# Experimental model of escape phenomenon in hamsters and the effectiveness of YM-53601 in the model

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**1** The aim of this study was to establish an experimental model of the escape phenomenon, in which plasma cholesterol, initially reduced by a 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase inhibitor such as pravastatin, increases again on long-term administration. We also evaluated the efficacy of YM-53601 ((*E*)-2-[2-fluoro-2-(quinuclidin-3-ylidene) ethoxy]-9*H*-carbazole monohydrochloride), a squalene synthase inhibitor, in this model.

**2** Pravastatin inhibited cholesterol biosynthesis in hamster primary hepatocytes ($IC_{50}$, 14 nM). After pre-treatment with pravastatin, in contrast, almost no effect on cholesterol biosynthesis was seen.

**3** In hamsters fed a high fat diet, 3 mg $kg^{-1}$ pravastatin for 9 days decreased plasma non-HDL cholesterol (total cholesterol – high density lipoprotein cholesterol) ($P<0.01$), but this effect was lost between 17 and 27 days of treatment, accompanied by an increase in HMG-CoA reductase activity. No such increase in plasma non-HDL cholesterol was seen with YM-53601 at 30 mg $kg^{-1}$ after 9 ($P<0.001$), 17 ($P<0.01$) or 27 ($P<0.001$) days of treatment. Replacement of pravastatin with YM-53601 caused a decrease in plasma non-HDL cholesterol by 53% ($P<0.001$) and in HMG-CoA reductase activity.

**4** This animal model thus satisfactorily replicates the escape phenomenon observed in humans and may therefore be useful in evaluation of lipid-lowering agents, specifically comparison of HMG-CoA reductase inhibitors. Further, YM-53601 may be useful in the treatment of hypercholesterolemia without induction of the escape phenomenon.

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## Introduction

Long-term administration of inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (EC 1.1.1.34), the rate limiting enzyme in cholesterol biosynthesis, is reported to be associated with the escape phenomenon, in which plasma cholesterol levels are initially reduced but return to pre-treatment levels (Naruse *et al.*, 1994; Pazzucconi *et al.*, 1995). As a result, this effect may be due to the induction of HMG-CoA reductase expression by a feedback system, wherein the inhibition of the enzyme is countered by homeostatic maintenance of the amount of the cholesterol pool in the liver (Stone *et al.*, 1989). From this result, it has been speculated that the efficacy of the compound might be attenuated or lost, and researchers have sought a compound that has both strong efficacy and high tolerance. Although changes in enzyme expression have been examined in animals, the escape phenomenon has only been observed clinically and no animal model is available (Fujioka & Tsujita, 1997; Fujioka *et al.*, 1995). Among rodents, only guinea-pigs show a hypocholesterolemic effect of statins on plasma cholesterol (Conde *et al.*, 1996).

The mechanism by which HMG-CoA reductase inhibitors induce the escape phenomenon is said to involve their inhibition of not only cholesterol but also mevalonate derivatives such as farnesol. Mevalonate derivatives are known to regulate the protein levels of cholesterol biosynthesis enzymes through translation after transcription (Goldstein & Brown, 1990). On this basis, research has focused on new target molecules that do not inhibit the biosynthesis of these derivatives. An example is squalene synthase, which works downstream in the cholesterol biosynthesis pathway.

As no suitable animal model has been available, however, it has not been possible to compare squalene synthase inhibitors with HMG-CoA reductase inhibitors directly. We therefore developed a model using hamsters fed a high-fat diet in which plasma cholesterol increases after reduction by pravastatin, one of the HMG-CoA reductase inhibitors. Using this model, we also compared the effect of YM-53601 ((*E*)-2-[2-fluoro-2-(quinuclidin-3-ylidene) ethoxy]-9*H*-carbazole monohydrochloride), a novel squalene synthase inhibitor that shows a strong hypercholesterolemic effect in hamsters (Ugawa *et al.*, 2000), with pravastatin. To our knowledge, the present report is the first to describe an animal model of the escape phenomenon and to compare the

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effects of inhibitors of HMG-CoA reductase and squalene synthase.

# Methods

## Materials

YM-53601 and NB-598 (Horie *et al.*, 1990), a squalene epoxidase inhibitor, were synthesized at the Chemistry Laboratories, Yamanouchi Pharmaceutical Co., Ltd. (Tokyo, Japan). The HMG-CoA reductase inhibitor pravastatin was purchased from Sankyo Co., Ltd. (Tokyo, Japan). Farnesyl pyrophosphate (FPP), [$^3$H]-FPP (15 Ci mmol$^{-1}$) and [$^{14}$C]-acetate (55 mCi mmol$^{-1}$) were obtained from American Radiolabelled Chemicals Inc (MO, U.S.A.).

## Preparation of hepatocytes

Hepatocyte preparations were produced from male Syrian golden hamsters weighing approximately 80 g. Cells were prepared as described previously (Seglen, 1976) and plated out as a suspension in foetal bovine serum (FBS)-containing Dulbecco's modified eagle medium (DMEM) at a density of $4 \times 10^5$ cells ml$^{-1}$ in 12-well plates. The cells were allowed to form a mono-layer (2 h) and treated in various ways according to the experimental protocol (see below).

## Measurement of cholesterol biosynthesis

After isolation, the cells were plated in 10% FBS-containing DMEM for 24 h and then washed. The medium was replaced with DMEM containing 5% human lipoprotein-deficient serum (LPDS) in the presence or absence of pravastatin (1 – 100 nM final concentration) added as a solution in dimethylsulphoxide (DMSO). After 18 h, pravastatin was added to the medium of cells without pretreatment and [$^{14}$C]-acetate (1 $\mu$Ci, 55 mCi mmol$^{-1}$) was added to the medium of both. The cells were then incubated for 6 h. After removal of the medium, the cells were lysed by adding 15% KOH and the lysate was saponified in 15% KOH in ethanol at 75°C for 1 h. Samples were extracted with petroleum ether under alkaline conditions and the amount of [$^{14}$C]-cholesterol was measured by scintillation counting following separation by thin layer chromatography in petroleum ether : diethylether : acetate (80 : 20 : 1). The amount of protein was measured by the method of Lowry *et al.* (1951).

## Evaluation of hepatic disorder in hamsters

Male Syrian golden hamsters purchased from Hamri (Ibaraki, Japan) were 13 weeks old and weighed approximately 150 g at the start of the study. A standard low cholesterol diet (CE-2 from CLEA Japan Inc., Tokyo, Japan) and water were provided *ad libitum*. They were given a 3-, 10- or 30-mg oral dose of pravastatin per kg of body weight once daily for 11 days. Pravastatin was suspended in 0.5% methylcellulose. The no-treatment control group was given an equal volume of the 0.5% methylcellulose vehicle. Blood specimens were obtained 2 h after the last dose from animals which had been fasted 18 h. Plasma samples were analysed for aspartate aminotransferase (AST) and alanine amino-transferase (ALT) using a Hitachi 7250 Automatic analyser (Tokyo, Japan).

## Evaluation of hypocholesterolemic effect

Male Syrian golden hamsters weighing approximately 140 g were fed a diet containing 0.5% cholesterol and 10% coconut oil. Hamsters were allotted to one of four groups depending on plasma non-HDL cholesterol level as measured before feeding of the high-fat diet. In the first experiment, pravastatin or YM-53601 at doses of 3 or 30 mg kg$^{-1}$, respectively, was given once a day for 27 days. Both drugs were suspended in 0.5% methylcellulose. The no-treatment control group was given an equal volume of the 0.5% methylcellulose vehicle. Blood specimens were obtained from the femoral vein using a glass capillary at 2 h after the last compound dose from animals which had been fasted 18 h. Plasma non-HDL cholesterol levels were measured at days 0, 9, 17 and 27 for all animals. Plasma non-HDL cholesterol levels were calculated from total cholesterol and HDL cholesterol as measured enzymatically by the Cholesterol-C Test Wako and HDL Cholesterol Test Wako (Osaka, Japan), respectively.

In the second experiment, pravastatin at a dose of 3 mg kg$^{-1}$ was given once daily for 17 days, followed by YM-53601 at 30 mg kg$^{-1}$ body weight orally once daily for a further 10 days. The no-treatment control group was given an equal volume of the 0.5% methylcellulose vehicle. Plasma non-HDL cholesterol levels were measured as above at days 0, 9, 17 and 27.

## Preparation of microsomes from hamster livers

Microsomes were prepared from the livers of hamsters fed a diet containing 0.5% cholesterol and 10% coconut oil and given pravastatin or YM-53601 at a dose of 3 or 30 mg kg$^{-1}$, respectively, for 9, 17 and 27 days, or given pravastatin for the first 17 days and YM-53601 for the following 10 days. Livers were also collected from control groups given an equal volume of the 0.5% methylcellulose solution for 0, 9, 17 and 27 days. All livers were prepared as previously described (Burton *et al.*, 1995). The tissues were homogenized in 0.1 M potassium phosphate buffer (pH 7.4) containing (mM): MgCl$_2$ 5, nicotinamide 30, using a glass homogenizer. Homogenates were centrifuged at $500 \times g$ for 5 min at 4°C and the resulting supernatants further centrifuged at $8000 \times g$ for 15 min at 4°C. Microsomes were then isolated from this second supernatant by ultra-centrifugation at $100,000 \times g$ for 60 min at 4°C. The microsome precipitates were washed four times and suspended in the above mixed buffer (approximately 25 mg ml$^{-1}$), and stored in aliquots at $-80$°C for up to 2 months. Protein was assayed by the method of Lowry *et al.* (1951).

## Measurement of HMG-CoA reductase activity

HMG-CoA reductase activity was measured by a modification of the method of Burton *et al.* (1995). After pre-incubation of microsomes (200 $\mu$g protein) with 10 units inorganic pyrophosphatase from *E. coli*, the reaction was started by adding 0.1 M potassium phosphate buffer (pH 7.4) containing (mM): EDTA 10, DTT 10, NADPH 5,

144 MBq mmol$^{-1}$ [$^{14}$C]-HMG-CoA and HMG-CoA 0.091. The reaction was carried out at 37°C for 60 min and then terminated by the addition of 2N HCl. Synthesized [$^{14}$C]-mevalonolactone was measured by scintillation counting following separation by thin layer chromatography in benzene : acetone (1 : 1).

### Measurement of squalene synthase activity

Squalene synthase activity in microsomes was assayed using the technique of Amin *et al.* (1992) with modifications. Briefly, the assay was performed in 50 mM HEPES buffer (pH 7.5) containing (mM): NaF 11, MgCl$_2$ 5.5, DTT 3, NADPH 1, FPP 5 $\mu$M, [$^3$H]-FPP (0.017 $\mu$M, 15 Ci mmol$^{-1}$), 10 $\mu$M NB-598 and 1 mM sodium pyrophosphate deca-hydrate. After pre-incubation of these components at 30°C for 5 min, the reaction was started by the addition of microsomes (10 $\mu$g protein), continued at 30°C for 20 min and then terminated by the addition of 100 $\mu$l of a 1 : 1 solution of 40% KOH : ethanol. Synthesized [$^3$H]-squalene was extracted in petroleum ether and counted in Aquasol-2 (Packard, Netherlands) using a Beckman liquid scintillation counter.

### Statistical analysis

Results are presented as mean ± s.e.mean. For study of the effects of drugs on plasma non-HDL cholesterol, AST, ALT and on HMG-CoA reductase and squalene synthase activities, drug-treatment values were compared with those of the vehicle-treatment control using Dunnett's multiple comparison tests. IC$_{50}$ values were calculated using linear regression analysis by the probit method in a RS/1 computer program (Domain Solutions Corp., U.S.A.). Differences between treatments in cholesterol biosynthesis were estimated by parallel line assay using the Statistical Analysis System (SAS).

### Ethical considerations

All experiments were performed in accordance with the regulations of the Animal Ethical Committee of Yamanouchi Pharmaceutical Co., Ltd.

# Results

### Pravastatin showed little inhibitory effect on cholesterol biosynthesis in vitro with pre-incubation

Figure 1 shows that pravastatin inhibited cholesterol biosynthesis in a dose-dependent manner in hamster primary hepatocytes after 6 h of treatment. The IC$_{50}$ value for pravastatin was 14 nM. After 18 h pre-treatment with pravastatin, in contrast, no effect on cholesterol biosynthesis was seen at 30 nM. At a pravastatin concentration of 100 nM, the inhibitory effect without 18 h pre-treatment was 89%, but only 17% on pre-treatment for 18 h.

### Pravastatin increases plasma AST and ALT in hamsters

In order to find the highest dosage of pravastatin that could be used without causing hepatic disorder, the effect of

pravastatin on plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels was confirmed in hamsters fed a normal diet and orally administered the compound. As shown in Table 1, no difference in plasma AST levels was seen between the control group and animals given pravastatin at 3 mg kg$^{-1}$ for 11 days. In contrast, animals given pravastatin at 10 and 30 mg kg$^{-1}$ showed marked and significant increases in plasma AST levels of two and six times the control group values, respectively. With regard to ALT, plasma levels were maintained at the same level as the control group on oral administration at 3 mg kg$^{-1}$ for 11 days, but, similar to the results with AST, were significantly elevated in hamsters given 10 and 30 mg kg$^{-1}$ for 11 days, by four and seven times control group values, respectively.
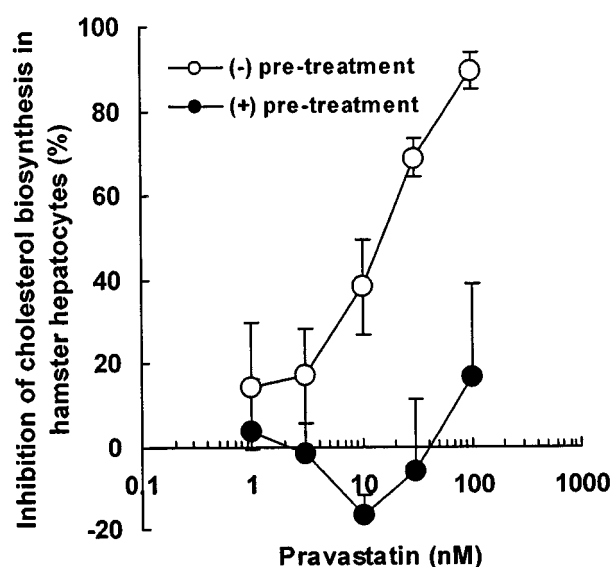


**Figure 1** Effect of pravastatin on cholesterol biosynthesis in hamster primary hepatocytes. Hepatocytes were incubated for 18 h in the presence (+) or absence (−) of pravastatin pre-treatment and then labelled with [$^{14}$C]-acetate (1 $\mu$Ci) for 6 h with pravastatin at the indicated concentrations. See Methods section for details. Data are presented as the mean of assays performed in triplicate ± s.e.mean of per cent inhibition versus the DMSO-treated cells. The IC$_{50}$ value of (−) pre-treatment was 14 nM by RS/1 computer program. The difference between these treatments was statistically significant by parallel line assay in the Statistical Analysis System ($P < 0.01$).

**Table 1** Effect of pravastatin on plasma AST and ALT in hamsters fed a normal diet

| Pravastatin (mg kg$^{-1}$) | AST (IU l$^{-1}$) | ALT (IU l$^{-1}$) |
| --- | --- | --- |
| 0 | 26 ± 2 | 55 ± 11 |
| 3 | 29 ± 2 | 62 ± 9 |
| 10 | 51 ± 9 | 207 ± 47* |
| 30 | 145 ± 45*** | 407 ± 85*** |

Data are represented as mean ± s.e.mean ($n = 3$ or 5). Pravastatin was administered once daily. All groups received a normal diet. Statistical analysis was carried out using Dunnett's multiple comparison test in order to compare with control. *$P < 0.05$ and ***$P < 0.001$ versus control.
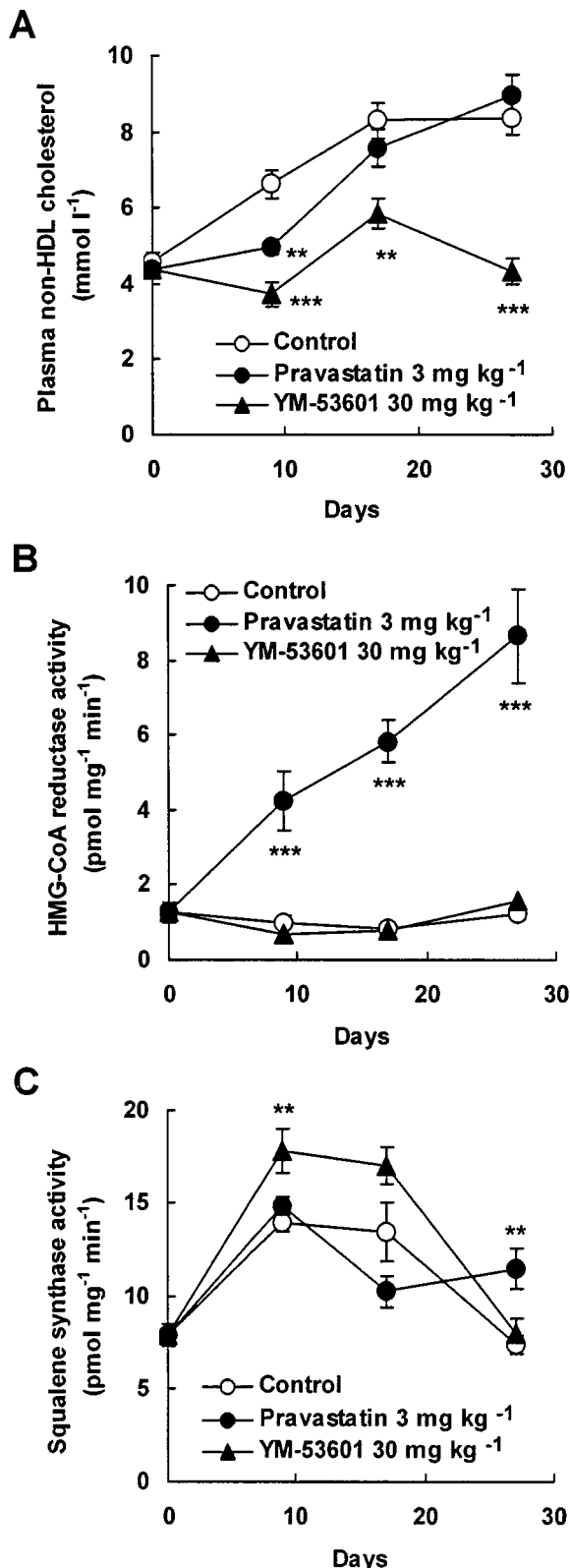
**Figure 2** Effect of YM-53601 and pravastatin on plasma concentrations of non-HDL cholesterol and on activity of HMG-CoA reductase and squalene synthase in hamsters fed a high-fat diet. Effects of pravastatin and YM-53601 on plasma non-HDL cholesterol (A) and activities of HMG-CoA reductase (B) and squalene synthase (C) were evaluated. Values are expressed as mean ± s.e.mean ($n = 5 - 7$). See Methods section for details. Statistical analysis was carried out using Dunnett's multiple comparison test

*Pravastatin initially reduces plasma cholesterol but it increases again on long-term administration, accompanied by induction of HMG-CoA reductase activity*

Figure 2A shows the effects of pravastatin and YM-53601 on plasma non-HDL cholesterol levels in hamsters fed a high cholesterol diet. In control hamsters, (open circle), plasma non-HDL cholesterol increased in a time-dependent manner. Pravastatin at 3 mg kg$^{-1}$ significantly decreased plasma non-HDL cholesterol compared with control by 25% at day 9. Levels increased to those of the control at day 17, and finally tended to be greater than the control at day 27, although the change was not significant. In contrast, non-HDL cholesterol levels with YM-53601 30 mg kg$^{-1}$ were significantly lower than those of the control throughout administration.

Next, to understand why pravastatin's effect on plasma non-HDL cholesterol levels changed during administration, we measured HMG-CoA reductase activity of microsomes prepared from livers of hamsters administered pravastatin or YM-53601 at 3 or 30 mg kg$^{-1}$, respectively, for 0, 9, 17 or 27 days. In control hamsters, activity remained unchanged. In contrast, pravastatin increased activity in a time-dependent manner. YM-53601 did not affect activity (Figure 2B).

Similarly, we measured squalene synthase activity of the above microsomes. As shown in Figure 2C, unlike the case with HMG-CoA reductase activity, no increase by pravastatin was seen in squalene synthase activity up to day 17. At day 27, however, pravastatin increased activity significantly. In contrast, YM-53601 induced squalene synthase activity at day 9, but no effect was seen at days 17 and 27.

Figure 3A shows that pravastatin at a dose of 3 mg kg$^{-1}$ significantly decreased plasma non-HDL cholesterol levels by 18% of the control value on 9 days' oral administration. At day 17, levels returned to those of the control group. Following pravastatin treatment for 17 days, YM-53601 was substituted at a dose of 30 mg kg$^{-1}$. At day 27 (i.e. day 10 of YM-53601), plasma non-HDL cholesterol decreased by 53% compared with control. The decrease in the plasma non-HDL cholesterol level with YM-53601 was greater than that with pravastatin.

HMG-CoA reductase activity in microsomes from livers of animals treated with pravastatin at 3 mg kg$^{-1}$ increased in a time-dependent manner until day 17. In contrast, HMG-CoA reductase activity in control microsomes showed similar levels during the experiment. After replacement with YM-53601 at 30 mg kg$^{-1}$, HMG-CoA reductase activity returned to the same level as in the control group (Figure 3B).

Squalene synthase activity in microsomes prepared from livers from animals treated with pravastatin show no significant changes compared with control group values. At 10 days after replacement with YM-53601, control and YM-53601 group activity was the same (Figure 3C).

In the experiment in Figure 2, plasma HDL cholesterol was significantly increased by pravastatin on day 9 only by 23% compared to control animals. In the experiment in Figure 3, pravastatin tended to increase plasma HDL cholesterol on day 9 only by 11%, although the change was not significant. Except for day 9, pravastatin did not raise this. On the other

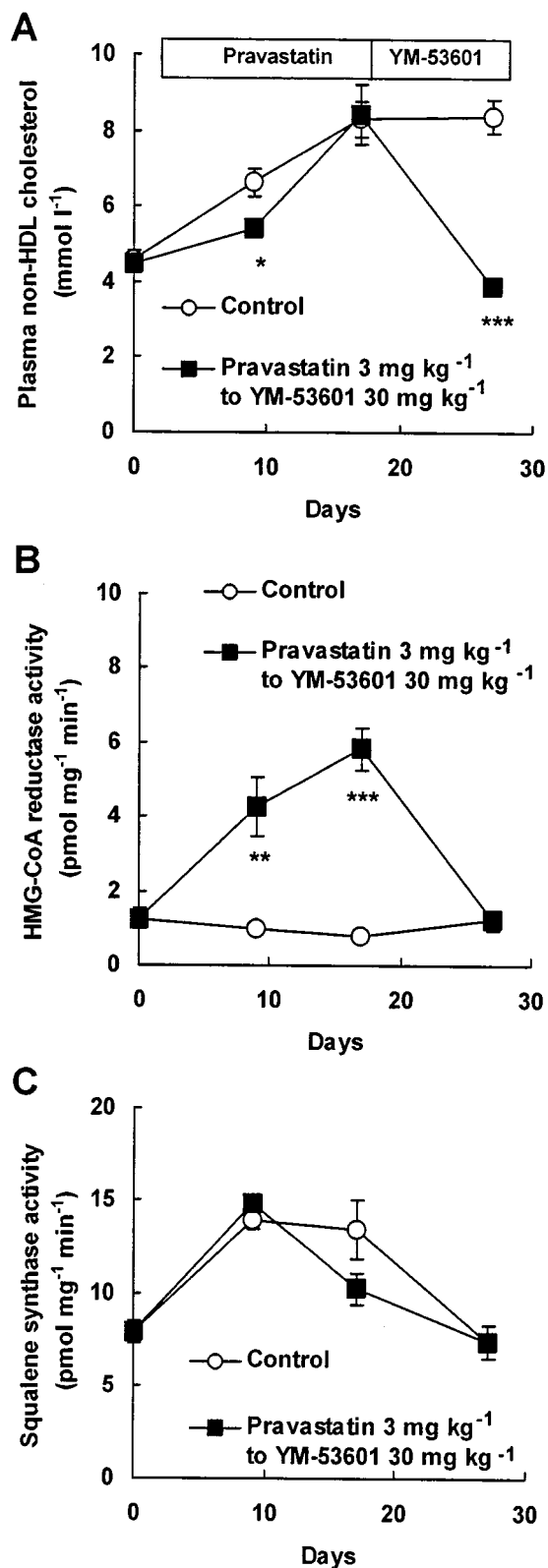versus the control group on each indicated day. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus respective controls.

**Figure 3** Decrease in plasma non-HDL cholesterol and activities of HMG-CoA reductase and squalene synthase in hamsters pre-treated with pravastatin. Effects of pravastatin and YM-53601 on plasma non-HDL cholesterol (A), activities of HMG-CoA reductase (B) and squalene synthase (C) in hamsters fed a high-fat diet were evaluated. The values are expressed as mean±s.e.mean ($n = 7$). See Methods section for details. Statistical analysis was carried out using Dunnett's multiple comparison test versus control group in each indicated day. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus each control.

hand, YM-53601 showed no increase in plasma HDL cholesterol in either experiment (data not shown).

## Discussion

This study set out to establish an experimental model of the escape phenomenon and to use this model to evaluate the effect of YM-53601, a squalene synthase inhibitor, in comparison with that of pravastatin, a widely used HMG-CoA reductase inhibitor.

The hamster was selected because plasma lipid composition in this species is similar to that of humans (Spady & Dietschy, 1985). Pravastatin, a HMG-CoA reductase inhibitor (statin) in wide clinical use for the treatment of hypercholesterolemia, was used as reference compound because this drug produces the escape phenomenon in humans, wherein total plasma cholesterol levels may increase after first showing a significant decrease (Naruse *et al.*, 1994).

Plasma non-HDL cholesterol levels in individual hamsters fell during pravastatin treatment but HMG-CoA reductase was up-regulated approximately 7 fold compared to the control group. Continued administration of pravastatin lead to enhanced enzyme activities and subsequently to an increase in plasma non-HDL cholesterol to the same levels of the control group. No re-reduction in plasma cholesterol level compared with control was seen on longer administration; on the contrary, HMG-CoA reductase activity was further enhanced by pravastatin treatment. This animal model thus satisfactorily replicates the escape phenomenon observed in humans. Use of a high-fat diet may result in decreased cholesterol biosynthesis activity through down regulation in response to adequate or excessive diet-derived cholesterol in the liver. This may explain why pravastatin induces a decrease in plasma non-HDL cholesterol. All rodents apart from guinea-pigs normally have high cholesterol biosynthesis activity (Spady & Dietschy, 1983). This activity is easily induced, and would seem to prevent the observation of a cholesterol-lowering effect. The absence of this effect in guinea-pigs may therefore justify their use in the present model.

Pravastatin attenuated inhibition of cholesterol biosynthesis in hamster primary hepatocytes by pre-treatment (IC$_{50}$ values >100 nM or 14 nM, with or without pre-treatment, respectively). Further, pravastatin is reported to induce HMG-CoA reductase (Fujioka & Tsujita, 1997; Fujioka *et al.*, 1995). The present result may therefore be derived from enzyme induction by the positive feedback system itself. In other words, long-treatment with pravastatin *in vitro* showed a similar effect to that *in vivo* and in previous reports. Moreover, the effect of HMG-CoA reductase inhibitors on enzyme expression or activity is also seen in mononuclear leukocytes of normal subjects (Stone *et al.*, 1989). As enzyme induction is known to lead to the escape phenomenon, drugs have been sought that do not produce induction and the escape phenomenon.

Unlike HMG-CoA reductase, squalene synthase acts downstream in cholesterol biosynthesis. YM-53601 belongs to a novel class of lipid-lowering agents that inhibit squalene synthase activity in cholesterol biosynthesis in animal models (Ugawa *et al.*, 2000). In pre-clinical studies in hamsters, guinea-pigs and rhesus monkeys, YM-53601 reduced plasma

concentrations of non-HDL cholesterol equally or to a greater degree than pravastatin. YM-53601 thus appears to be a potentially useful agent for the treatment of hypercholesterolemia.

In the present animal model of the escape phenomena, YM-53601 showed a consistent plasma cholesterol-lowering effect during treatment. YM-53601 weakly up-regulated squalene synthase activity and did not affect HMG-CoA reductase. A possible explanation is that, given the level at which squalene synthase inhibitors act in the biosynthesis pathway, the cholesterol inhibition system is less prone to positive regulatory feedback mechanisms as compared with statins. Squalene synthase inhibitors may consequently provide greater lipid lowering efficacy than statins. Accumulation of farnesyl pyrophosphate as a result of squalene synthase inhibition is known to down regulate HMG-CoA reductase mRNA expression (Correll *et al.*, 1994; Meigs *et al.*, 1996). However, a previous report indicates that other squalene synthase inhibitors have a strong effect on HMG-CoA reductase up-regulation (Amin *et al.*, 1997). One possible explanation why YM-53601 did not induce HMG-CoA reductase activity in comparison with other squalene synthase inhibitors might be the difference in their action on squalene synthase. Squalene synthase catalyzes the head to head condensation of two molecules of FPP to form squalene. In the first reaction, two molecules of FPP react to form the stable cyclopropylcarbinyl diphosphate intermediate, presqualene pyrophosphate, with concomitant release of a proton and a molecule of inorganic pyrophosphate. In the second half of the reaction, presqualene pyrophosphate undergoes heterolysis, isomerization, and reduction with NADPH to form squalene (Poulter, 1990; Poulter & Rilling, 1981). A variety of structurally distinct, reversible, competitive, first half-reaction squalene synthase inhibitors such as P-3622, CP-210172, CP-295697 and CP-

294838 reduce cholesterogenesis by up to 90% with no appreciable increase in HMG-CoA reductase activity. In contrast, zaragozic acid A, an irreversible squalene synthase inhibitor, exhibits a high degree of HMG-CoA reductase modulation, producing increases in activity at concentrations that produce cholesterolgenesis inhibition (Petras *et al.*, 1999). YM-53601 may belong to the former group showing reversible, competitive, first half-reaction squalene synthase inhibition. These enzyme regulation aspects should be investigated in more detail.

In conclusion, we have developed an animal model of the escape phenomenon observed in humans using hamsters fed a high-fat diet. In this model, YM-53601 consistently decreased plasma non-HDL cholesterol without inducing HMG-CoA reductase activity. YM-53601 also decreased plasma non-HDL cholesterol in hamsters whose enzyme activities had been induced by pravastatin and returned HMG-CoA reductase activity to the control level. It should be noted that the substition of pravastatin by YM-53601 was effective in decreasing plasma cholesterol and HMG-CoA reductase activity, suggesting that an increase in HMG-CoA reductase activity might counteract the lipid-lowering effect of pravastatin. We therefore suggest that, because it does not induce HMG-CoA reductase activity, YM-53601 may decrease plasma cholesterol levels in both hypercholesterolemic patients who have been affected by the escape phenomenon as well as those who have not.

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