ELSEVIER

International Journal of Pharmaceutics 229 (2001) 75–86

international journal of pharmaceutics

www.elsevier.com/locate/ijpharm

Potency of select statin drugs in a new mouse model of hyperlipidemia and atherosclerosis

Thomas P. Johnston [a,*], Lien B. Nguyen [b], Waihei A. Chu [a], Sarah Shefer [b]

[a] *Division of Pharmaceutical Sciences, School of Pharmacy, University of Missouri, Kansas City, MO, 64110-2499, USA*
[b] *Department of Medicine and Liver Center, University of Medicine and Dentistry of New Jersey, New Jersey Medical School, Newark, NJ, USA*

Received 25 January 2001; received in revised form 28 June 2001; accepted 12 July 2001

Abstract

Poloxamer-407 (P-407) is a nonionic surfactant that induces atheroma formation in the aortas of C57BL/6 mice with long-term (14 weeks) administration. The objectives of the present study were to determine the mechanism(s) responsible for the induction of hypercholesterolemia as well as to determine whether this animal model may be of potential use in rank ordering the efficacy (lipid lowering) of various statin drugs. The effect of long-term (16 weeks) administration of P-407 on the catalytic activities of rate-limiting enzymes of cholesterol biosynthesis [HMG-CoA reductase (HMGR)] and catabolism [microsomal cholesterol 7α-hydroxylase (C7αH) and mitochondrial sterol 27 hydroxylase (S27H)] was assessed in C57BL/6 mice. Effects of P-407 on these enzymes were compared in mice fed an atheroma-inducing diet (high-cholesterol, supplemented with cholic acid) and animals maintained on a basal diet and injected with saline (controls) after 16 weeks. The mean value for the activities of C7αH in P-407-injected mice was 24.3 ± 3.8 pmol min^{-1} mg^{-1} and was significantly ($P < 0.05$) less than the mean value determined for sham-injected control animals (37.0 ± 14.3 pmol min^{-1} mg^{-1}). In contrast, the mean values for the catalytic activities of S27H and HMGR did not change with P-407 administration. Neither C7αH nor S27H activity in mice fed the high-cholesterol diet differed from values for control animals, whereas the mean HMGR activity was drastically reduced (-94%, $P < 0.05$). The hypercholesterolemic effect of P-407 is not due to altered cholesterol biosynthesis, but is mediated by reduced cholesterol catabolism due to decreased activity of the rate limiting enzyme (C7αH) in the classic bile acid synthetic pathway. Plasma triglyceride lowering resulting from the oral administration of equal doses of various statin drugs appeared, in general, to be positively correlated with their relative aqueous solubility and paralleled the efficacy of these agents to lower low-density-lipoprotein-associated cholesterol (LDL-C) in humans. The plasma triglyceride lowering effect of the five statin drugs tested produced the following rank order; pravastatin sodium (-44%) > atorvastatin calcium (-36%) > simvastatin (-33%) > lovastatin (-25%) > fluvastatin sodium (-19%). While reductions in plasma total cholesterol following administration of the statin drugs was not as profound as that observed with triglycerides, the relative rank order or trend was preserved. The percent reduction in plasma triglycerides in the present model appears to be a useful parameter with which to predict the relative reduction in plasma LDL-C expected for these agents in humans.

Keywords: Atherosclerosis; Hypercholesterolemia; Bile acids; 7α-hydroxylase; Sterol 27-hydroxylase; 3-hydroxy-3-methylglutaryl coenzyme A reductase; Statin drugs

* Corresponding author. Tel.: +1-816-235-1624; fax: +1-816-235-5190.
E-mail address: johnstont@umkc.edu (T.P. Johnston).

0378-5173/01/$ - see front matter
PII: S0378-5173(01)00834-1

1. Introduction

Because heart disease accounts for approximately 50% of all deaths in Western countries like the United States, research continues to be intensified so as to elucidate mechanisms responsible for atherosclerosis and stroke-related illnesses as well as develop new treatment strategies. The pathogenesis of atherosclerotic heart disease has received much attention, and significant advances have been made with regard to diagnosis and treatment. However, atherosclerosis is a multifactorial disease state. As such, each contributing variable in the development of atherosclerotic lesions has to be carefully identified, explained, and treatment solutions suggested.

One of the most significant advances in drug therapy during the twentieth century was development of the statin class of drugs. These agents inhibit the activity of 3-hydroxy-3-methylglutaryl Coenzyme A reductase (HMGR) as well as induce upregulation of LDL receptors on the cell surface (Witztum, 1996). HMGR is the rate-limiting enzyme associated with de novo cholesterol biosynthesis. As such, these drugs are enormously effective in reducing plasma concentrations of low-density-lipoprotein associated cholesterol (LDL-C). LDL-C has clearly been shown to play a role in the formation of atherosclerotic lesions subsequent to its oxidation in vivo. The oxidized form of LDL-C (ox-LDL) is thought to damage vascular endothelial cells and initiate the atherogenic cascade. The role of ox-LDL in atherosclerosis has now been formulated as one of two theories for the pathogenesis of atherosclerosis, namely, the 'modified (oxidized) LDL-C' theory.

The development of atherosclerosis has been studied in a variety of animal models which simulate the human condition. Recently, mouse models of atherogenesis have gained popularity because precise genetic alterations are possible which assist in identifying key factors involved with atherosclerosis. However, these 'knockout' mouse models, such as the LDL-receptor- and ApoE-knockout models, have limitations as well as advantages. It should be noted that no one animal model of atherosclerosis is ideal, but rather, each have their own distinct advantages and drawbacks. As an example, the popular diet-induced mouse model of atherosclerosis using the high-cholesterol, cholic acid-containing diet only forms lesions in female mice while most human heart disease is manifested in men. Additionally, gene knockout models, while helping to identify various factors involved in atherosclerotic lesion development, are a rather drastic departure from normal physiology and the human condition.

A new mouse model of atherosclerosis has recently been developed in our laboratory (Wout et al., 1992; Johnston and Palmer, 1993a; Johnston et al., 1993b; Porter et al., 1995; Nash et al., 1996; Li et al., 1996; Johnston and Palmer, 1997a,b; Palmer et al., 1997, 1998; Johnston et al., 1999, 2000). This new model of hyperlipidemia and atherosclerosis involves the administration of a nonionic surfactant called poloxamer 407 (P-407) to C57BL/6 mice. The precise degree of hypertriglyceridemia and hypercholesterolemia desired is easily achieved by the dose of P-407 administered. The effect of short-term (up to 72 h) treatment with P-407 has previously been characterized with regard to the catalytic activity of HMGR (rate limiting enzyme of cholesterol biosynthesis) as well as heparin-releasable (capillary-bound) and intracellular lipoprotein lipase (LPL). In addition, the effect of long-term (30 weeks) administration of P-407 to mice has been characterized with regard to mRNA levels of HMGR (Johnston et al., 1999). The P-407 mouse model of hyperlipidemia and atherosclerosis has also been used to screen the efficacy of several antihyperlipidemic drugs of various classes (Porter et al., 1995; Nash et al., 1996; Johnston and Palmer, 1997b; Johnston et al., 2000). In addition, the distribution of individual lipoproteins associated with triglycerides and cholesterol such as LDL, VLDL, IDL, and HDL following administration of P-407 to mice have been reported (Johnston et al., 1999). More recently, the P-407 mouse model of atherogenesis was shown to produce atherosclerotic lesions without the requirement of dietary cholic acid in female C57BL/6 mice at a rate and size comparable to those induced by the classic fat-fed model (Palmer et al., 1998). This is a significant advantage since it has been suggested that inclusion of

cholate in the diet may, in and of itself, cause inflammation to the vascular endothelium and initiate the atherogenic cascade (Breslow, 1996). The long-term (1 year) administration of P-407 to mice did not affect either hepatic total cholesterol concentrations (Palmer et al., 1998) or plasma concentrations of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (Johnston et al., 1999, 2000) and did not result in either morbidity or mortality when compared to control mice (Palmer et al., 1998). The most recent finding with the P-407-induced mouse model of atherosclerosis was that established aortic lesions could be completely regressed by concomitant administration of a typical 'statin' drug (atorvastatin) for 4 months (Johnston et al., 2000).

The purpose of the present study was two-fold. First, we sought to determine the mechanism by which mice are maintained in a hypercholesterolemic state in this new mouse model of hyperlipidemia and atherosclerosis. Secondly, since the currently-marketed statin drugs differ in their relative lipid-lowering potency, once a steady-state level of both plasma cholesterol and triglycerides had been obtained, the therapeutic efficacy (reduction in both plasma triglycerides and cholesterol) of five different statin drugs was evaluated in this new animal model.

2. Experimental procedures

Lithium heparin blood collection tubes were obtained from B&D (Franklin Lakes, NJ). Poloxamer 407, N.F. (Pluronic F-127; BASF Corporation, Parsippany, NJ) solution for intraperitoneal injection was prepared by combining the agent with double deionized water to a final concentration of 15 mg/ml. The solution was refrigerated overnight to facilitate dissolution of the P-407 by the cold method of incorporation (Schmolka, 1991). Plasma samples were obtained following centrifugation using a Beckman model GS-15R centrifuge (Palo Alto, CA). Pravastatin sodium (Pravachol®), atorvastatin calcium (Lipitor®), simvastatin (Zocor®), lovastatin (Mevacor®), and fluvastatin sodium (Lescol®) were provided as a gift from the inpatient pharmacy of the University of Kansas Medical Center, Kansas City, Kansas, and used as received. Each commercially-obtained statin drug underwent trituration in a mortar and pestle and was formulated in an aqueous carboxymethylcellulose suspension vehicle (1.5% in water) so that 0.5 ml of the formulation provided a dose of 70 mg/kg. Mice were maintained on a low-fat diet (Formulab diet 5008; PMI Nutrition International, Inc., Brentwood, MO) which consisted of 6.5% fat, 280 ppm cholesterol, linoleic acid (1.4%), linolenic acid (0.09%), arachidonic acid (0.01%), omega-3 fatty acids (0.3%), total saturated fatty acids (2.5%), and total monounsaturated fatty acids (2.3%). Mice which were maintained on the high-fat chow received Diet TD-88051 (HarlanTeklad, Madison, WI) which consisted of 12% fat, high-protein casein (7.5%), dextrose (2.5%), sucrose (1.6%), dextrin (0.6%), cocoa butter (7.5%), cholesterol (1.3%), sodium cholate (0.5%), cellulose (1.3%), Teklad mineral and vitamin mix (1.1%), and choline chloride (0.1%).

Five to six week-old, female C57BL/6 mice were obtained from Harlan Laboratories (Indianapolis, IN) and weighed an average of 14.7 ± 1.2 gm at the time of the study. Mice in all groups were housed (3 animals/cage) under controlled conditions at temperatures between 21 and 23 °C with a reverse 12–12 h light–dark cycle (19:00–07:00 h light). The animals were provided unrestricted access to water and the appropriate mouse chow described above for at least 2 weeks before the experiments to minimize any potential effects of changes in feeding schedules and diurnal rhythms. All procedures for P-407 administration and subsequent blood or tissue collection were in accordance with the institution's guide for the care and use of laboratory animals, and the treatment protocol was approved by the Animal Care Committee at the University of Missouri, Kansas City.

2.1. P-407 and statin administration

Twenty-seven mice were randomly assigned to three groups. The first group of mice (P) were administered 0.5 gm/kg P-407 by intraperitoneal (i.p.) injection every third day for 120 days and

consumed a normal chow diet. This dosing regimen was based on previous studies wherein a single 0.5 gm/kg dose of P-407 administered to C57BL/6 mice by i.p. injection caused an elevation in both plasma cholesterol and triglycerides 2 h post-dosing and a subsequent return of both lipids to normal plasma concentrations after approximately 120 h (Palmer et al., 1998). Using this dosage regimen with free access to mouse chow and water, we have previously demonstrated that plasma concentrations of total cholesterol and triglycerides may be maintained at approximately 1200 ± 225 mg/dl and 2800 ± 460 mg/dl, respectively, for up to 1 year (Palmer et al., 1998). Moreover, we have previously shown that administration of P-407 to female C57BL/6 mice for 1 year using the above dosing regimen did not result in either morbidity (other than the intended hyperlipidemia and subsequent atheroma formation) or mortality (Palmer et al., 1998; Johnston et al., 1999). For comparative purposes, the second treatment group (HF) consisted of mice that were fed the high-cholesterol 'Paigen' diet containing cholic acid and known to result in atheroma formation in this species (Jokinen et al., 1985; Ishida and Paigen, 1989; Paigen et al., 1990, 1994; Palmer et al., 1998). The third group of mice (C) were allowed to consume a standard chow diet and were injected with normal saline i.p. every third day for 120 days and served as sham-injected controls. Lastly, an extra group of 30 mice (**S**) were treated identically to mice contained in Group P (Group 1) and served as the group that would be administered one of the five statin drugs (6 mice/statin drug) evaluated in this study. Mice in Group S were studied separately so that administration of statin drugs would not interfere with the determination of the enzymatic activity of HMGR for mice in Groups P, HF, and C. After 120 days of P-407 administration using the treatment regimen described for mice in Group P, mice in Group S (6 mice/statin drug) were administered a 0.5 gm/kg dose of P-407 by i.p. injection at 07:00 h and then a 70 mg/kg dose of an individual statin by oral gavage at 18:00 h. A second and third dose of the individual statin drug being evaluated was administered orally at 18:00 h the following day and 2 days subsequent to the intraperitoneal injection of P-407, respectively. Dosing of statin drugs at 18:00 h was selected because it is well established that these agents exhibit maximum efficacy when dosed prior to sleep when HMGR attains its maximum biological activity (Ma et al., 1986). A final dose of P-407 was administered to mice in Group S according to schedule (07:00 h) 3 days following the previous injection of P-407 and all the mice sacrificed at 16:00 h that same day. No attempt was made to fast the mice overnight to minimize the contribution of postprandial lipid to the plasma triglyceride concentrations because statin administration occurred daily over a 3 day period. Moreover, this experiment was designed to assess the reduction in plasma triglyceride concentrations relative to the steady-state concentration achieved after 120 days of P-407 administration.

2.2. Animal sacrifice and tissue collection

At 120 days, all mice in each of Groups P, HF, and C were sacrificed and the livers immediately excised and then frozen in an acetone/dry ice mixture and stored at -80 °C until the time of analysis. Blood samples from mice in Group S were obtained by peri-orbital bleeding and then immediately centrifuged at 10 000 rpm to obtain the plasma. Plasma samples were stored at -20 °C until the time of analysis for total cholesterol and triglycerides.

2.3. Determination of plasma cholesterol and triglycerides

Plasma samples were assayed for total cholesterol by using standard enzymatic assay kits. Cholesterol was measured by the technique described by Allain et al. (1974). Plasma was assayed for triglycerides by the method of Bucolo and David (1973). All assays were performed in duplicate. No attempt was made to determine the plasma concentrations of very-low-density, low-density, and high-density lipoproteins associated with cholesterol, i.e. VLDL-, LDL-, or HDL-cholesterol, since these data (the lipoprotein distribution following P-407 treatment) have been reported previously (Johnston et al., 1999).

2.4. Assays for HMGR, C7αH, and S27H

Liver specimens were homogenized in 4 vol. buffer (250 mmol/l sucrose, 10 mmol/l Tris, and 0.1 mmol/l disodium EDTA, pH 7.2) with a Potter–Elvehjem homogenizer. Microsomal and mitochondrial fractions were prepared from the homogenates by differential ultracentrifugation (2000–9000 × *g* for mitochondria and 10 000–100 000 × *g* for microsomes). Protein concentrations in the liver fractions were measured by the method of Lowry et al. (1951). Sterol concentrations in the microsomes and mitochondria were analyzed by gas liquid chromatography (Xu et al., 1995) and were incorporated into the calculation of radioactive specific activity of the cholesterol substrate in the radioisotope incorporation methods that were used for assays of C7αH and S27H activities. Thus, the dilution of radiolabeled substrate by endogenous cholesterol (a problem that was previously avoided by the acetone extraction of sterols prior to enzyme assay) was eliminated. Results with control samples measured by the modified methods are comparable to those obtained with acetone powder preparations of microsomes (Shefer et al., 1988) for C7αH and the previously validated method for mitochondrial S27H (Shefer et al., 1995). It was determined that P-407 did not interfere with activity assays for HMGR, C7αH, and S27H. It has previously been demonstrated that only approximately 3% of an administered dose of P-407 is distributed to the hepatic tissue of rats (Li et al., 1996) since urinary excretion of P-407 as the parent compound is the primary route of elimination.

2.5. C7αH

[4-^{14}C]Cholesterol (60 mCi/mmol, Dupont/New England Nuclear) was purified on a silicic acid column (Nicolau et al., 1974) and mixed with similar purified unlabeled cholesterol to a specific activity of 15 dpm/pmol. A 100-nmol aliquot of [4-^{14}C] cholesterol was evaporated under nitrogen, dissolved in 25% β-cyclodextrin (Trappsol, Pharmatec Inc, Alachua, FL) and diluted with buffer (100 mM K_2HPO_4/0.1 mM DDT, pH 7.4) for a final concentration of 0.8% β-cyclodextrin. The microsomal enzyme (50–200 μg microsomal protein) was added for incubation at 37 °C for 10 min in a total volume of 0.5 ml in the presence of 5 mM DTT, 30 mM nicotinamide. The reaction was initiated with addition of 0.6 μmol NADPH or NADPH generating system (3.4 mM $NADP^+$/30 mM glucose-6-phosphate/0.3 units of glucose-6-phosphate dehydrogenase) and was stopped with 110 μl 8.9 M KOH. Aliquots of butylated hydroxytoluene (5 μg dissolved in 25 μl ethanol), ^{3}H-labeled (10 000 dpm) and unlabeled (5 μg in 10 μl ethanol) 7α-hydroxycholesterol for internal standard and product marker, respectively, were added. The mixture was hydrolyzed for 60 min at 37 °C and extracted twice with 3 ml hexane. The dried extracts, dissolved in 0.3 ml solvent A (hexane/isopropanol 97:3, v/v), were loaded on hexane-equilibrated silica columns (Sep-Pak, Waters, Inc.), washed free of cholesterol with 4 ml solvent A, then eluted with 3 ml solvent B (hexane/isopropanol 80:20, v/v). The eluted labeled products were separated by thin layer chromatography (silica gel 60 plates from EM Science, Gibbstown, NJ, developed with ether) and quantitated by liquid scintillation spectroscopy, using Ecolume (ICN Radiochemicals, Irvine, CA).

2.6. S27H

An isotope incorporation method with consideration of endogenous cholesterol was used as described above except for the following: mictochondria was used as enzyme source; an NADPH system for mitochondria using isocitrate dehydrogenase (Shefer et al., 1995) instead of glucose 6-phosphate dehydrogenase was included; [^{3}H]-labeled and unlabeled 7-hydroxycholesterol were used as recovery standard and product markers, respectively; and thin layer plates were developed with chloroform/methanol 94:6, v/v.

2.7. HMGR

Briefly, 50 and 100 μg of hepatic microsomal protein were preincubated for 2 min at 37 °C with an NADPH-generating system (as described for C7αH) in total final volume of 100 μl buffer (50 mM K_2PHO_4/70 mM KCl/10 mM DTT/30 mM EDTA, pH 7.4). The reaction was started

with the addition of 15 μl ^{14}C-labeled substrate ([^{14}C]HMG-CoA purchased from Amersham, diluted with unlabeled HMG-CoA to a specific activity of 25 dpm/pmol and a final concentration of 300 μM). The mixture was incubated for 15 min at 37 °C, and the reaction was stopped with 15 μl 6 M HCl. About 10 000 cpm of [^{3}H] mevalonolactone and unlabeled mevalonolactone were added for recovery standard and product marker, respectively. After lactonization at 37 °C for 15 min and the addition of 1 ml ether, 0.2 g sodium sulfate, and 100 μl water, the products were extracted twice with ether and separated by TLC on Silica Gel 60 plates with benzene/acetone (1:1, v/v) as the solvent system. The immediate product (^{14}C-labeled mevalonolactone) was quantitated by scintillation spectrometry in EcoLume. It should be noted that values for HMGR activity reported in this study represent total enzyme activity since microsomal HMGR was fractionated and assayed in the absence of phosphatase inhibitors.

2.8. Data analysis

All results were expressed as the mean value ± the standard deviation. Mean values of the plasma cholesterol and triglyceride concentrations between statin treatment groups for mice contained in Group S as well as the enzymatic activity associated with either C7αH, S27H, or HMGR between Groups P, HF, and C were compared for significant differences using a one-way analysis of variance (ANOVA). A Tukey's post hoc analysis was used to identify significantly different mean values (Snedecor and Cochran, 1980) if a significant ($P < 0.05$) F value was calculated during the ANOVA.

3. Results

3.1. Hepatic enzyme activities

The hepatic enzymatic activities of HMGR, C7αH, and S27H for each treatment group are listed in Table 1. As expected, the high-fat diet significantly reduced HMGR activity (−94%, $P < 0.05$), but had no effect on the bile acid synthetic enzymes. In contrast, P-407 administration significantly reduced microsomal C7αH activity (to 24.3 ± 3.8 pmol min^{-1} mg^{-1} from 37.0 ± 14.3 pmol min^{-1} mg^{-1}, $P < 0.05$) in the classic bile acid synthetic pathway, but produced no significant change in mitochondrial S27H activity in the alternative bile acid pathway and had no effect on HMGR activity in the cholesterol biosynthetic pathway.

3.2. Plasma cholesterol and triglyceride concentrations following statin treatment

Of the statins tested, pravastatin demonstrated the greatest reduction from the steady-state plasma concentration of triglycerides [−44% vs the steady-state concentration (Palmer et al., 1998) of 2800 ± 460 mg/dl]. As shown in Fig. 1, the relative rank order of effectiveness (potency) of the five statins tested in this model of hyperlipidemia and atherosclerosis was pravastain > atorvastatin > simvastatin > lovastatin > fluvastatin. The percent reduction in plasma triglycerides relative to the steady-state concentration for triglycerides in this model was −44 ± 3.9% (pravastatin), −36 ± 3.2% (atorvastatin), −33 ± 2.8% (simvastatin), −25 ± 2.7% (lovastatin), and −19 ± 1.4% (fluvastatin) which corresponded to post-statin

Table 1
Effects of Dietary/P-407 treatment on HMGR, C7αH, and S27H activities in mouse liver

Treatment	HMGR (pmol/min/mg)	C7αH (pmol/min/mg)	S27H (pmol/min/mg)
Control	165.4 ± 85.8[a]	37.0 ± 14.3	23.1 ± 8.6
HF 'Paigen' Diet	10.5 ± 5.1[b]	28.3 ± 10.0	37.8 ± 14.0
P-407-treated	148.5 ± 11.9	24.3 ± 3.8[b]	35.4 ± 13.8

(Mean value ± SD)
[a] Duplicate measurements on at least 4 animals per group were included for each enzyme.
[b] Significantly ($P < 0.05$) different from the mean value associated with controls.

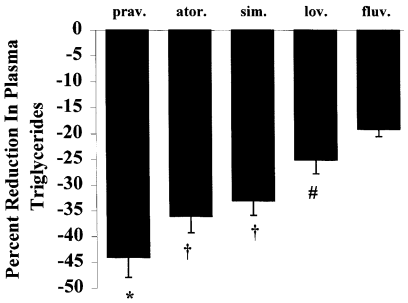

Fig. 1. The effect of statin drug therapy on the percent reduction in plasma triglycerides in C57BL/6 mice. Prav., ator., sim., lov., and fluv. represent abbreviations for pravastatin sodium, atorvastatin calcium, simvastatin, lovastatin, and fluvastatin sodium, respectively. * indicates a significantly ($P < 0.05$) greater percent reduction in plasma triglycerides relative to each of the other statin drugs tested. † indicates a significantly ($P < 0.05$) greater percent reduction in plasma triglycerides when either ator. or sim. is individually compared with either lov. or fluv., but no significant difference in the mean values between ator. and sim. # indicates a significantly ($P < 0.05$) greater percent reduction in plasma triglycerides for mice treated with lov. when compared to the same parameter for fluv.

treatment plasma triglyceride concentrations of approximately 1570 ± 110 mg/dl, 1790 ± 90 mg/dl, 1880 ± 80 mg/dl, 2100 ± 80 mg/dl, and 2270 ± 40 mg/dl, respectively. All post-statin treatment plasma triglyceride concentrations were significantly ($P < 0.05$) less than the steady-state value of 2800 ± 460 mg/dl for this model. Lovastatin was significantly ($P < 0.05$) more potent at reducing plasma triglycerides than fluvastatin; atorvastatin and simvastatin were significantly ($P < 0.05$) more effective than lovastatin, although there was no difference between these two drugs; and pravastatin was significantly ($P < 0.05$) more efficacious than all of the statins tested. Plasma total cholesterol concentrations followed the same relative rank order reduction from the cholesterol steady-state value [1200 ± 225 mg/dl; (Palmer et al., 1998)] as that observed with triglyceride lowering, albeit the reductions were more moderate in nature. The percent decrease in plasma total cholesterol concentrations relative to the steady-state value of 1200 ± 225 mg/dl were $-24 \pm 2.1\%$, $-19 \pm 1.7\%$, $-17 \pm 1.1\%$, $-11 \pm 0.8\%$, and $-8 \pm 0.6\%$ for pravastatin, atorvastatin, simvastatin, lovastatin, and fluvastatin, respectively (Fig. 2).

4. Discussion

The potency of the five statin drugs evaluated in this study appears to be in close agreement with the rank order observed in humans with one exception. Pravastatin exhibited the greatest plasma triglyceride lowering effect in this new mouse model of hyperlipidemia and atherosclerosis. It is of interest to note that pravastatin demonstrates the greatest aqueous solubility ($>$ 300 mg/ml) of the five statins tested. However, it should be noted that fluvastatin sodium is also water soluble, but its administration did not result in a plasma triglyceride lowering effect similar to pravastatin. Clinically, fluvastatin sodium has been found to be the least potent of the five statins evaluated (Jones et al., 1998) and that finding has been demonstrated in the present study as well. It has been suggested (Hamelin and Turgeon, 1998) that even though fluvastatin (octanol:water partition coefficient ≈ 25) is completely absorbed ($\approx 98\%$) following oral

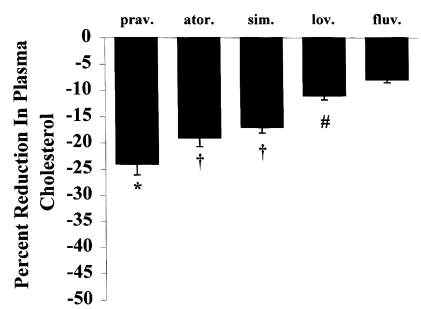

Fig. 2. The effect of statin drug therapy on the percent reduction in plasma cholesterol in C57BL/6 mice. Prav., ator., sim., lov., and fluv. represent abbreviations for pravastatin sodium, atorvastatin calcium, simvastatin, lovastatin, and fluvastatin sodium, respectively. *Indicates a significantly ($P <$ 0.05) greater percent reduction in plasma cholesterol relative to each of the other statin drugs tested. †Indicates a significantly ($P < 0.05$) greater percent reduction in plasma cholesterol when either ator. or sim. is individually compared with either lov. or fluv., but no significant difference in the mean values between ator. and sim. # Indicates a significantly ($P <$ 0.05) greater percent reduction in plasma cholesterol for mice treated with lov. when compared to the same parameter for fluv.

administration, its lipid-lowering effectiveness may be compromised relative to more hydrophilic pravastatin (octanol:water partition coefficient $\approx$ 0.6) because of its passive diffusion into hepatocytes, its extremely efficient metabolism ($>90\%$) into principally inactive metabolites, and its elimination (parent drug plus metabolites) directly into the bile. In contrast, pravastatin is not as completely absorbed as fluvastatin following oral administration, but the former is efficiently and extensively concentrated in hepatocytes (hepatoselective) via the sodium ion-independent organic anion transporter (Tsuji, 1999). Incorporation of pravastatin in peripheral tissues is limited by the lack of active transporter systems in peripheral cells (e.g. muscle cells, fibroblasts, human lens). Taken together, these findings may help to explain why fluvastatin was the least potent and pravastatin the most potent of the five statin drugs evaluated in our mouse model of hyperlipidemia.

Of the statin drugs investigated in the present study, only pravastatin and fluvastatin completely dissolved in the oral formulation and, as such, did not actually conform to the true definition of a drug suspension. Rather, these two statin formulations were each a true drug solution which contained 1.5% (w/v) carboxymethylcellulose (CMC) to simulate the composition of the other three statin suspensions. Thus, this would have resulted in the oral administration of a drug solution containing CMC in the case of pravastatin and fluvastatin and a drug suspension for atorvastatin, simvastatin, and lovastatin. The three other statins (atorvastatin, simvastatin, and lovastatin) are relatively water-insoluble with a rank-order aqueous solubility of atorvastatin > simvastatin > lovastatin. Both lovastatin and simvastatin are inactive lactones which must be hydrolyzed to the β-hydroxyacid form for activity. Nagasawa et al. (Nagasawa et al., 2000) has reported the octanol:pH 7 phosphate buffer partition coefficients for pravastatin and the β-hydroxyacid forms of lovastatin and simvastatin as 0.59, 50, and 115, respectively. Results from the present investigation provide suggestive evidence that the magnitude of the plasma triglyceride and cholesterol lowering effect for statin drugs in the P-407-induced mouse model of hyperlipidemia and atherosclerosis was positively correlated with aqueous solubility; the one exception being fluvastatin. While there is currently a sixth statin drug on the market in the U.S. (cerivastatin sodium; Baycol®), it was not evaluated in the present study. Cerivastatin is an entirely synthetic, enantiomerically pure HMGR inhibitor which is soluble in water and which has a recommended dose (0.4 mg once daily) that is 200-fold less than the maximum daily dose for the other five statins (80 mg daily).

The findings in the present study are also similar to the findings of Krause and Newton (Krause and Newton, 1995) using atorvastatin and lovastatin in rats. Using chow-fed rats, Krause and Newton demonstrated that plasma triglycerides were lowered to a greater extent by atorvastatin compared to lovastatin following oral administration of doses ranging from 25 to 100 mg/kg (Krause and Newton, 1995). These authors (Krause and Newton, 1995) concluded that normal rats could be used to assess the potential lipid-lowering effects of HMGR inhibitors in preclinical studies and that such data was positively correlated with the efficacy of these agents found in larger animal models (guinea pigs and rabbits) in which LDL is the major cholesterol transporting lipoprotein. Data obtained in the present study which determined the percent reduction in plasma triglycerides and total cholesterol following oral statin therapy to P-407-induced hyperlipidemic mice would appear to be predictive of the relative rank order for lipid-lowering observed with these agents in humans. Use of plasma triglyceride-lowering data in mice following statin therapy would appear to be a reasonable parameter to assess statin efficacy in humans because (1) the P-407-induced hyperlipidemic mouse model elevates plasma triglycerides much more profoundly than cholesterol; and (2) it is now well established that inhibitors of HMGR primarily lower plasma triglycerides and not plasma cholesterol in normal chow-fed rats by inhibiting hepatic triglyceride secretion (Yoshino et al., 1988; Khan et al., 1989, 1990; Yoshino et al., 1991; Kasim et al., 1992). This is unlike the situation in other species such as rabbits (La Ville et al., 1984; Tsujita et al., 1986; Quig and Zilversmit, 1988),

miniature pigs (Huff and Telford, 1989), and guinea pigs (Berglund et al., 1989; Matsunaga et al., 1991) in which there is no change in plasma triglycerides and cholesterol-lowering represents the major drug action. The exact mechanism(s) responsible for the triglyceride-lowering action of the statin drugs is still not known, but probably reflects the decrease in very-low-density-lipoprotein (VLDL) levels since the major lipid class associated with VLDL is endogenous triglycerides (Witztum, 1996). It is interesting to note that in the P-407 mouse model of hyperlipidemia, hypertriglyceridemia occurs as a result of P-407's ability to inactivate capillary-bound lipoprotein lipase (Johnston and Palmer, 1993a) and that the major lipoprotein present in plasma following P-407 administration is VLDL (Johnston et al., 1999). Regardless, statin therapy in the present animal model most likely caused a reduction in plasma triglycerides both by decreasing VLDL levels and by inhibiting hepatic triglyceride secretion similar to the action of these agents in rats.

The present study has also successfully demonstrated one possible mechanism for the hypercholesterolemic response observed in C57BL/6 mice following extended administration of P-407. Since we have previously demonstrated no direct effect of P-407 on hepatic HMGR activity both in vitro and when assayed in microsomes from P-407-treated animals (Johnston and Palmer, 1997a; Johnston et al., 1999) and also observed no permanent effect on hepatic cholesterol stores (Palmer et al., 1998), we hypothesized that the observed hypercholesterolemic effect was due to a cholesterol elimination disorder.

Cholesterol is normally eliminated into bile. The enzyme that catalyzes the biochemical reaction of cholesterol conversion to 7α-hydroxycholesterol is microsomal cholesterol 7α-hydroxylase (C7αH) [EC 1.14.13.17] which is considered to be rate controlling (Shefer et al., 1970; Myant and Mitropoulos, 1997). This bile acid synthetic pathway is designated as the classic pathway. The hepatic bile acid flux exerts negative feedback control on C7αH (Eriksson, 1957; Bergstrom and Danielsson, 1958; Shefer et al., 1990) so that depletion of the bile acid pool upregulates, and feeding of bile acids inhibits C7αH and bile acid synthesis via the classic pathway. However, it has been shown that bile acids may be synthesized via an alternative pathway as well (Björkhem, 1992; Javitt, 1994). The initial reaction is the formation of 27-hydroxycholesterol from cholesterol catalyzed by mitochondrial sterol 27-hydroxylase (S27H). The alternative pathway begins with the formation of 27-hydroxycholesterol that is then 7α-hydroxylated by microsomal oxysterol 7α-hydroxylase (27-hydroxycholesterol-7α-hydroxylase), an enzyme that is independent and different from C7αH, but both pathways lead to the formation of primary bile acids (cholic acid and chenodeoxycholic acid).

The present investigation shows that P-407 inhibits C7αH activity, but does not significantly affect the enzymatic activity of either S27H or HMGR. The lack of activity of P-407 towards HMGR in the present study supports our previous findings which demonstrated no direct effect of P-407 on HMGR activity both in vitro and when assayed in hepatic microsomal homogenates obtained after 30, 100, and 200 days of P-407 administration to mice (Johnston and Palmer, 1997a; Johnston et al., 1999). Since nonionic detergents have been shown to be rather inert with regard to their structure-perturbing and protein-denaturant properties, then presumably the long-term administration of P-407 affected the bile acid pool, although the present study did not eliminate the possibility of surfactant-mediated enzyme inactivation. It is unlikely that P-407 acts directly on the enzymes. If a direct effect of the surfactant on the enzyme had occurred in vivo, one would expect that the surfactant would exhibit the same effect on both C7αH and HMGR. That is, P-407 would tend to denature (inactivate) both enzymes rather than decrease the enzymatic activity of C7αH while leaving HMGR and S27H fully active. It should be noted that, while S27H is located in mitochondria, both C7αH and HMGR are microsomal enzymes exposed to the same endogenous concentration of P-407.

A more plausible explanation for the hypercholesterolemia observed following administration of P-407 to mice would be an increase in the bile

acid pool. P-407, being a surfactant, may act as a substrate stabilizer in rats and mice and cause an initial stimulation of both bile acid synthetic enzymes, followed by an expansion of the bile acid pool, which will, in turn, downregulate C7αH activity. Such a time-dependent expansion of the bile acid pool and inhibition of C7αH, but not S27H, have been demonstrated in the rabbit (Xu et al., 1998, 1999). Alternatively, P-407 may potentially interfere with bile acid secretion into the intestine. Interference with bile acid secretion would result in an expanded bile acid pool and subsequent reduction of C7αH activity, and an increase or no change in cholesterol elimination via the alternative bile acid pathway, which is not regulated by bile acids via the feedback mechanism (Nguyen et al., 1999).

In conclusion, we have shown that administration of P-407 to mice over a 4-month period resulted in a significant reduction in the activity of C7αH and no significant change in the activity of either S27H or HMGR. We hypothesize that this occurred due to an expansion of the bile acid pool due to either P-407's ability to inhibit bile acid secretion, add to the bile acid (detergent) pool, or modify the substrate (cholesterol) such that C7αH was unable, while S27H was still able, to convert the P-407-modified cholesterol to bile acids. The increased bile acid pool would then inhibit C7αH in the classic bile acid synthetic pathway and cause excess cholesterol to accumulate in the systemic circulation. In addition to determining a potential mechanism for the induced hypercholesterolemia, this mouse model which utilizes the percent reduction in plasma triglycerides would appear to be particularly effective in predicting the relative potency of existing and/or new statin drugs in humans. The rank order of statin-mediated, plasma triglyceride-lowering efficacy appeared to be positively correlated with the individual statin's aqueous solubility and may assist pharmaceutical scientists with a rapid screening tool for this class of drugs. It remains to be determined whether this new mouse model of hyperlipidemia will prove beneficial in screening the relative potency of a series of compounds in another antihyperlipidemic drug class.

Acknowledgements

This work was supported in part by a Grant-In-Aid from the American Heart Association, Kansas Affiliate, Inc., awarded to T.P. Johnston, and a Grant-In-Aid from the American Heart Association, New Jersey, Inc., awarded to L.B. Nguyen and the National Institutes of Health grant DK26756, awarded to S. Shefer.

References

Allain, C.A., Poor, L.S., Chan, C.S., Richmond, W., Fu, P.C., 1974. Enzymatic determination of total serum cholesterol. Clin. Chem. 20, 470–476.

Berglund, L.F., Sharkey, M.F., Elam, R.L., Witztum, J.L., 1989. Effects of lovastatin therapy on guinea pig low density lipoprotein composition and metabolism. J. Lipid Res. 30, 1591–1597.

Bergstrom, S., Danielsson, H., 1958. On the regulation of the bile acid formation in the rat liver. Acta Physiol. Scand. 43, 1–7.

Björkhem, I., 1992. Mechanism of degradation of the steroid side chain in the formation of bile acids. J. Lipid Res. 33, 455–471.

Breslow, J.L., 1996. Mouse models of atherogenesis. Science 272, 685–688.

Bucolo, G., David, H., 1973. Quantitative determination of serum triglycerides by the use of enzymes. Clin. Chem. 19, 476–479.

Eriksson, S., 1957. Biliary excretion of bile acids and cholesterol in bile fistula rats. Proc. Soc. Exp. Biol. Med. 94, 578–582.

Hamelin, B.A., Turgeon, J., 1998. Hydrophilicity/lipophilicity: relevance for the pharmacology and clinical effects of HMG-CoA reductase inhibitors. Trends Pharmacol. Sci. 19, 26–37.

Huff, M.V., Telford, D.E., 1989. Regulation of low density lipoprotein apoprotein B metabolism by lovastatin and cholestyramine in miniature pigs: effects on LDL composition and synthesis of LDL subfractions. Metabolism 38, 256–262.

Ishida, B.Y., Paigen, B., 1989. Atherosclerosis in the mouse. In: Lusis, A.J., Sparkes, S.R. (Eds.), Genetic Factors in Atherosclerosis—Approaches and Model Systems. Monographs in Human Genetics. Karger, Basel, pp. 189–222.

Javitt, N.B., 1994. Bile acid synthesis from cholesterol: Regulatory and auxiliary pathways. FASEB J. 8, 1308–1311.

Johnston, T.P., Palmer, W.K., 1993a. Mechanism of poloxamer 407-induced hypertriglyceridemia in the rat. Biochem. Pharmacol. 46, 1037–1042.

Johnston, T.P., Beris, H., Kennedy, J.L., 1993b. Effects on splenic, hepatic, hematological, and growth parameters following high-dose poloxamer 407 administration to rats. Int. J. Pharm. 100, 279–284.

Johnston, T.P., Palmer, W.K., 1997a. Effect of poloxamer 407 on the activity of microsomal 3-hydroxy-3-methylglutaryl CoA reductase in rats. J. Cardiovasc. Pharmacol. 29, 580–585.

Johnston, T.P., Palmer, W.K., 1997b. The effect of pravastatin on hepatic 3-hydroxy-3-methylglutaryl CoA reductase obtained from poloxamer 407-induced hyperlipidemic rats. Pharmacotherapy 17, 342–347.

Johnston, T.P., Baker, J.C., Hall, D., Jamal, A.S., Emeson, E.E., Palmer, W.K., 1999. Potential downregulation of HMG-CoA reductase following chronic administration of P-407 in C57BL/6 mice. J. Cardiovasc. Pharmacol. 34, 831–842.

Johnston, T.P., Baker, J.C., Hall, D., Jamal, A.S., Palmer, W.K., Emeson, E.E., 2000. Regression of poloxamer 407-induced atherosclerotic lesions in C57BL/6 mice using atorvastatin. Atherosclerosis 149, 303–313.

Jokinen, M., Clarkson, T., Prichard, R., 1985. Animal models in atherosclerosis research. Exp. Mol. Path. 42, 1–28.

Jones, P., Kafonek, S., Laurora, I., Hunninghake, D., 1998. Comparative dose efficacy study of atorvastatin versus simvastatin, pravastatin, lovastatin, and fluvastatin in patients with hypercholesterolemia. Am. J. Cardiol. 81, 582–587.

Kasim, S.E., LeBoeuf, R.C., Khilnani, S., Tallapaka, L., Dayananda, D., Jen, K.-LC, 1992. Mechanisms of triglyceride-lowering effect of an HMG-CoA reductase inhibitor in a hypertriglyceridemic animal model, the Zucker obese rat. J. Lipid Res. 33, 1–11.

Khan, B., Wilcox, H.G., Heimberg, M., 1989. Cholesterol is required for secretion of very-low-density lipoprotein by rat liver. Biochem. J. 259, 807–814.

Khan, B.V., Fungwe, T.V., Wilcox, H.G., Heimberg, M., 1990. Cholesterol is required for the secretion of the very-low-density lipoprotein: in vivo studies. Biochim. Biophys. Acta 1044, 297–304.

Krause, B.R., Newton, R.S., 1995. Lipid-lowering activity of atorvastatin and lovastatin in rodent species: triglyceride-lowering in rats correlates with efficacy in LDL animal models. Atherosclerosis 117, 237–244.

La Ville, A., Moshy, R., Turner, P.R., Miller, N.E., Lewis, B., 1984. Inhibition of cholesterol synthesis reduces low-density-lipoprotein apoprotein B production without decreasing very-low-density-lipoprotein apoprotein B synthesis in rabbits. Biochem. J. 219, 321–326.

Li, C., Palmer, W.K., Johnston, T.P., 1996. Disposition of poloxamer 407 following intraperitoneal injection to rats. J. Pharm. Biomed. Anal. 14, 659–665.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265–275.

Ma, P.T.S., Gil, G., Südhof, T.C., Bilheimer, D.W., Goldstein, J.L., Brown, M.S., 1986. Mevinolin, an inhibitor of cholesterol synthesis, induces mRNA for low density lipoprotein receptor in livers of hamsters and rabbits. Proc. Natl. Acad. Sci. USA 83, 8370–8374.

Matsunaga, A., Sasaki, J., Takada, Y., Hidaka, K., Arakawa, K., 1991. Effect of simvastatin on receptor mediated metabolism of low density lipoprotein in guinea pigs. Atherosclerosis 90, 31–43.

Myant, N.B., Mitropoulos, K.A., 1997. Cholesterol 7α-hydoxylase. J. Lipid Res. 18, 135–153.

Nagasawa, K., Muraki, Y., Matsuda, T., Ohnishi, N., Yokoyama, T., 2000. Inhibitory effect of statins on fetal bovine serum-induced proliferation of rat cultured mesangial cells and correlation between their inhibitory effect and transport characteristics. J. Pharm. Sci. 89, 1594–1604.

Nash, V.L., Johnston, T.P., Palmer, W.K., 1996. Effects of nicotinic acid on poloxamer 407-induced hyperlipidemia. Pharmacotherapy 16, 10–15.

Nguyen, L.B., Xu, G., Shefer, S., Tint, G.S., Batta, A.K., Salen, G., 1999. Comparative regulation of hepatic sterol 27-hydroxylase and cholesterol 7α-hydroxylase activities in the rat, guinea pig, and rabbit: Effects of cholesterol and bile acids. Metabolism 48, 1542–1548.

Nicolau, G., Shefer, S., Salen, G., Mosbach, E.H., 1974. Determination of hepatic cholesterol 7α-hydroxylase activity in man. J. Lipid Res. 15, 146–151.

Paigen, B., Ishida, B.Y., Verstuyft, J., Winters, R.B., Albee, D., 1990. Atherosclerosis susceptibility differences among progenitors of recombinant inbred strains of mice. Arteriosclerosis 10, 316–323.

Paigen, B., Plump, A.S., Rubin, E.M., 1994. The mouse as a model for human cardiovascular disease and hyperlipidemia. Curr. Opin. Lipidol. 5, 258–264.

Palmer, W.K., Emeson, E.E., Johnston, T.P., 1997. The poloxamer 407-induced hyperlipidemic atherogenic animal model. Med. Sci. Sports Exerc. 29, 1416–1421.

Palmer, W.K., Emeson, E.E., Johnston, T.P., 1998. Poloxamer 407-induced atherogenesis in the C57BL/6 mouse. Atherosclerosis 136, 115–123.

Porter, J., Carter, B.L., Johnston, T.P., Palmer, W.K., 1995. Effect of pravastatin on poloxamer 407-induced hyperlipidemia in rats. Pharmacotherapy 15, 92–98.

Quig, D.W., Zilversmit, D.B., 1988. Dissociation between cholesterol secretion and plasma lipid transfer activity in rabbits. FASEB J. 2, 2712–2717.

Schmolka, I.R., 1991. Poloxamers in the pharmaceutical industry. In: Tarch, P.J. (Ed.), Polymers for Controlled Drug Delivery. CRC Press, Boca Raton, pp. 189–215.

Shefer, S., Hauser, S., Berkersky, I., Mosbach, E.H., 1970. Biochemical site of regulation of bile acid biosynthesis in the rat. J. Lipid Res. 11, 404–411.

Shefer, S., Salen, G., Nguyen, L.B., Batta, A.K., Packin, V., Tint, G.S., Hauser, S., 1988. Competitive inhibition of bile acid synthesis by endogenous cholestanol, and sitosterol in sitosterolemia with xanthomatosis: Effect of cholesterol 7 alpha hydroxylase. J. Clin. Invest. 82, 1833–1839.

Shefer, S., Nguyen, L., Salen, G., Batta, A.K., Brooker, D., Zaki, F.G., Rani, I., Tint, G.S., 1990. Feedback regulation of bile acid synthesis in the rat. J. Clin. Invest. 85, 1191–1198.

Shefer, S., Kren, B.T., Salen, G., Steer, C.J., Nguyen, L.B., Chen, T., Tint, G.S., Batta, A.K., 1995. Regulation of bile acid synthesis by deoxycholic acid in the rat: Different effects on cholesterol 7α-hydoxylase and sterol 27-hydroxylase. Hepatology 22, 1215–1221.

Snedecor, G.W., Cochran, W.G., 1980. One-way classifications; analysis of variance. In: Snedecor, G.W., Cochran, W.G. (Eds.), Statistical Methods. Iowa State University Press, Ames, pp. 215–233.

Tsuji, A., 1999. Tissue selective drug delivery utilizing carrier-mediated transport systems. J. Controlled Rel. 62, 239–244.

Tsujita, Y., Kuroda, M., Shimada, Y., Tanzawa, K., Arai, M., Kaneko, I., Tanaka, M., Masuda, H., Tarumi, C., Watanabe, T., Fujii, S., 1986. CS-514, a competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase: tissue-selective inhibition of sterol synthesis and hypolipidemic effect on various animal species. Biochim. Biophys. Acta 877, 50–57.

Witztum, J.L., 1996. Drugs used in the treatment of hyperlipoproteinemias. In: Hardman, J.G., Limbird, L.E., Molinoff, P.B., Ruddon, R.W. (Eds.), The Pharmacological Basis of Therapeutics, 9th. McGraw-Hill, New York, pp. 875–897.

Wout, Z., Pec, E.A., Johnston, T.P., 1992. Poloxamer 407-mediated changes in plasma cholesterol and triglycerides following intraperitoneal injections to rats. J. Parent. Sci. Technol. 46, 192–200.

Xu, G., Salen, G., Shefer, S., Ness, G.C., Nguyen, L.B., Parker, T.S., Chen, T.S., Zhao, Z., Donnelly, T.M., Tint, G.S., 1995. Unexpected inhibition of cholesterol 7α-hydroxylase by cholesterol in New Zealand white and Watanabe heritable hyperlipidemic rabbits. J. Clin. Invest. 95, 1497–1504.

Xu, G., Salen, G., Shefer, S., Tint, G.S., Nguyen, L.B., Parker, T.T., Chen, T.S., Roberts, J., Kong, X., Greenblatt, D., 1998. Regulation of classic and alternative bile acid synthesis in hypercholesterolemic rabbits: Effects of cholesterol feeding and bile acid depletion. J. Lipid Res. 39, 1608–1615.

Xu, G., Salen, G., Shefer, S., Tint, G.S., Nguyen, L.B., Chen, T.S., Greenblatt, D., 1999. Increasing dietary cholesterol induces different regulation of classic and alternative bile acid synthesis. J. Clin. Invest. 103, 89–95.

Yoshino, G., Kazumi, T., Kasama, T., Iwai, M., Iwatani, I., Matsuba, K., Matsushita, M., Baba, S., 1988. Effect of CS-514 (pravastatin) on VLDL-triglyceride kinetics in rats. Atherosclerosis 73, 191–198.

Yoshino, G., Matsushita, M., Iwai, M., Morita, M., Matsuba, K., Hirano, T., Furukawa, S., Kazumi, T., Baba, S., 1991. Effect of pravastatin on newly-secreted very low density lipoprotein composition in rats. Hor. Metab. Res. 23, 193–201.