Stevastelins, a novel group of immunosuppressants, inhibit dual-specificity protein phosphatases

Takuya Hamaguchi[1], Akira Masuda[2], Tomio Morino[2] and Hiroyuki Osada[1]

Background: Since the molecular target of the immunosuppressive reagents FK506 and cyclosporin A was revealed to be protein phosphatase PP2B (calcineurin), many researchers have been screening the protein phosphatase inhibitors from microbial metabolites to develop new immunosuppressive reagents. We isolated stevastelin B, which is composed of valine, threonine, serine and 3,5-dihydroxy-2,4-dimethyl stearic acid, and stevastelin A, which is a sulphonylated derivative of stevastelin B. To understand the action mechanism of stevastelins A and B, we synthesized a series of stevastelin derivatives and investigated their structure–activity relationships.

Results: A series of stevastelin derivatives have been systematically synthesized. Stevastelin B inhibited gene expression that is dependent on interleukin-2 (IL-2) or IL-6 promoters *in situ*, but it had no inhibitory activity against any protein phosphatases *in vitro*. In contrast, stevastelin A, which is a sulphonylated derivative of stevastelin B, inhibited the phosphatase activity of a dual-specificity phosphatase, VH1-related human protein (VHR), *in vitro*, but it had no inhibitory activity against gene expression or cell-cycle progression *in situ*.

Conclusions: Stevastelin B is a novel immunosuppressant. It inhibited IL-2 or IL-6 dependent gene expression but did not inhibit the phosphatase activity of calcineurin. The structure–activity relationships show that the acidic functional group on the threonine residue and the stearic acid moiety in the stevastelin molecule are important for inhibitory effects on the dephosphorylation activity of VHR *in vitro*. Stevastelin B might be sulphonylated or phosphorylated after incorporation into the target cell, and then it interacts with protein tyrosine phosphatases and regulates cell-cycle progression.

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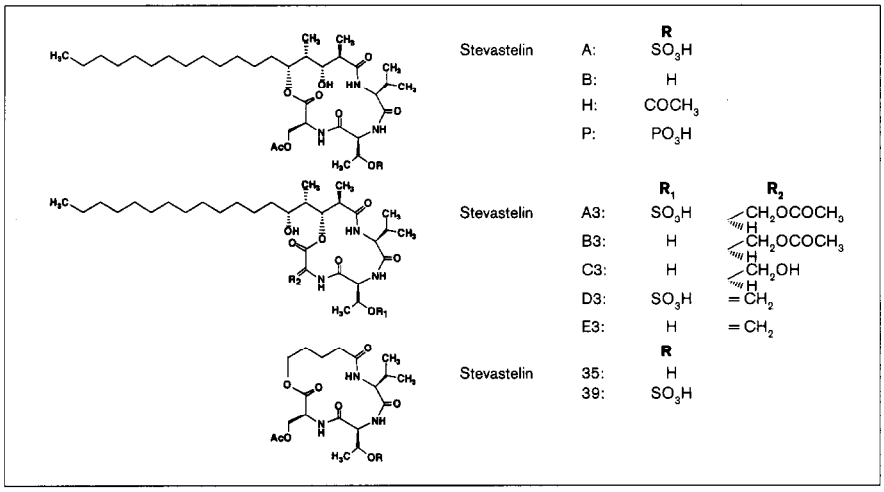
Introduction

Cyclosporin A (CsA) [1,2] and FK506 [3,4] are known to be immunosuppressive compounds that have similar inhibitory effects on T lymphocytes [5]. Both drugs bind to immunophilins, cyclophilin and FKBP, respectively, and inhibit the phosphatase activity of calcineurin (PP2B), which is a Ca^{2+}-dependent and calmodulin-dependent serine/threonine phosphatase [6,7]. In addition, the drugs inhibit the transcription of the interleukin-2 (IL-2) gene, which is essential for the immune response [8,9]. CsA and FK506 also inhibit cell-cycle progression at the G1 phase in normal human T lymphocytes [10,11]. To discover new immunosuppressive reagents from microbial metabolites, we have been screening new phosphatase inhibitors which directly inhibit the PP2B activity or other phosphatase activities. Protein phosphatases are grouped as protein serine/threonine phosphatases (PPases), protein tyrosine phosphatases (PTPases) and dual-specificity phosphatases (DSPases) [12,13]. It is known that protein phosphatases are involved in the process of cytokine signalling from the cell membrane to the nucleus of mammalian cells. In particular, DSPases, including VH1-related human protein (VHR), cdc25, HVH1 and 3CH134, are known to be involved in many aspects of cellular responses, such as cell-cycle progression [14,15], MAP kinase inactivation [16], and growth factor stimulation [17]. Thus, we have been screening inhibitors from microbial metabolites against PPases (PP1 and PP2B), a PTPase (CD45) and a DSPase (VHR).

Recently, stevastelins were isolated by us from the cultured broth of *Penicillium* sp. NK374186 [18,19]. Stevastelins are composed of valine, threonine, serine and a 3,5-dihydroxy-2,4-dimethylstearic acid moiety (Fig. 1). They have growth-inhibition activities against OKT3-stimulated human T cell proliferation, but their biological properties have been unclear.

Here, we report that some of the stevastelins inhibited the dephosphorylation activity of VHR, and some inhibited cell-cycle progression. To clarify the structure–activity relationships of the stevastelins we prepared a series of stevastelin derivatives and evaluated their inhibitory activities both on protein phosphatases and on the cell cycle. We find that the functionality at the threonine

Figure 1



Structures of stevastelins. In stevastelins A3, B3, C3, D3 and E3, cyclization occurred at the C-3 carbon of stearic acid (defined as 'ester position 3'). Other stevastelins had the 'ester position 5'.

residues of stevastelins is particularly important for their inhibitory activities.

Results

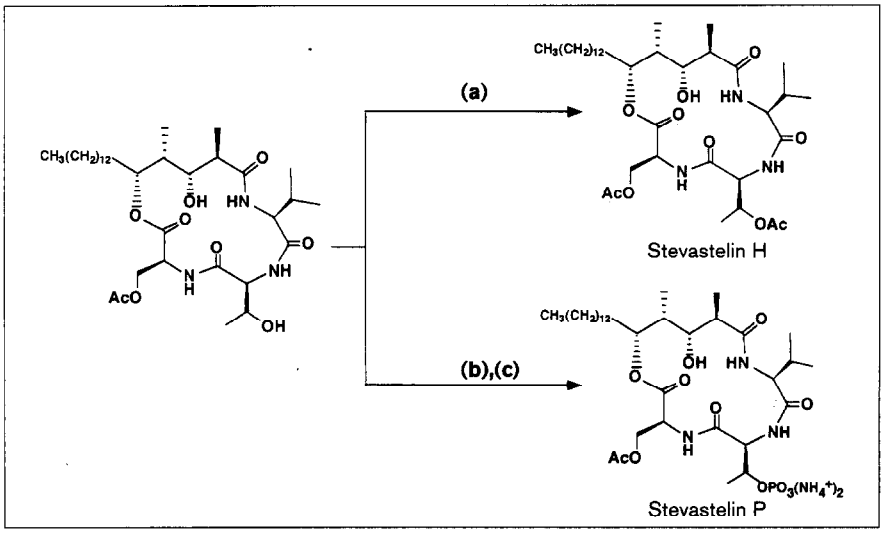
Preparation of stevastelins

Stevastelins A, B, A3, B3, C3, D3 and E3 (Fig. 1) were purified from the fermentation broth of *Penicillium* sp. NK374186 as described previously [18,19]. Stevastelins H and P were obtained by acetylation and phosphorylation, respectively, of the threonine residue of stevastelin B (Fig. 2). Stevastelins 35 and 39 were synthesized using the general chemical methods summarized in Figure 3. Compound **1** was synthesized using the valine and threonine derivatives and coupling agents, and compound **2** was synthesized from 5-hydroxypentanoic acid and *O*-acetylserine by esterification. Stevastelins 35 and 39 have no long-chain aliphatic hydrocarbon group.

Immunosuppressive effects of stevastelins

Stevastelins B, B3, C3 and E3 were originally discovered as inhibitors of the IL-2 and IL-6 promoter-dependent β-galactosidase gene expressions in Jurkat cells, and stevastelins A, A3, and D3 were isolated as the congeners. The inhibitory effects of stevastelins on gene expression in Jurkat cells are summarized in Table 1. Stevastelins B, B3, C3 and E3 (B-type) have a free hydroxyl group in their threonine residues and showed inhibitory activities on gene expression. In contrast, stevastelins A, A3 and D3 (A-type), which have *O*-sulphonylated threonine residues, did not show any inhibitory activities.

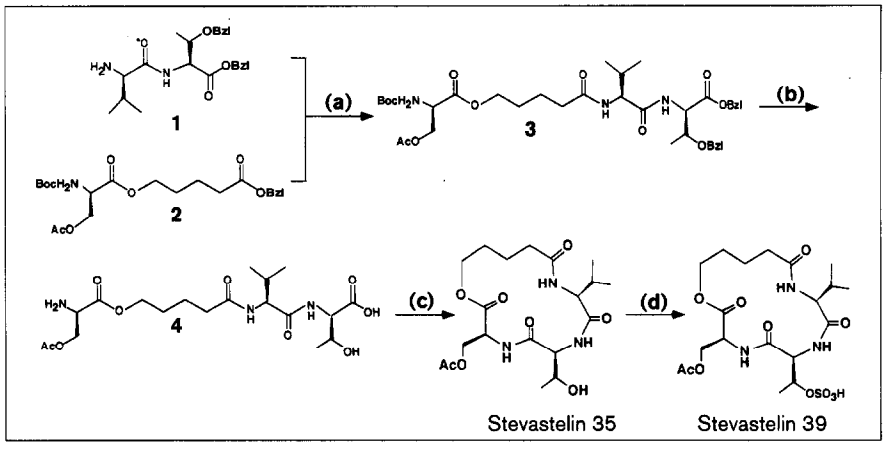
Figure 2



Synthesis of stevastelins H and P. Reagents and conditions: **(a)** $(CH_3CO)_2O$, pyridine, r.t.; **(b)** 1*H*-tetrazole, *N,N* diethyl-1,5-dihydro-2,4,3-benzodioxaphosphepin-3-amine, CH_2Cl_2, r.t., then H_2O; and **(c)** m-chloroperbenzoic acid (mCPBA), CH_2Cl_2, −40°C.

Figure 3

Synthesis of stevastelins 35 and 39. Reagents and conditions: **(a)** HOBt (1-hydroxybenzotriazole hydrate), Et_3N, EDC (1-(3-dimethylaminopropyl)-3-ethylcarbodiimide), DMF, −20°C; **(b)** Pd-C, H_2, EtOH, 60°C; **(c)** HOBt, Et_3N, EDC, DMF, −20°C; and **(d)** sulphur trioxide trimethylamine complex, DMF, 50°C.



To confirm the involvement of the hydroxyl group in the threonine residue in stevastelin B in the biological activity of the compound, we prepared stevastelins P and H which are *O*–phosphorylated and *O*-acetylated derivatives of stevastelin B, respectively. *O*-Acetylation of the threonine residue in stevastelin H slightly reduced the inhibitory activities, and *O*-phosphorylation of this residue (stevastelin P) resulted in the loss of inhibitory activity *in situ*. The acidic functionality of the threonine residue may interfere with the membrane permeability of stevastelins.

Two totally synthesized derivatives, stevastelins 35 and 39, that lack a long-chain aliphatic hydrocarbon group did not have inhibitory activities, regardless of the acidic functionality of the threonine residue. Thus, both the free threonine hydroxyl group and a long-chain aliphatic hydrocarbon group are required for the inhibitory activities against the promoter-dependent gene expression *in situ*.

Table 1

Inhibition of IL-2 and IL-6 promoter-dependent β-galactosidase gene expression by stevastelins.

		Activity [IC_{50} (μM)]	
Stevastelin	Thr-OH	IL-2*	IL-6†
A	sulfonyl	>25	>25
A3	sulfonyl	>25	>25
D3	sulfonyl	>25	>25
P	phosphoryl	>25	>25
39	sulfonyl	>25	>25
H	acetyl	11.5	5.7
B	free	4.6	3.1
B3	free	4.6	3.1
C3	free	4.9	4.9
E3	free	5.0	3.4
35	free	>25	>25
Cyclosporin A		0.042	1.7

*IL-2 promoter-dependent β-galactosidase gene expression was induced by ionomycin plus phorbol myristate acetate (PMA) for 5 h.
†IL-6 promoter-dependent β-galactosidase gene expression was induced by lipopolysaccharide (LPS) for 4 h.

Inhibitory effects of stevastelins on protein phosphatase *in vitro*

To clarify the molecular target for the inhibitory activity of stevastelin B, we investigated the inhibitory effects of stevastelins on protein phosphatases including PP1, PP2A, PP2B, CD45 and VHR. Interestingly, although stevastelins A, A3, and D3 had no inhibitory activity *in situ*, they had strong inhibitory activity against VHR *in vitro* (Table 2). As shown in Figure 4, the inhibition of the dephosphorylation activity of VHR by stevastelin A (IC_{50}=2.7 μM) was more potent than that by stevastelins B (IC_{50}=19.8 μM), stevastelin 35 (IC_{50}>1000 μM) and stevastelin 39 (IC_{50} > 1000 μM).

In contrast, stevastelin B, which caused strong inhibition *in situ*, was about sixfold less active *in vitro* than the analogues that had a modified threonine residue. The acidic functionalities of the threonine residues of stevastelins A (*O*-sulphonyl) and P (*O*-phosphoryl) were found to be essential for inhibitory activities towards VHR *in vitro*. Stevastelins A and A3 are cyclized at the 'ester position 5' and 'ester position 3' (Fig. 1), respectively, which had no influence on the inhibitory activity. Stevastelins 35 and 39, which have no stearic acid moiety, had no inhibitory activity both *in situ* and *in vitro*. Stevastelin P had weak inhibitory activity (IC_{50}= 0.61 μM) against CD45, but other stevastelin derivatives had no activities.

Effects of stevastelins on the cellular phosphoproteins of Ball-1 cells

Next, we investigated the effects of stevastelin derivatives on the tyrosine-phosphorylation level of cellular phosphoproteins in a human B cell leukemia cell line, Ball-1. Cell lysates prepared from Ball-1 cells were treated with various compounds and analyzed by sodium dodecyl

Table 2

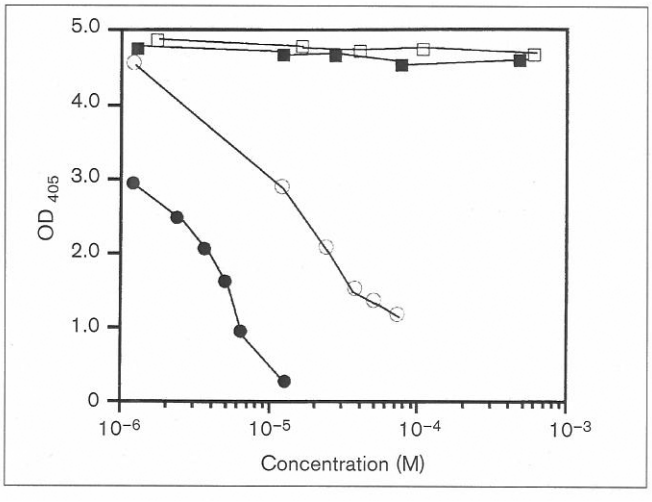
Inhibitory potencies of stevastelins to protein phosphatases.

Group	Stevastelin	Activity* [IC_{50}(μM)]		
		VHR	CD45	PPases†
	A	2.7	>1000	>1000
A-type	A3	3.6	>1000	>1000
potent	D3	1.7	>1000	>1000
inhibitors	P	4.0	160	>1000
	B	19.8	>1000	>1000
B-type	B3	13.7	>1000	>1000
weak	C3	16.0	>1000	>1000
inhibitors	E3	13.6	>1000	>1000
	H	24.0	>1000	>1000
Inactive	35	>1000	>1000	>1000
inhibitors	39	>1000	>1000	>1000

*Protein phosphatases were prepared as described in the Materials and methods section. Inhibitory activities of stevastelins against phosphatase activities to *p*Npp (PP1, PP2A, VHR), ^{32}P-labelled casein (PP2B), and phosphotyrosine (CD45). †PPases represent the inhibitory effects of stevastelins.

sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). The phosphotyrosine level was analyzed by western blotting with an anti-phosphotyrosine antibody (Fig. 5). Stevastelins A (lane 4) and P (lane 6) markedly increased the level of tyrosine phosphorylation of some cellular phosphoproteins as did sodium orthovanadate (vanadate; lane 2) and RK-682 (lane 3). This showed that stevastelins A and P at high concentration inhibited cellular PTPases *in vitro*. In contrast, stevastelin B showed no inhibitory effect towards cellular PTPases in cell-free lysate even at high concentration (lane 5).

Figure 4

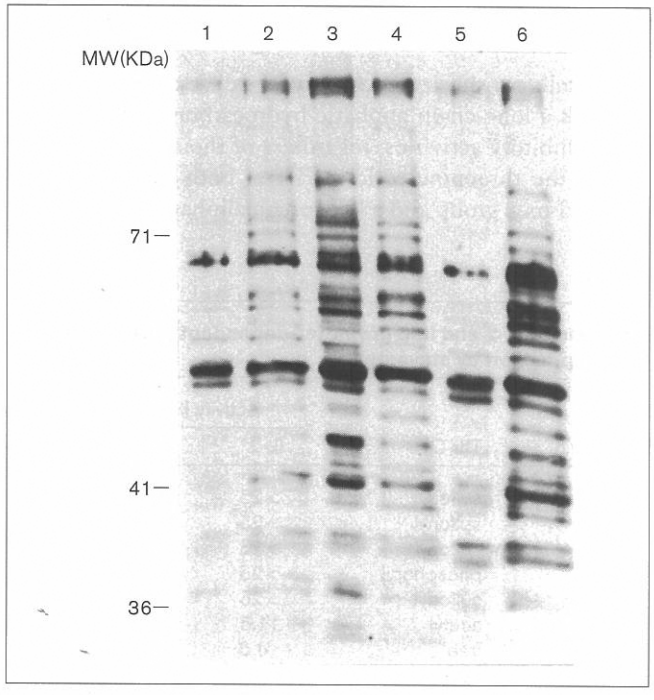


The effects of stevastelins on the dephosphorylation activity of GST–VHR. Inhibitory activity of stevastelin A (●), B (○), 35 (■) and 39 (□). GST–VHR was prepared as described in the Materials and methods section.

Effects of stevastelins on cell-cycle progression

Immunosuppressants as well as protein-phosphatase inhibitors are known to interfere with the cell-cycle progression

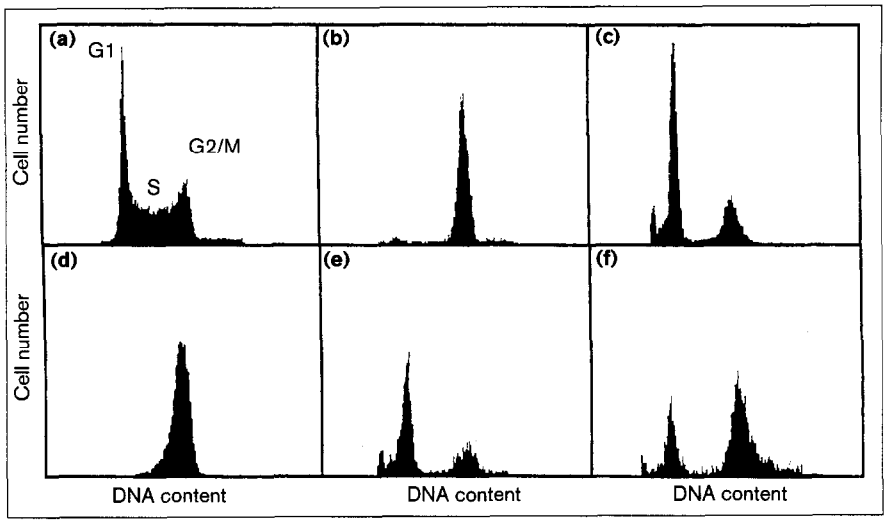
Figure 5



The effects of phosphatase inhibitors on tyrosine phosphorylation in Ball-1 cells. Protein was loaded on a gel (3 μg per lane) for western blotting with anti-phosphotyrosine antibody. Phosphatase inhibitors vanadate (460 μM, lane 2), RK-682 (230 μM, lane 3), stevastelin A (340 μM, lane 4), stevastelin B (340 μM, lane 5) and stevastelin P (340 μM, lane 6) were added to Ball-1 cell lysates. Lane 1 is the control without inhibitor.

Figure 6

The influence of stevastelins on the cell-cycle progression of the tsFT210 cell line. **(a)** tsFT210 cells were cultured at 32°C for 17 h, **(b)** raised to 39°C for 17 h and **(c)** then lowered to 32°C for 4 h after adding inhibitors. Cells were treated with **(d)** vanadate (165 μM), **(e)** stevastelin A (40 μM), and **(f)** stevastelin B (45 μM), and analyzed by flow cytometry.



of mammalian cells. We therefore investigated the inhibitory effects of stevastelins on the cell-cycle progression of tsFT210 cells. (The tsFT210 cell is a temperature-sensitive cdc2 mutant of the mammary carcinoma cell line, FM3A.) The cells grew normally at the permissive temperature (32°C; Fig. 6a) but were arrested in the G2 phase at the nonpermissive temperature (39°C; Fig. 6b). When the temperature was lowered to 32°C after incubation at 39°C for 17 h, the cell cycle progressed into the G1 phase (Fig. 6c). As shown in Figure 6, stevastelin B (Fig. 6f) inhibited the cell-cycle transition from G2/M to G1 phase like vanadate (Fig. 6d), but stevastelin A had no inhibitory effect (Fig. 6e).

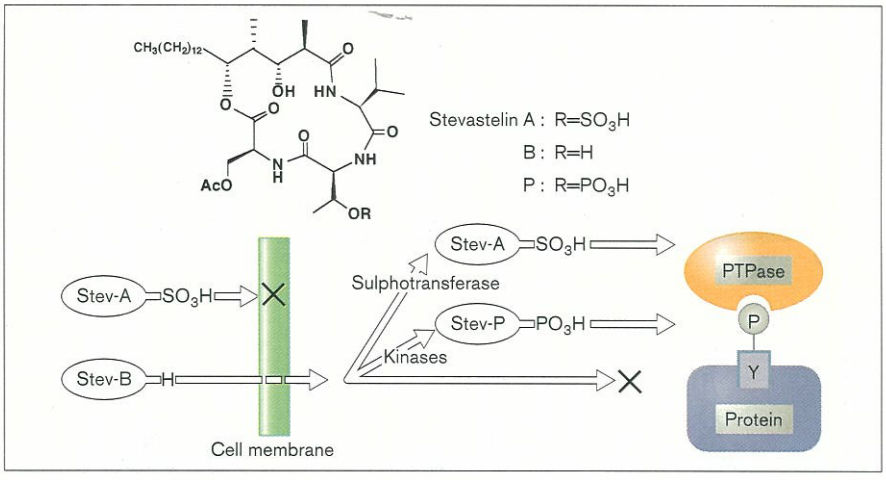
Discussion

FK506 and CsA are valuable reagents not only as immunosuppressants but also as biochemical tools with which to investigate the signal transduction mechanism. It is known that FK506 and CsA bind to immunophilins (FKBPs and cyclophilins) first, then respective FK506–FKBP and CsA–cyclophilin complexes inhibit the serine/threonine phosphatase activity of calcineurin [8,20], and finally, they inhibit the nuclear transport of a cytoplasmic subunit of NFAT in human T cells [21]. These findings stimulated the exploitation of new immunosuppressive compounds from microbial metabolites.

Other specific phosphatase inhibitors are also useful for investigating the signal transduction mechanism in mammalian cells. Indeed, PPase inhibitors, okadaic acid [22] and tautomycin [23] were used to elucidate the physiological role of serine/threonine phosphorylation in signal transduction. In contrast, only vanadate [24], phenylarsine oxide (PAO) [25], dephostatin [26] and RK-682 [14] have been demonstrated to be PTPase inhibitors or dual-specificity phosphatase inhibitors. We have established two bioassay methods to find new immunosuppressants and have started screening for inhibitors of IL-2 and IL-6 dependent gene expression in Jurkat cells and for inhibitors of protein phosphatases. During screening, stevastelin B was originally isolated as an inhibitor of the IL-2 or IL-6 dependent gene expression in Jurkat cells [18], but at that time, stevastelin A was only recognized as an inactive congener of stevastelin B. Now, we have shown that stevastelin A is a phosphatase inhibitor; so, we examined the biological properties and structure–activity relationships of stevastelin derivatives in this study.

B-type stevastelins (B, B3, C3 and E3) were more potent inhibitors of IL-2 and IL-6 dependent gene expression than A-type stevastelins (A, A3, and D3) in Jurkat cells. The two types only differ structurally by the acidic functional group at the threonine residue. To confirm whether the functional group at the threonine residue was involved in the inhibitory action, we synthesized stevastelins H and P from stevastelin B. As a result, it was revealed that the phosphorylation of the hydroxyl group at the threonine residue in stevastelin B may interfere with the membrane permeability and resulted in the loss of its immunosuppressive activity *in situ* (Table 1). In contrast, stevastelin derivatives that possessed an acidic functional group on the threonine residue, and a stearic acid moiety in their structures showed strong inhibition of VHR *in vitro*. These data suggest that A-type stevastelins have poor membrane permeability due to the acidic functionality of the threonine residue, but B-type stevastelins easily permeate the cell membrane. In particular, a long-chain aliphatic hydrocarbon group was important because the totally synthesized compounds, stevastelins 35 and 39, that lacked an aliphatic hydrocarbon chain did not have

Figure 7



Tentative model for the inhibition of phosphatases by stevastelins. Stevastelin A is an inhibitor of VHR, but it may hardly permeate the cell membrane. In contrast, stevastelin B may permeate the cell membrane and arrest cell cycle at the G2 phase. Stevastelin B is converted to an activated form such as stevastelin P by cellular enzyme(s) and interacts with VHR or unidentified PTPases, which regulate the G2/M transition.

any inhibitory activities *in vitro* or *in vivo*, regardless of the acidic functionality of the threonine residue.

Next, we investigated the inhibitory effect of stevastelins on the cell cycle of tsFT210 cells. Because tsFT210 cells are temperature-sensitive mutants of cdc2 kinase [27], the cells are suitable for the cell-cycle analysis of G2/M transition [28]. Stevastelin A had an inhibitory activity towards VHR *in vitro* (Fig. 4) but not towards the cell-cycle progression of tsFT210 cells (Fig. 6e) because it has poor membrane permeability. In contrast, stevastelin B had less activity than stevastelin A *in vitro*, but it inhibited the cell cycle at the G2/M transition (Fig. 6f). These observations suggest that stevastelin B may be phosphorylated or sulphonylated by cellular enzyme(s) after incorporation into the target cells and converted to a form such as stevastelin P or A that is active against phosphatases (Fig. 7). Consequently, the reason why stevastelin H has less of an effect than stevastelin B is presumably because the acetyl group at the threonine residue of stevastelin H interferes with its *in situ* conversion into stevastelin A or P. To investigate this conversion by any cellular serine/threonine kinases, we added stevastelin B to Ball-1 intact cells or cell lysates, which were labelled with ^{32}P–ATP, and analyzed the existence of stevastelin P in the cytosol fraction by thin layer chromatography or autoradiography. We could not detect either stevastelins B or P, however, because a detectable amount of stevastelin B was cytotoxic to Ball-1 intact cells. According to the data shown in Figure 5, it may take time to convert stevastelin B to an active form such as stevastelin P or A in the cell lysate. The acid functionality group at the threonine residue of stevastelins might be needed for inhibitory activity not only towards VHR but also towards cellular PTPase of Ball-1. It is possible that stevastelin B binds to immunophilins and inhibits calcineurin activity *in situ*. This possibility was excluded, however, by results from experiments using the yeast *Saccharomyces cerevisiae* [29]. FK506 and CsA recovered the growth of *S. cerevisiae* on an agar plate containing a high concentration of Ca^{2+}, but all stevastelin derivatives had no effect on the PP2B-dependent growth control in *S. cerevisiae* (data not shown).

Stevastelins are considered to be a novel group of immunosuppressants because their immunosuppressive activity is not due to the inhibition of calcineurin. Thus, stevastelins are potentially useful as bioprobes to elucidate a new signal transduction pathway in eukaryotic cells.

Significance

We have isolated stevastelins A and B from fungal metabolites. To understand their molecular action, a series of derivatives were systematically synthesized and their structure–biological activity relationships were investigated.

Stevastelin B, which was originally isolated as an inhibitor of IL-2 and IL-6 dependent gene expression, did not inhibit calcineurin in yeast or any protein phosphatase activities *in vitro*. Conversely, stevastelin A, a sulphonylated derivative of stevastelin B, potently inhibited the activity of a dual-specificity phosphatase, VHR, *in vitro* but did not show any inhibitory effects *in vivo*.

According to the structure–biological activity relationships, it is suggested that the acidic functional group on the threonine residue and the stearic acid moiety are important for the inhibitory action of stevastelins on VHR *in vitro*. The acidic functional group on the threonine residue in the stevastelin molecule may interfere with permeation of the cell membrane. Thus, stevastelin B is considered to be phosphorylated by cellular enzyme(s) and converted to stevastelins A or P, which have an inhibitory effect on cellular protein phosphatases. These

findings give a clue to understanding the cause of differential activity among stevastelin derivatives and suggest that a dual-specificity phosphatase could be a relevant molecular target for immunosuppressive activity.

Materials and methods

Synthesis of stevastelins H and P

Stevastelin B (42.6 mg, 0.065 mmol) was dissolved in pyridine containing acetic anhydride (30.7 μl, 0.325 mmol). The reaction mixture was stirred at room temperature for 20 h and concentrated. The residue was purified by silica gel column chromatography to give stevastelin H (36.0 mg, 79% yield).

Stevastelin P was obtained by phosphorylation of the threonine residue of stevastelin B. Stevastelin B (61.2 mg, 0.093 mmol) and 1*H*-tetrazole (32.6 mg, 0.465 mmol) were combined and dissolved in CH_2Cl_2 (2 ml). *N,N*-diethyl-1,5-dihydro-2,4,3-benzodioxaphosphepin-3-amine (24.1 μl, 0.112 mmol) was added, and the reaction mixture was stirred at room temperature for 30 min under an argon atmosphere. Then, more *N,N*-diethyl-1,5-dihydro-2,3,4 benzodioxaphosphepin-3-amine (12.0 μl, 0.056 mmol) was added. After a further 20 min reaction under the same condition, H_2O (50 μl) was added and the reaction mixture was stirred for 10 min. The reaction mixture was cooled at −40°C, 3-chloroperbenzoic acid (140 mg, 0.811 mmol) was added, and it was stirred at room temperature for 10 min. $Na_2S_2O_3$ (10%, w/v) was added to the mixture, which was then extracted with ethyl acetate. The organic layer was washed successively with a saturated NaCl solution and an aqueous $NaHCO_3$ solution, dried over Na_2SO_4, and concentrated on a rotary evaporator. The residue was purified by silica gel column chromatography to give the desired product (39.4 mg, 51% yield). The product (29.1 mg, 0.035 mmol) was dissolved in 80% aqueous EtOH (5 ml), 10% palladium on charcoal (10.0 mg) was added, and it was hydrogenolyzed for 40 min. The reaction mixture was filtered through Celite. Concentrated ammonia was added to the filtrate, and the resultant mixture was concentrated. The residue was dissolved in H_2O (1 ml) and lyophilized to give stevastelin P ammonium salt (25.9 mg, 96% yield).

Synthesis of stevastelins 35 and 39

Compounds **1** (1.14 g, 2.37 mmol) and **2** (710 mg, 2.04 mmol) were combined and dissolved in *N,N*-dimethylformamide (DMF, 20 ml). 1-Hydroxybenzotriazole hydrate (HOBt; 331 mg, 2.45 mmol), triethylamine (285.2 μl, 2.04 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC; 783.6 mg, 4.09 mmol) were added at −20°C and stirred for 24 h at room temperature. The reaction mixture was concentrated *in vacuo* and extracted with ethyl acetate. The organic layer was washed successively with 5% (w/v) citric acid, saturated NaCl solution, and concentrated on a rotary evaporator. The residue was purified by silica gel column chromatography to give compound **3** (1.50 g, 99% yield). The product **3** (1.50 g, 2.07 mmol) was dissolved in EtOH (20 ml), 10% palladium on charcoal (160 mg) was added and the mixture hydrogenolyzed at 60°C for 3.5 h. The reaction mixture was filtered through Celite and concentrated. Syrup (612 mg) was dissolved in CH_2Cl_2, trifluoroacetic acid (TFA; 2 ml) was added and it was stirred for 1 h at room temperature. The reaction mixture was concentrated *in vacuo* with toluene to give compound **4** (600 mg, 55% yield).

The product **4** (410 mg, 0.77 mmol) dissolved in DMF (150 ml) was added to 1-hydroxybenzotriazole hydrate (147.2 mg, 1.08 mmol), triethylamine (102.7 μl, 0.73 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (209.0 mg, 1.09 mmol) at −20°C, and then stirred for 72 h at room temperature. The reaction mixture was concentrated *in vacuo* and extracted with ethyl acetate. The organic layer was washed successively with 5% (w/v) citric acid, a saturated NaCl solution, and concentrated on a rotary evaporator to give stevastelin 35 (95 mg, 28% yield).

Stevastelin 35 (6.2 mg, 0.014 mmol) was suspended in DMF, treated with sulphur trioxide trimethylamine complex (8.0 mg, 0.053 mmol) and stirred for 1 h at 50°C. After removing the solvent *in vacuo*, the residue was purified by silica gel column chromatography to give stevastelin 39 (8.3 mg, 100% yield).

Cell culture

A human B cell leukemia cell line, Ball-1, was obtained from Cell Bank (RIKEN) and maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37°C in RPMI–1640 medium supplemented with 10% fetal bovine serum (Gibco). A temperature-sensitive (ts) cdc2 mutant of the mammary carcinoma cell line, FM3A, tsFT210 cell [27], was obtained from F Hanaoka (RIKEN) and cultured at 32°C in RPMI–1640 medium supplemented with 10% calf serum (Hyclone).

Immunosuppressive activity

IL-2 promoter-dependent expression was measured by a β-galactosidase reporter gene using the Jurkat cell line, NFIL-2B6.1 [9]. A cell line for measuring IL-6 promoter-dependent expression was established in this study. In brief, human IL-6 promoter segment (1.3 Kb DNA fragment between Bam H1 and Xho1 restriction sites) was connected to bacterial β-galactosidase gene and transferred to the mouse macrophage cell line, J774.1 [30]. Transfectants were selected by their G418 resistance. Expression of β-galactosidase gene of these clones was induced by 1 μg ml^{-1} lipopolysaccharide (LPS). For assay, the inducer and sample were simultaneously added to cell cultures, and after 4 hours, β-galactosidase activity was measured as described previously [9].

Phosphatase assay

PP1 and PP2A were purchased from Upstate Biotechnology Incorporated (Lake Placid, NY, USA.). Dephosphorylation of *p*-nitrophenylphosphate (*p*Npp) by PP1 and PP2A was measured in an assay buffer containing 20 mM MOPS (pH 7.5), 60 mM 2-mercaptoethanol, 0.1 M NaCl, 1 mg ml^{-1} bovine serum albumin and 50 % (v/v) glycerol. PP2B (calcineurin) was partially purified from *Saccharomyces cerevisiae* [31]. Phosphatase activity of PP2B to the ^{32}P–casein substrate was measured as described previously [29]. Glutathionine-*S*-transferase (GST)–VHR fusion protein (VHR) was prepared as described [32]. Bacterially expressed GST–VHR (1 μg ml^{-1}) was incubated at 37°C for 30 min with 10 mM *p*Npp in an assay buffer containing 25 mM MOPS (pH 6.5), 5 mM EDTA, and 1 mM dithiothreitol (100 μl final volume). A CD45 fraction was prepared from Ball-1 cells by the method described [33], and its PTPase activity to phosphotyrosine was measured by calorimetric assay [34].

Cell cycle assay

The general procedure of cell-cycle analysis was previously reported [28]. Briefly, tsFT210 cells were cultured in a 12-well plate at a concentration of 2 x 10^5 cells per well with 1 ml of RPMI–1640 medium supplemented with 10% fetal bovine serum at 32°C for 1 day. To test G2/M to G1 transition, cells were cultured at 39°C for 17 h, and then the temperature was lowered to 32°C for 4 h after adding the inhibitors. The cells were then washed with phosphate buffered saline (PBS), stained with propidium iodide (Sigma) and analyzed by flow cytometry (Coulter, Epics Profile II, Hialeah, FL, USA).

Immunoblotting

Ball-1 cell lysates (400 μg ml^{-1}) were treated with phosphatase inhibitors for 1 h at 37°C. The lysates (3 μg protein per lane) were subjected to 10% SDS PAGE, electrophoretically transferred to a nitrocellulose membrane, and incubated for 1 h with a monoclonal antibody to phosphotyrosine (1G2, Chemicon International Inc., Temecula, CA, USA). The membrane was incubated with 0.5 μg ml^{-1} of horseradish peroxidase conjugated anti-mouse IgG (Amersham, UK) and visualized with a chemiluminescence system (ECL, Amersham).

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