitamin D3 by human CYP27A1 and CYP24A1. Drug Metab Dispos 33: 778–784.

Akiyoshi-Shibata M, Sakaki T, Ohyama Y, Noshiro M, Okuda K, Yabusaki Y (1994). Further oxidation of hydroxycalcidiol by calcidiol 24-hydroxylase. A study with the mature enzyme expressed in *Escherichia coli*. Eur J Biochem 224: 335–343.

Bard M, Bruner DA, Pierson CA, Lees ND, Biermann B, Frye L, et al. (1996). Cloning and characterization of ERG25, the *Saccharomyces cerevisiae* gene encoding C-4 sterol methyl oxidase. Proc Natl Acad Sci USA 93: 186–190.

Brown AJ, Zhang F, Cooke NE, Ritter CS (2013). The role of the serum vitamin D binding protein in the actions of the vitamin D analog eldecalcitol (ED-71) on bone and mineral metabolism. Calcif Tissue Int 93: 163–171.

Gachotte D, Barbuch R, Gaylor J, Nickel E, Bard M (1998). Characterization of the *Saccharomyces cerevisiae* ERG26 gene encoding the C-3 sterol dehydrogenase (C-4 decarboxylase) involved in sterol biosynthesis. Proc Natl Acad Sci USA 95: 13794–13799.

Gachotte D, Sen SE, Eckstein J, Barbuch R, Krieger M, Ray BD, et al. (1999). Characterization of the *Saccharomyces cerevisiae* ERG27 gene encoding the 3-keto reductase involved in C-4 sterol demethylation. Proc Natl Acad Sci USA 96: 12655–12660.

Gaylor JL, Mason HS (1968). Investigation of the component reactions of oxidative sterol demethylation. Evidence against participation of cytochrome P-450. J Biol Chem 243: 4966–4972.

He M, Kratz LE, Michel JJ, Vallejo AN, Ferris L, Kelley RI, et al. (2011). Mutations in the human SC4MOL gene encoding a methyl sterol oxidase cause psoriasiform dermatitis, microcephaly, and developmental delay. J Clin Invest 121: 976–984.

He M, Smith LD, Chang R, Li X, Vockley J (2013). The role of sterol-C4-methyl oxidase in epidermal biology. Biochim Biophys Acta 1841: 331–335.

Ito H, Fukuda Y, Murata K, Kimura A (1983). Transformation of intact yeast cells treated with alkali cations. J Bacteriol 153: 163–168.

Kawata S, Trzaskos JM, Gaylor JL (1986). Affinity chromatography of microsomal enzymes on immobilized detergent-solubilized cytochrome b5. J Biol Chem 261: 3790–3799.

Kusudo T, Sakaki T, Abe D, Fujishima T, Kittaka A, Takayama H, et al. (2004). Metabolism of A-ring diastereomers of 1alpha,25-dihydroxyvitamin D3 by CYP24A1. Biochem Biophys Res Commun 321: 774–782.

Li L, Kaplan J (1996). Characterization of yeast methyl sterol oxidase (ERG25) and identification of a human homologue. J Biol Chem 271: 16927–16933.

Matsumoto T (2012). Osteoporosis treatment by a new active vitamin D3 compound, eldecalcitol, in Japan. Curr Osteoporos Rep 10: 248–250.

Matsumoto T, Kubodera N (2007). ED-71, a new active vitamin D3, increases bone mineral density regardless of serum 25(OH)D levels in osteoporotic subjects. J Steroid Biochem Mol Biol 103: 584–586.

Matsumoto T, Miki T, Hagino H, Sugimoto T, Okamoto S, Hirota T, et al. (2005). A new active vitamin D, ED-71, increases bone mass in osteoporotic patients under vitamin D supplementation: a randomized, double-blind, placebo-controlled clinical trial. J Clin Endocrinol Metab 90: 5031–5036.

Matsumoto T, Takano T, Yamakido S, Takahashi F, Tsuji N (2010). Comparison of the effects of eldecalcitol and alfacalcidol on bone and calcium metabolism. J Steroid Biochem Mol Biol 121: 261–264.

Matsumoto T, Ito M, Hayashi Y, Hirota T, Tanigawara Y, Sone T, et al. (2011). A new active vitamin D3 analog, eldecalcitol, prevents the risk of osteoporotic fractures–a randomized, active comparator, double-blind study. Bone 49: 605–612.

Miller WL, Gaylor JL (1970). Investigation of the component reactions of oxidative sterol demethylation. Oxidation of a 4 alpha-methyl sterol to a 4 alpha-carboxylic acid during cholesterol biosynthesis. J Biol Chem 245: 5369–5374.

Miller WL, Brady DR, Gaylor JL (1971). Investigation of the component reactions of oxidative demethylation of sterols. Metabolism of 4 -hydroxymethyl steroids. J Biol Chem 246: 5147–5153.

Miyamoto K, Murayama E, Ochi K, Watanabe H, Kubodera N (1993). Synthetic studies of vitamin D analogues. XIV. Synthesis and calcium regulating activity of vitamin D3 analogues bearing a hydroxyalkoxy group at the 2 beta-position. Chem Pharm Bull (Tokyo) 41: 1111–1113.

Munetsuna E, Kawanami R, Nishikawa M, Ikeda S, Nakabayashi S, Yasuda K, et al. (2014). Anti-proliferative activity of 25-hydroxyvitamin D3 in human prostate cells. Mol Cell Endocrinol 382: 960–970.

Ohyama Y, Ozono K, Uchida M, Shinki T, Kato S, Suda T, et al. (1994). Identification of a vitamin D-responsive element in the 5′-flanking region of the rat 25-hydroxyvitamin D3 24-hydroxylase gene. J Biol Chem 269: 10545–10550.

Okano T, Tsugawa N, Masuda S, Takeuchi A, Kobayashi T, Takita Y, et al. (1989). Regulatory activities of 2 beta-(3-hydroxypropoxy)-1 alpha, 25-dihydroxyvitamin D3, a novel synthetic vitamin D3 derivative, on calcium metabolism. Biochem Biophys Res Commun 163: 1444–1449.

Ono Y (2013). Multifunctional and potent roles of the 3-hydroxypropoxy group provide eldecalcitol's benefit in osteoporosis treatment. J Steroid Biochem Mol Biol 139: 88–97.

Ono Y, Watanabe H, Taira I, Takahashi K, Ishihara J, Hatakeyama S, et al. (2006). Synthesis of putative metabolites of 1alpha,25-dihydroxy-2beta-(3-hydroxypropoxy)vitamin D (3) (ED-71). Steroids 71: 529–540.

Ritter CS, Brown AJ (2011). Suppression of PTH by the vitamin D analog eldecalcitol is modulated by its high affinity for the serum vitamin D-binding protein and resistance to metabolism. J Cell Biochem 112: 1348–1352.

Sakaki T, Sawada N, Nonaka Y, Ohyama Y, Inouye K (1999a). Metabolic studies using recombinant *Escherichia coli* cells producing rat mitochondrial CYP24 CYP24 can convert 1alpha,25-dihydroxyvitamin D3 to calcitroic acid. Eur J Biochem 262: 43–48.

Sakaki T, Sawada N, Takeyama K, Kato S, Inouye K (1999b). Enzymatic properties of mouse 25-hydroxyvitamin D3 1 alpha-hydroxylase expressed in *Escherichia coli*. Eur J Biochem 259: 731–738.

Sakaki T, Sawada N, Komai K, Shiozawa S, Yamada S, Yamamoto K, et al. (2000). Dual metabolic pathway of 25-hydroxyvitamin D3 catalyzed by human CYP24. Eur J Biochem 267: 6158–6165.

Sakaki T, Yasuda K, Kittaka A, Yamamoto K, Chen TC (2013). CYP24A1 as a potential target for cancer therapy. Anticancer Agents Med Chem 14: 97–108.

Sawada N, Kusudo T, Sakaki T, Hatakeyama S, Hanada M, Abe D, et al. (2004). Novel metabolism of 1 alpha,25-dihydroxyvitamin D3 with C24-C25 bond cleavage catalyzed by human CYP24A1. Biochemistry 43: 4530–4537.

Soars MG, Burchell B, Riley RJ (2002). In vitro analysis of human drug glucuronidation and prediction of in vivo metabolic clearance. J Pharmacol Exp Ther 301: 382–390.

Tsugawa N, Nakagawa K, Kurobe M, Ono Y, Kubodera N, Ozono K, et al. (2000). In vitro biological activities of a series of 2 beta-substituted analogues of 1 alpha,25-dihydroxyvitamin D3. Biol Pharm Bull 23: 66–71.

Urushino N, Nakabayashi S, Arai MA, Kittaka A, Chen TC, Yamamoto K, et al. (2007). Kinetic studies of 25-hydroxy-19-nor-vitamin D3 and 1 alpha,25-dihydroxy-19-nor-vitamin D3 hydroxylation by CYP27B1 and CYP24A1. Drug Metab Dispos 35: 1482–1488.

Urushino N, Yasuda K, Ikushiro S, Kamakura M, Ohta M, Sakaki T (2009). Metabolism of 1alpha,25-dihydroxyvitamin D2 by human CYP24A1. Biochem Biophys Res Commun 384: 144–148.

Yasuda K, Ikushiro S, Kamakura M, Ohta M, Sakaki T (2010). Metabolism of sesamin by cytochrome P450 in human liver microsomes. Drug Metab Dispos 38: 2117–2123.

Yasuda K, Ikushiro S, Kamakura M, Takano M, Saito N, Kittaka A, et al. (2013). Human cytochrome P450-dependent differential metabolism among three 2alpha-substituted-1alpha,25-dihydroxyvitamin D(3) analogs. J Steroid Biochem Mol Biol 133: 84–92.