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Comparing myotoxic effects of squalene synthase inhibitor, T-91485, and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors in human myocytes

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Abstract

TAK-475 is a squalene synthase inhibitor, rapidly metabolized to T-91485 *in vivo*. We investigated the myotoxicities of T-91485 and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors in a human rhabdomyosarcoma cell line, RD, and in human skeletal myocytes. In differentiated RD cells, T-91485, atorvastatin (ATV) and simvastatin acid (SIM) inhibited cholesterol biosynthesis, with IC_{50} values of 36, 2.8 and 3.8 nM, respectively. ATV and SIM decreased the intracellular ATP content, with IC_{25} values (concentrations giving a 25% decrease in intracellular ATP content) of 0.61 and 0.44 μM, respectively. Although T-91485 potently inhibited cholesterol synthesis in RD cells, the IC_{25} value exceeded 100 μM. In human skeletal myocytes, T-91485, ATV and SIM concentration-dependently inhibited cholesterol biosynthesis, with IC_{50} values of 45, 8.6 and 8.4 nM, respectively. ATV and SIM decreased intracellular ATP content, with IC_{25} values of 2.1 and 0.72 μM, respectively. Although T-91485 potently inhibited cholesterol synthesis, the IC_{25} value exceeded 100 μM. Myotoxicity induced by ATV was prevented by mevalonate or geranylgeranyl-PP, but not by squalene in skeletal cells. Furthermore, T-91485 attenuated the myotoxicity of ATV. These findings suggest that TAK-475 and T-91485 may not only be far from myotoxic, they may also decrease statin-induced myotoxicity in lipid-lowering therapy.

Keywords: TAK-475; HMG-CoA reductase inhibitor; Rhabdomyosarcoma cell line RD; Skeletal muscle cell; Geranylgeranyl-PP; Squalene synthase inhibitor

1. Introduction

HMG-CoA reductase inhibitors, known as statins, are widely used for the treatment of patients with hypercholesterolemia [1–3]. Although statins have shown little serious toxicity to date, some side effects, such as skeletal myopathy or hepatotoxicity, have been reported [4]. These are said to be due to the inhibition of mevalonate synthesis, which likely reduces isoprenylated metabolites such as ubiquinone, isopentenyl tRNA, dolichols and isoprenylated proteins [5–7]. Among these toxicities, myotoxicity, albeit rarely [8], is the most serious adverse effect of statins, reflected in increased serum levels of creatinine phosphokinase and symptoms of muscle weakness and myalgia, finally progressing to rhabdomyolysis and renal failure [9,10]. Recently, the HMG-CoA reductase inhibitor, cerivastatin, was withdrawn from the world market because of its high frequency of rhabdomyolysis [11,12].

Squalene synthase converts farnesyl pyrophosphate to squalene in the cholesterol biosynthesis pathway (Fig. 1). Since squalene synthase inhibitors do not reduce the synthesis of isoprenylated metabolites, they may not have the toxicity of HMG-CoA reductase inhibitors, such as myotoxicity. Flint *et al.* [6] indicated that BMS-187745, a squalene synthase inhibitor, did not induce myotoxicity in primary cultures of rat myotubes, whereas HMG-CoA reductase inhibitors did.

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Abbreviations: farnesyl-PP, farnesyl pyrophosphate; geranylgeranyl-PP, geranylgeranyl pyrophosphate; ATV, atorvastatin; SIM, simvastatin acid; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LDH, lactate dehydrogenase; CPK, creatinine phosphokinase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum.

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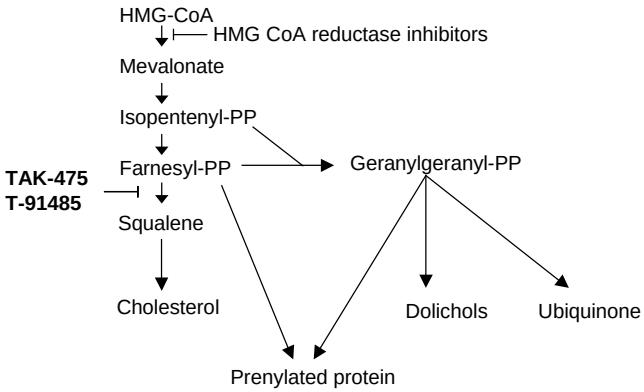

Fig. 1. Cholesterol biosynthesis pathway and its inhibitors.

Recently, we discovered a novel squalene synthase inhibitor, TAK-475, 1-[[(3*R*,5*S*)-1-(3-acetoxy-2,2-dimethylpropyl)-7-chloro-5-(2,3-dimethoxyphenyl)-2-oxo-1,2,3,5-tetrahydro-4,1-benzoxazepin-3-yl]acetyl]piperidine-4-acetic acid, which lowered plasma lipid levels in various animals [13–15]. After oral administration to rats, TAK-475 was absorbed and rapidly hydrolyzed into a pharmacological active metabolite, T-91485 (Fig. 2). In rat liver and plasma, we could detect T-91485, but not TAK-475 [13]. In this study, we investigated the myotoxic effects of T-91485 and statins in human rhabdomyosarcoma cell line RD and in human skeletal myocytes.

2. Material and methods

2.1. Materials

TAK-475 and its pharmacologically active metabolite, T-91485, were synthesized in our laboratories. The following materials were purchased: atorvastatin calcium and simvastatin in lactone form (Wako Pure Chemical Co. Ltd.), DL-mevalonolactone (Sigma), [2-^{14}C] sodium acetate (Amersham Pharmacia Biotech Ltd.), FBS, DMEM (Gibco-BRL Co. Ltd.), geranylgeranyl-PP, farnesyl-PP (BIOMOL Co.), SkGM medium (Bio Whittaker Co.), ATP-Lite M (Packard Co.), and Apo-ONE^{TM} homogenous caspase-3/7 assay kit (Promega Co.). Other chemicals were purchased from Wako Pure Chemical Co. Ltd. Simvastatin in lactone form and DL-mevalonolactone were dissolved in

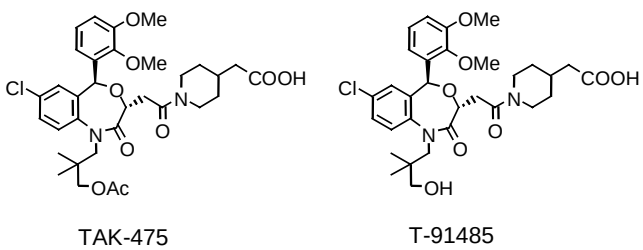

Fig. 2. The chemical structures of squalene synthase inhibitors TAK-475 and T-91485.

0.1 M NaOH to give the acid form, and neutralized with 0.1 M HCl [16,17].

2.2. Cell culture

Human rhabdomyosarcoma cell line RD was purchased from the American Type Culture Collection. RD cells were maintained in DMEM containing 10% FBS at 37° under 5% CO_2. RD cell differentiation was initiated at confluence by replacing the growth medium with DMEM containing 1% FBS, and the cells were maintained in this medium for 7 days [18,19]. Human skeletal muscle cells were purchased from Bio Whittaker Co. The cells were maintained in SkGM medium (Bio Whittaker Co.) at 37° under 5% CO_2.

2.3. Measurement of cholesterol biosynthesis

RD cells were seeded into 96-well plates in DMEM containing 10% FBS. After the cultures reached confluence, the medium was changed to DMEM containing 1% FBS. After 7 days, the cells were treated with drugs for 1 hr, followed by the addition of [2-^{14}C] sodium acetate (57.0 mCi/mmol, 2 μCi per well) and incubated for 2 hr. After saponification of the cell cultures, the non-saponified lipid was extracted with petroleum ether, and the sterol fraction was precipitated using digitonin solution according to the method of Kuroda and Endo [20]. The radioactivity of the digitonin-precipitable sterols was measured with a liquid scintillation counter (Wallac 1414 Winspectral v1.30). Human skeletal muscle cells were seeded into 96-well plates in SkGM medium. After the cultures reached confluence, cholesterol biosynthesis was measured as above, except that SkGM medium was used.

2.4. Estimation of cytotoxicity

Cell viability was estimated by measuring intracellular ATP concentrations with the firefly luciferin/luciferase reaction (ATP-Lite M; Packard). RD cells were seeded into 96-well plates in DMEM containing 10% FBS at 37° under 5% CO_2. After the cultures reached confluence, the medium was changed to DMEM containing 1% FBS. After 7 days, the cells were treated with drugs. After a further 3 days, intracellular ATP concentrations were measured. The cytotoxicity to RD cells was also estimated by measuring LDH and CPK levels in the culture medium. LDH and CPK levels were measured enzymatically with a biochemical autoanalyzer (Hitachi Auto Analyzer 7070). Human skeletal muscle cells were seeded into 96-well plates in SkGM medium. After the cultures reached confluence, the cells were treated with drugs. After a further 3 days, intracellular ATP concentrations were measured as above. Apoptosis was evaluated by measuring the activities of caspase-3 and -7 (Apo-ONE^{TM} homogenous caspase-3/7 assay kit; Promega).

2.5. Statistical analysis

Statistical analysis was performed by two-way analysis of variance (ANOVA), followed by Student's *t*-test for comparison with respective controls. To evaluate the effects of T-91485 on ATV cytotoxicity, the concentration-dependent cytotoxicity of ATV was analyzed using the one-tailed Williams' test, and the effects of T-91485 on reducing ATV cytotoxicity were analyzed by Student's *t*-test. If necessary, multiple comparisons were corrected by Holm's method. The IC_{50} and IC_{25} values for each drug were determined from dose–response curves generated by least-squares linear regression. Data are represented as the mean ± SEM.

3. Results

3.1. Effects of T-91485, ATV and SIM on cholesterol biosynthesis in differentiated RD cells and human skeletal myocytes

In differentiated RD cells, T-91485, ATV and SIM inhibited cholesterol biosynthesis, with IC_{50} values of 36, 2.8 and 3.8 nM, respectively (Table 1). We also investigated the effects of T-91485, ATV and SIM on cholesterol biosynthesis in skeletal muscle cells. In these cells, T-91485, ATV and SIM inhibited cholesterol biosynthesis, with IC_{50} values of 45, 8.6 and 8.4 nM, respectively (Table 2).

3.2. Cytotoxicity of T-91485, ATV and SIM in differentiated RD cells

ATP is a marker for cell viability, because it is present in all metabolically active cells. We, therefore, determined the intracellular ATP levels in order to estimate cell viability. In differentiated RD cells, ATV and SIM potently decreased the intracellular ATP content, with IC_{25} values of 0.61 and 0.44 μM, respectively, whereas T-91485 did not decrease the intracellular ATP content, even at 100 μM, although it potently inhibited cholesterol synthesis in these cells (Fig. 3). As shown in Fig. 4, we also measured CPK and LDH levels in cultured medium as other markers of cytotoxicity. These were increased by ATV and SIM even at 0.1–0.3 μM, while T-91485 at 100 μM had no effect.

Table 1
Inhibitory effects of T-91485, ATV and SIM on cholesterol biosynthesis in differentiated RD cells

Drugs	IC_{50} (nM)	95% confidence interval
T-91485	36	12–120
ATV	2.8	2.0–3.7
SIM	3.8	2.6–5.6

Table 2
Effects of T-91485, ATV and SIM on cholesterol biosynthesis in human skeletal myocytes

Drugs	IC_{50} (nM)	95% confidence interval
T-91485	45	33–60
ATV	8.6	7.2–10
SIM	8.4	6.3–11

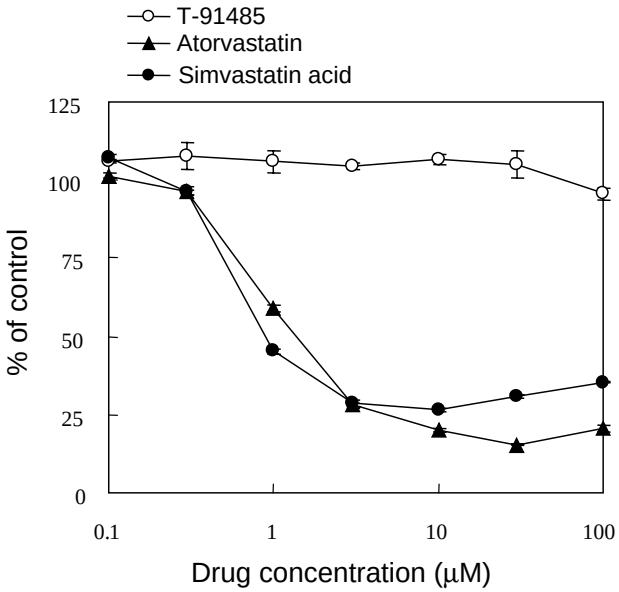

Fig. 3. Effects of T-91485, ATV and SIM on intracellular ATP levels in differentiated RD cells. Differentiated RD cells were incubated with drugs in DMEM + 1% FBS for 3 days, and the intracellular ATP content was measured with the firefly luciferin/luciferase reaction. Data represent the means ± SEM (N = 3).

3.3. Cytotoxicity of T-91485, ATV and SIM in human skeletal muscle cells

In human skeletal muscle cells, ATV and SIM decreased the intracellular ATP content, with IC_{25} values of 2.1 and 0.72 μM, respectively (Fig. 5). Although T-91485 slightly decreased the intracellular ATP content at 100 μM, the IC_{25} value exceeded 100 μM. We also confirmed that T-91485 was much less toxic than ATV and SIM by using WST-1, a new water-soluble respiration indicator [21] (data not shown). Since HMG-CoA reductase inhibitors have shown to induce apoptosis in several types of cells [22–24], we examined whether ATV and T-91485 induces apoptotic cell death in these cells or not. As a result, ATV induced apoptotic cell death even at 0.1 μM, while T-91485 at 100 μM did not induce it (data not shown).

3.4. Effects of mevalonate, geranylgeranyl-PP, farnesyl-PP and squalene on statin cytotoxicity in human skeletal myocytes

To clarify the mechanism of ATV and SIM myotoxicity, we investigated the effects of mevalonate, geranylgeranyl-PP, farnesyl-PP and squalene on statin cytotoxicity in

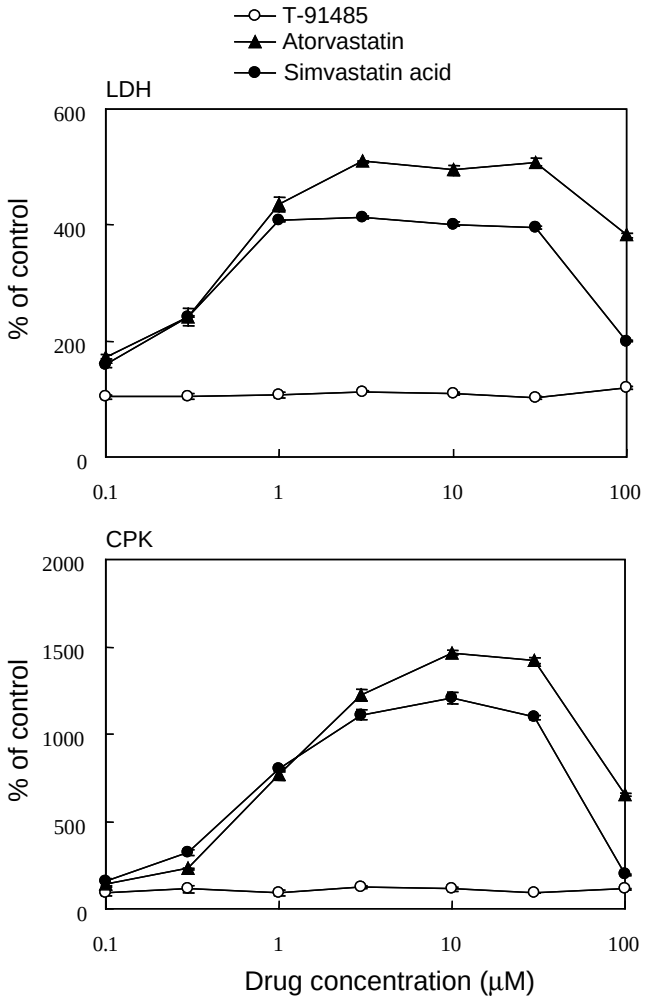

Fig. 4. Effects of T-91485, ATV and SIM on medium LDH and CPK levels in differentiated RD cells. Differentiated RD cells were incubated with drugs in DMEM + 1% FBS for 3 days, and medium LDH and CPK levels were measured enzymatically. Data represent the means ± SEM (N = 3).

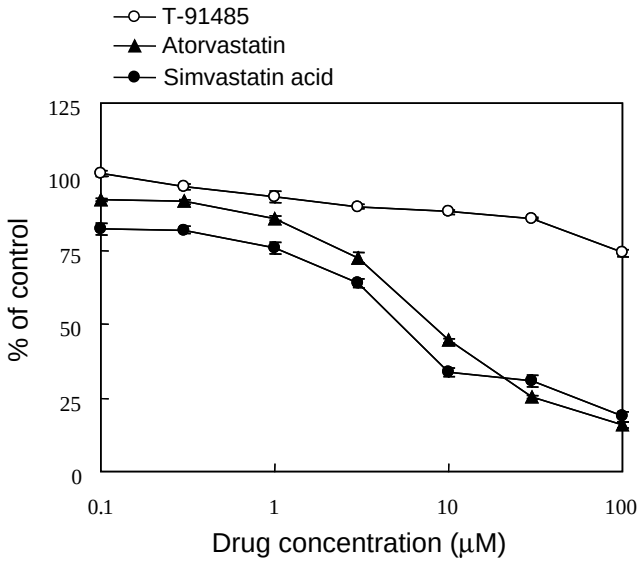

Fig. 5. Effects of T-91485, ATV and SIM on intracellular ATP levels in human skeletal myocytes. Human skeletal muscle cells were incubated with drugs in SkGM medium for 3 days, and the intracellular ATP content was measured with the firefly luciferin/luciferase reaction. Data represent the means ± SEM (N = 3).

human skeletal myocytes (Fig. 6). As shown in Fig. 6A, SIM (10 μM)- and ATV (10 μM)-induced decreases in intracellular ATP content changed from 40.1 and 51.7% of the control level to 89.0 and 83.3% of the control level, respectively, by supplementation with 100 μM mevalonate. As shown in Fig. 6B, SIM (10 μM)- and ATV (10 μM)-induced decreases in intracellular ATP content changed from 46.2 and 52.0% of the control level to 72.3 and 67.8% of the control level, respectively, by supplementation with 10 μM geranylgeranyl-PP. As shown in Fig. 6C, SIM (10 μM)- and ATV (10 μM)-induced decreases in intracellular ATP content changed from 44.7 and 53.8% of the control level to 54.1 and 58.0% of the control level, respectively, by supplementation with 10 μM farnesyl-PP. The effect of farnesyl-PP at 30 μM on the ATV cytotoxicity (10 μM) was obscure (data not shown). As shown in Fig. 6D, treatment with 10 μM squalene did not affect the cytotoxicity of ATV (10 μM) or SIM (10 μM). Treatment with squalene even at 100 μM also did not attenuate the cytotoxicity (data not shown).

3.5. Effects of T-91485 on ATV cytotoxicity in human skeletal myocytes

As T-91485 inhibited squalene synthase, which converts farnesyl-PP to squalene in the cholesterol biosynthesis pathway (Fig. 1), T-91485 may increase intracellular farnesyl-PP and geranylgeranyl-PP levels. Since the addition of geranylgeranyl-PP and farnesyl-PP attenuated statin cytotoxicity as mentioned above, we expected that T-91485 would also attenuate statin cytotoxicity. We investigated whether or not T-91485 attenuated the ATV cytotoxicity in human skeletal myocytes. First, we simultaneously added T-91485 (10 μM) and ATV (10 or 100 μM) to cultured cells. As shown in Fig. 7A, ATV (100 μM)- and ATV (10 μM)-induced decreases in intracellular ATP content changed from 33.6 and 55.2% of the control level to 41.4 and 59.1% of the control level, respectively, by supplementation with 10 μM T-91485.

Second, we investigated the pre-treatment effects of T-91485 on ATV cytotoxicity. After the cultured cells were incubated with T-91485 (10 μM) for 1 day, we removed the old medium and added fresh medium containing ATV (10 or 100 μM) without T-91485 to the cell cultures. As shown in Fig. 7B, ATV (100 μM)-and ATV (10 μM)-induced decreases in intracellular ATP content significantly changed from 35.0 and 56.8% of the control level to 45.0 and 76.3% of the control level, respectively, by pre-treatment with 10 μM T-91485.

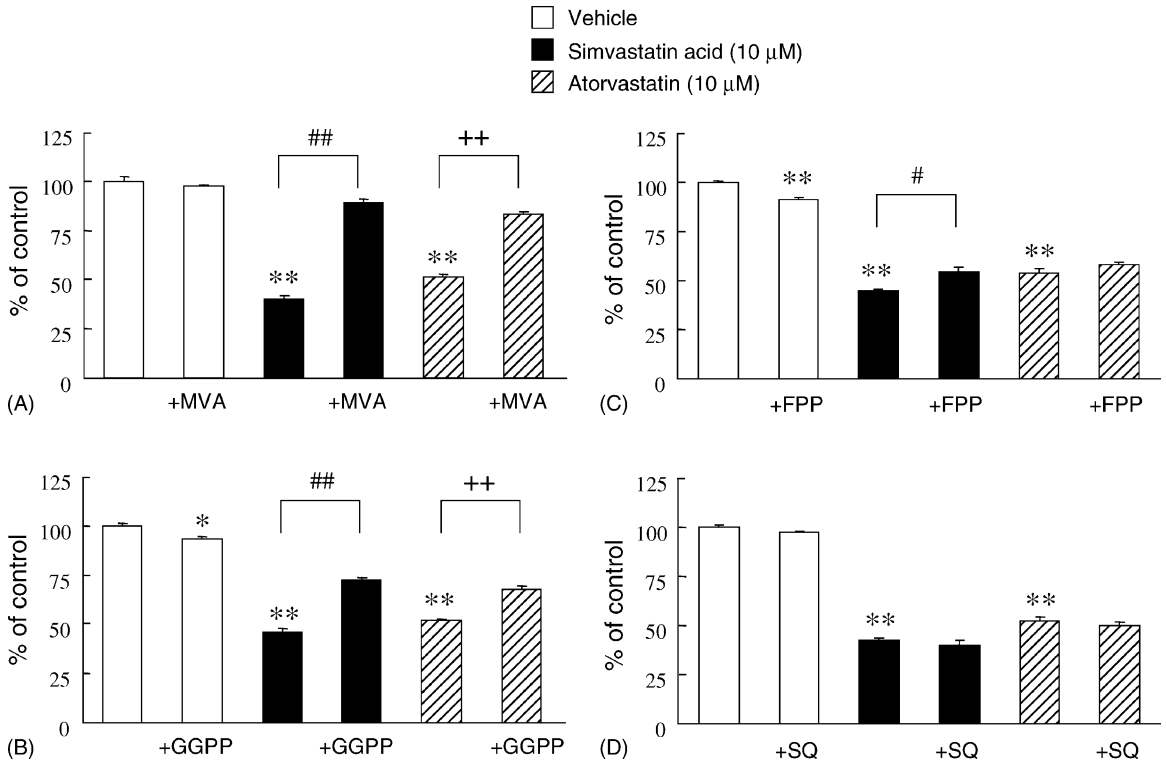

Fig. 6. Effects of mevalonate (MVA), geranylgeranyl-PP (GGPP), farnesyl-PP (FPP) and squalene (SQ) on statin cytotoxicity in human skeletal myocytes. Human skeletal muscle cells were incubated with ATV supplemented with MVA (A), GGPP (B), FPP (C) or SQ (D) in SkGM medium for 3 days, and the intracellular ATP content was measured with the firefly luciferin/luciferase reaction. Data represent the means ± SEM (N = 3). $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control without ATV or SIM by Student's *t*-test. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. the respective simvastatin-treated control by Student's *t*-test. $^{++}P < 0.01$ vs. the respective ATV-treated control by Student's *t*-test.

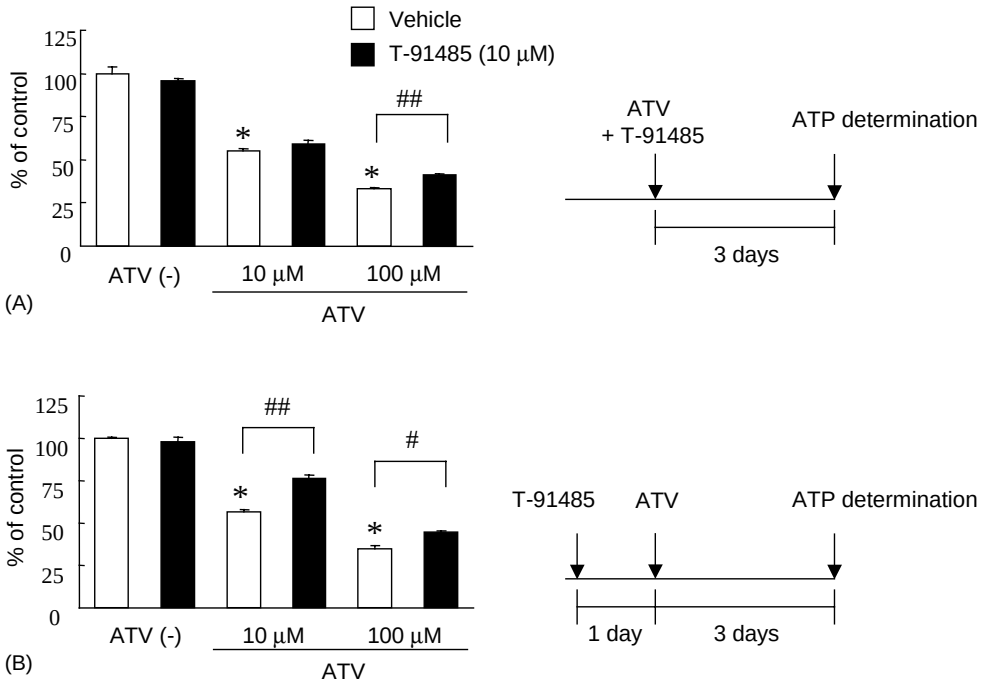

Fig. 7. Effects of T-91485 on ATV cytotoxicity in human skeletal myocytes. Human skeletal muscle cells were incubated with ATV and T-91485 in SkGM medium for 3 days (A). Human skeletal muscle cells were preincubated with T-91485 in SkGM medium for 1 day, and then the medium was changed to a fresh ATV-containing medium without T-91485 for 3 days (B). The intracellular ATP content was measured with the firefly luciferin/luciferase reaction. Data represent the means ± SEM (N = 3). $^{*}P < 0.025$ vs. control without ATV by the one-tailed Williams' test, and $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. the respective ATV-treated control by Student's *t*-test.

4. Discussion

In differentiated RD cells, the *in vitro* myotoxicity of squalene synthase inhibitor T-91485 was at least 100-fold less than that of ATV or SIM, although T-91485 inhibited cholesterol synthesis 9–13-fold less than ATV or SIM. Similarly, in human skeletal myocytes, the *in vitro* myotoxicity of T-91485 was at least 50-fold less than ATV or SIM, although T-91485 inhibited cholesterol synthesis about 5-fold less than ATV or SIM. Flint *et al.* [6] demonstrated that another squalene synthase inhibitor, BMS-187745, has a safety margin superior to those of pravastatin and simvastatin in rat primary skeletal muscle cells. In this study, the addition of mevalonate abrogated the myotoxicity of ATV and SIM in human skeletal myocytes, suggesting that this toxicity was caused by the mechanism of HMG-CoA reductase inhibition. On the other hand, squalene did not ameliorate the myotoxicity of ATV or SIM, suggesting that the reduction of downstream products of squalene, such as cholesterol, is not involved in their toxicity. Geranylgeranyl-PP effectively attenuated the myotoxicity of HMG-CoA reductase inhibitors. These findings suggest that the myotoxicity of HMG-CoA reductase inhibitors is caused by the reduced production of geranylgeranyl-PP derivatives, such as ubiquinone or geranylgeranyl proteins. Nakahara *et al.* [25] demonstrated that the oral administration of simvastatin- or pravastatin-induced myotoxicity, accompanied with decreased muscle ubiquinone levels in rabbits. Although Laaksonen *et al.* [26,27] indicated that the muscle ubiquinone concentration did not change in patients after treatment with simvastatin, they evaluated the ubiquinone concentration in the muscles of only about 20 patients, which seems to be too small a number to evaluate the low frequency of statin-induced myotoxicities. It was also reported that supplementation with ubiquinone ameliorated the myotoxicity of HMG-CoA reductase inhibitors in patients [5]. Marked increases in creatinine kinase levels following physical exertion have been reported in some patients receiving HMG-CoA reductase inhibitors [28,29]. These results suggest that a lack of muscular energy metabolism caused by ubiquinone depletion may be related to statin-induced myotoxicity. The inhibition of protein geranylgeranylation may also trigger myotoxicity. Recently, fluvastatin was reported to decrease RhoA protein in the cell membrane, and induced apoptosis in rat neonatal cardiac myocytes [30]. Martin *et al.* [31] reported that the inhibition of RhoGTPase geranylgeranylation by statins activates PPARα. It has been reported that other lipid-lowering agents called fibrates, well-known synthetic agonists of PPARα, also cause rhabdomyolysis in patients [32,33]. Maiguma *et al.* [34] reported that both statins and fibrates reduced the viability of human embryonal rhabdomyosarcoma cells. Although the mechanism of myotoxicity induced by HMG-CoA reductase inhibitors remains to be clarified, our results suggest that the depletion of geranylgeranyl-PP derivatives may cause myotoxicity.

In this study, Farnesyl-PP also slightly attenuated the myotoxicity of HMG-CoA reductase inhibitors. Farnesyl-PP is not effectively converted to geranylgeranyl-PP in the presence of HMG-CoA reductase inhibitors, because they inhibit the supply of a required mevalonate metabolite, isopentenyl-PP (Fig. 1). Flint *et al.* [6] demonstrated that not only geranylgeraniol but also farnesol effectively reduced statin-induced cytotoxicity in rat primary skeletal myocytes. They speculated that the metabolites of both geranylgeraniol and farnesol are required to abrogate the toxicity. Since 50–100 μM farnesol effectively attenuated the pravastatin (742 μM)-induced cytotoxicity in their results, high concentrations of farnesyl-PP or farnesol may attenuate the cytotoxicity more effectively. Ten to thirty micromoles farnesyl-PP used in this study seems to be relatively low. In our preliminary result, at a concentration of 5 μM, farnesol had no effect on statin-induced myotoxicity, and farnesol at 50 μM had potent cytotoxicity in our human cells (data not shown). Further examination is needed to clarify whether the depletion of farnesyl-PP derivatives causes myotoxicity or not.

We also investigated the effect of T-91485 on ATV-induced toxicity on human skeletal myocytes. As T-91485 inhibited squalene synthase, which converts farnesyl-PP to squalene (Fig. 1), T-91485 may increase intracellular farnesyl-PP and geranylgeranyl-PP levels in these cells. In fact, squalestatin I, a squalene synthase inhibitor, was reported to increase farnesyl-PP and geranylgeranyl-PP levels in rat liver [35]. Our preliminary results also indicated that the oral administration of T-91485 increased geranylgeraniol levels in the muscles of rats (data not shown). In co-treatment with T-91485 and ATV, T-91485 slightly attenuated ATV-induced myotoxicity, suggesting that T-91485 can increase farnesyl-PP and/or geranylgeranyl-PP, even though ATV inhibits upstream of the pathway. In pre-treatment with T-91485, ATV-induced myotoxicity was effectively attenuated. Since pre-treatment with T-91485 can substantially increase geranylgeranyl-PP levels in human skeletal cells, T-91485 attenuates cytotoxicity more effectively. TAK-475 at doses of 30–100 mg/kg lowered plasma non-high-density lipoprotein cholesterol levels by 23–43% in common marmosets [14]. In these doses, the plasma maximum concentration of T-91485 was estimated as about 2–8 μM (data not shown). Therefore, the concentration of T-91485 used in this study is not so different from levels *in vivo*.

We have demonstrated that a novel squalene synthase inhibitor, T-91485, which is a pharmacologically active metabolite of TAK-475, had little toxic effect on human-derived muscle cells, although it potently inhibited cholesterol biosynthesis. Furthermore, T-91485 attenuated the *in vitro* myotoxicity of ATV. These findings suggest that the squalene synthase inhibitors TAK-475 and T-91485 may not only be far from myotoxic, but also decrease statin-induced myotoxicity in lipid-lowering therapy.

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