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The cholesterol absorption inhibitor, ezetimibe, decreases diet-induced hypercholesterolemia in monkeys

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Abstract

Ezetimibe (1-(4-fluorophenyl)-(3*R*)-[3-(4-fluorophenyl)-(3*S*)-hydroxypropyl]-(4*S*)-(4-hydroxyphenyl)-2-azetidinone) potently and selectively inhibits the intestinal absorption of cholesterol, thereby reducing plasma cholesterol in preclinical models of hypercholesterolemia. In rhesus monkeys fed a diet containing 375 mg/day of cholesterol, 0.1 mg/kg of ezetimibe completely prevented the doubling of plasma cholesterol normally induced under these dietary conditions ($ED_{50} = 0.0005$ mg/kg). Low-density lipoprotein cholesterol (LDL) was dose-dependently reduced, while high-density lipoprotein cholesterol (HDL) and plasma triglyceride were unchanged. A single dose of an ezetimibe analog administered to cynomolgus monkeys fed a single cholesterol-containing meal caused a significant reduction (−69%) of cholesterol in chylomicrons during the postprandial phase without affecting triglyceride content. In rhesus monkeys, apolipoprotein (apo) B_{48} concentrations in chylomicrons did not differ between control and the ezetimibe analog, but apo B_{100} was significantly reduced in LDL (−41%). These data indicate that these cholesterol absorption inhibitors reduce cholesterol content in chylomicrons, which indirectly leads to a decrease in LDL cholesterol and particle number. © 2001 Published by Elsevier Science B.V.

Keywords: LDL (low density lipoprotein); Chylomicron; Apo B_{48}; Apo B_{100}; Postprandial; (Rhesus monkey); Cynomolgus monkey

1. Introduction

There is increasing evidence that reductions in plasma cholesterol lead to reductions in deaths from cardiovascular disease (4S: Scandinavian Simvastatin Survival Study Group, 1994; Shepherd et al., 1995; Sacks et al., 1996; LIPID: The Long-Term Intervention with Pravastatin in Ischaemic Disease, 1998). We have previously described the discovery of a novel class of cholesterol absorption inhibitors, ezetimibe (SCH58235: 1-(4-fluorophenyl)-(3*R*)-[3-(4-fluorophenyl)-(3*S*)-hydroxypropyl]-(4*S*)-(4-hydroxyphenyl)-2-azetidinone)) and an analog of ezetimibe, SCH48461 ((3*R*,4*S*)-1,4-bis-(4-methoxyphenol)-3-(3-phenylpropyl)-2-azetidinone; Fig. 1), that lower total plasma cholesterol in preclinical models of hypercholesterolemia (van Heek et al., 1997, 2000; Rosenblum et al., 1998; Salisbury et al., 1995). This class of compounds inhibits the intestinal absorption of both dietary and biliary cholesterol. In the present studies, we characterized the effect of ezetimibe on low density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol in a rhesus monkey model of hypercholesterolemia induced by a high fat, cholesterol containing diet. Previous studies also demonstrated in rodent models that ezetimibe and its analogs inhibit the transport of cholesterol from the intestinal lumen through the intestinal wall, ultimately diminishing the appearance of intestinally derived cholesterol in the plasma (van Heek et al., 1997, 2000). This raised the interesting question of what effect ezetimibe would have on the cholesterol content of chylomicrons in the postprandial state. Therefore, the composition of postprandial chylomicrons, as well as the concentration of apo B_{48} and apo B_{100} in chylomicrons and LDL during the postprandial period, was determined in cynomolgus and rhesus monkeys, respectively. Monkeys, rather than rodents, were utilized in these experiments because the outcomes would

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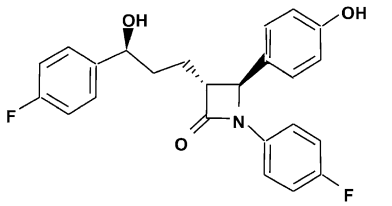
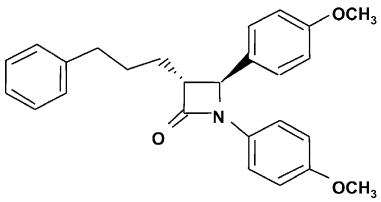


Ezetimibe (SCH58235)



SCH48461

Fig. 1. Structure of ezetimibe (SCH58235) and the ezetimibe analog, SCH48461. Ezetimibe: (1-(4-fluorophenyl)-(3*R*)-[3-(4-fluorophenyl)-(3*S*)-hydroxypropyl]-(4*S*)-(4-hydroxyphenyl)-2-azetidinone). SCH48461: (3*R*,x4*S*)-1,4-bis-(4-methoxyphenol)-3-(3-phenylpropyl)-2-azetidinone.

be more likely to predict what will occur in the human population.

Ezetimibe is rapidly progressing through clinical trials. Preliminary data indicated that ezetimibe and its analog, SCH48461, significantly lowered LDL cholesterol and raised HDL cholesterol (ezetimibe only) in hypercholesterolemic humans (Lipka et al., 2000; Bergman et al., 1995). Whether ezetimibe will have an effect on the cholesterol content of postprandial lipoproteins in humans is not yet known, but may have important implications for the development of atherosclerosis. Over two decades ago, Zilversmit (1979) proposed that atherogenesis may occur during the postprandial period since most people of the developed world consume a high-cholesterol, high-fat diet and are mostly in a postprandial state. In this seminal paper, Zilversmit (1979) suggested that postprandial lipids and chylomicron remnants in particular might be a risk factor for atherogenesis. Experimental data and data from a large number of case-control studies supporting this hypothesis have been accumulating (for reviews, please see Mamo, 1995; Zilversmit, 1995; Karpe, 1999; Ros, 2000). If the data in monkeys presented here translates to humans, ezetimibe may provide a novel pharmacological tool to decrease the cholesterol content of postprandial lipoproteins, thereby decreasing the potential atherogenicity of these particles.

2. Materials and methods

Ten adult male and female rhesus monkeys were divided into two groups of five based on body weight and fasting basal plasma cholesterol (Wako, Osaka, Japan). Rhesus monkeys were then fed 150 g/day of a chow-based diet containing 0.25% (w/w; 375 mg/day) cholesterol, 15% (w/w) hydrogenated coconut oil and 7.5% (w/w) olive oil with or without 0.1 mg/kg/day of ezetimibe for 20 days (Research Diets, New Brunswick, NJ). At the end of 20 days, the ezetimibe-treated group was switched to control chow, while the original control group was switched to the diet containing 0.1 mg/kg/day ezetimibe. Every 6–7 days throughout the experiment, fasting blood samples were obtained for total cholesterol and triglyceride (Sigma, St. Louis, MO). At day 0, 20 and 35 (15 days after the crossover), an aliquot of fresh plasma was subjected to sequential ultracentrifugation for the separation of the following lipoprotein classes (Havel et al., 1955): $d < 1.019$ (very low density lipoprotein + intermediate density lipoprotein, VLDL + IDL), $1.019 < d < 1.063$ g/ml (LDL) and $1.063 < d < 1.225$ g/ml (HDL). Cholesterol and triglyceride were determined on these fractions as well.

A full dose–response of ezetimibe (0.0003–0.01 mg/kg/day) was conducted in male and female rhesus monkeys (total $n = 28$) over the course of two separate experiments with overlapping doses ($n = 5–10$/group). Fasting blood samples were obtained for total cholesterol, VLDL + IDL cholesterol, LDL cholesterol and HDL cholesterol as described above. Ezetimibe was delivered admixed in the diet at 0, 0.0003, 0.001, 0.003, or 0.01 mg/kg/day for 3 weeks (Research Diets). After 3 weeks, total cholesterol, VLDL + IDL, LDL and HDL cholesterol were determined again.

Six cynomolgus monkeys were utilized to determine the effect of a single dose of the ezetimibe analog, SCH48461, on postprandial chylomicrons. Monkeys ($n = 3$/group), which had been maintained on chow, were fasted for 20 h, then given a 150-g of the diet described above with or without SCH48461 (10 mg/kg). Five hours into the postprandial period, a blood sample (25–30 ml) was taken under anesthesia (Telazol, 1–3 mg/kg, intramuscular), plasma was separated and chylomicrons were isolated from a known volume of plasma (10–12 ml). Briefly, plasma samples was overlayed with 26 ml of $d = 1.006$ g/ml solution and were centrifuged at $25{,}000 \times g$ for 30 min at 25°C. The chylomicron layer (top 1/3) was removed and concentrated in Amicon Centriprep-30 concentrators until the volume of the retentate was 1 ml. Triglyceride was determined directly on these fractions (Sigma GPO-Trinder-50). However, due to the turbidity caused by the high concentration of triglycerides, free cholesterol and cholesteryl ester were isolated by thin layer chromatography, were saponified and cholesterol was determined by the method of Rudell and Morris (1973).

In a separate study in eight rhesus monkeys ($n = 4$/group), the concentrations of apo B_{48} and apo B_{100} were determined in chylomicrons and LDL 4 h into the postprandial period after 19 days of feeding the diet described above with or without SCH48461 (10 mg/kg/day). Lipoproteins (chylomicrons, VLDL + IDL, and LDL) were isolated from 2 ml of plasma by sequential density ultracentrifugation as described above. Apolipoproteins of the

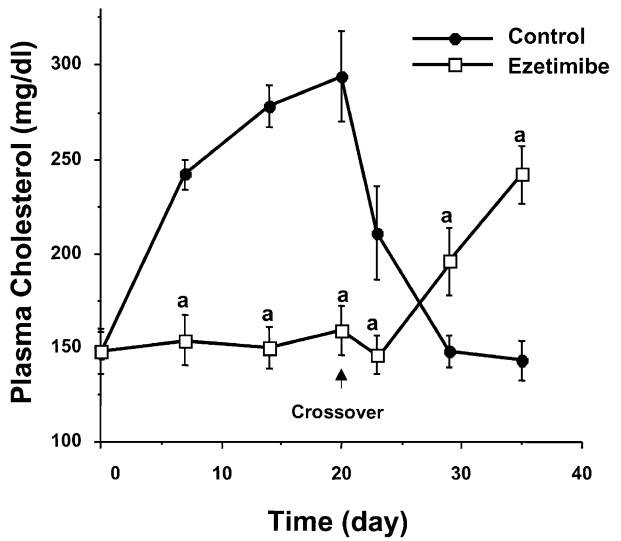


Fig. 2. Effect of ezetimibe (0.1 mg/kg) on plasma cholesterol in high fat, cholesterol-fed rhesus monkeys followed by a crossover. Rhesus monkeys were fed a diet containing 375-mg cholesterol/day and 22.5% fat (w/w) with or without ezetimibe (0.1 mg/kg/day). Plasma cholesterol concentrations were determined every 6–7 days. At 20 days, the diets of the two groups were switched (indicated by the arrow and "crossover"). Values are means ± S.E.M. ($n = 5$/group). [a]$P <$ at least 0.05 compared to control group (or treated group after crossover).

fractions were analyzed by 3–27% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Purified human LDL purchased from AKZO (Rockville, MD) was used as a standard and was treated identically to samples before loading on the gels. Gels were Coomassie stained and quantified by scanning densitometry. Data are expressed as micrograms of apo B_{48} or apo B_{100} per fraction.

Statistical comparisons were made using one way analysis of variance and unpaired Student's *t*-tests.

All studies were conducted in an Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited facility following protocols approved by the Schering-Plough Research Institute's Animal Care and Use Committee. The procedures were performed in accordance with the principles and guidelines established by the National Institutes of Health (NIH) for the care and use of laboratory animals.

3. Results

The chemical structures of ezetimibe and its analog, SCH48461, are shown in Fig. 1. SCH48461 was the first of this class of cholesterol absorption inhibitors to enter human clinical trials (Bergman et al., 1995). The ED_{50}, the dose at which the rise in plasma cholesterol is inhibited by 50% in cholesterol-fed rhesus monkeys, was 0.2 mg/kg. Ezetimibe was subsequently discovered and found to be

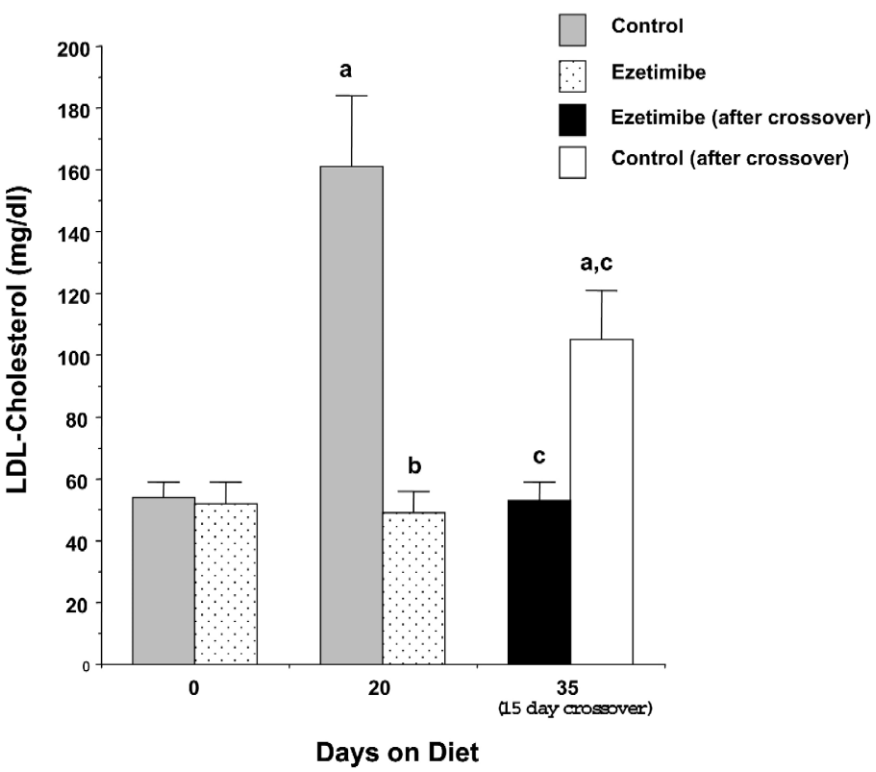


Fig. 3. Effect of ezetimibe (0.1 mg/kg) on LDL cholesterol in high fat, cholesterol-fed rhesus monkeys followed by a crossover. Rhesus monkeys were fed a diet containing 375-mg cholesterol/day and 22.5% fat (w/w) with or without ezetimibe (0.1 mg/kg/d). At 20 days, the diets of the two groups were switched. LDL cholesterol concentrations were determined at day 0, 20 and 35 (15 days after the crossover). Values are means ± S.E.M. ($n = 5$/group). [a]$P < 0.05$ compared to baseline value. [b]$P < 0.05$ compared to cholesterol-fed control group. [c]$P < 0.05$ compared to 20-day pre-crossover values.

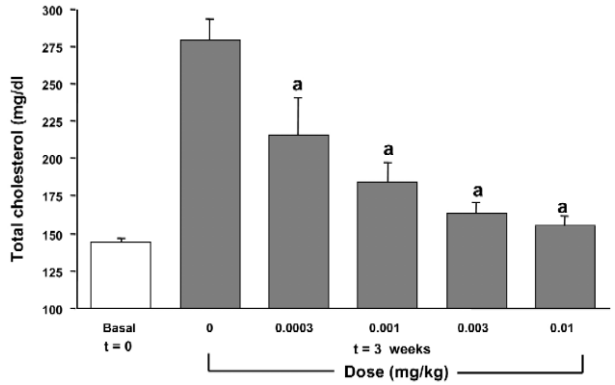


Fig. 4. Effect of a dose–response of ezetimibe on plasma cholesterol in high fat, cholesterol-fed rhesus monkeys. Rhesus monkeys were fed a diet containing 375 mg cholesterol/day and 22.5% fat (w/w) with or without ezetimibe (0.0003–0.01 mg/kg/d). Data shown are the plasma cholesterol values for all rhesus monkeys before the onset of the experiment ($n = 28$; white bar) and the plasma cholesterol concentrations 3 weeks after the treatments indicated (gray bars). Values are means ± S.E.M. ($n = 5$–10/group). [a]$P <$ at least 0.05. Statistical analysis was done by comparing monkeys on high fat, cholesterol diet treated with ezetimibe to monkeys on the same diet without ezetimibe.

400 times more potent in cholesterol-fed rhesus monkeys (van Heek et al., 1997), and thus became the clinical candidate that is presently in clinical trials. Since much of the early detailed preclinical work was conducted with SCH48461, experiments using both ezetimibe and SCH48461 are presented here.

Total plasma cholesterol in rhesus monkeys fed a high-fat diet containing 375 mg cholesterol per day for 20 days rose from 148 mg/dl to 294 mg/dl (Fig. 2). Ezetimibe (0.1 mg/kg/day) completely prevented this rise in plasma cholesterol. At 20 days, the treatments of the two groups were switched (indicated by "crossover" in Fig. 2). The pre-established hypercholesterolemia induced by dietary cholesterol was reduced to baseline levels by 9 days of treatment with 0.1 mg/kg/day ezetimibe. In the monkeys that had been receiving ezetimibe, plasma cholesterol re-

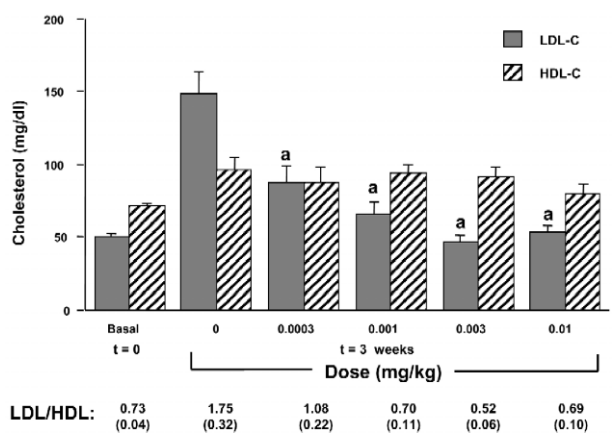


Fig. 5. Effect of a dose–response of ezetimibe on LDL and HDL cholesterol in high fat, cholesterol-fed rhesus monkeys. Rhesus monkeys were fed a diet containing 375 mg cholesterol/d and 22.5% fat (w/w) with or without ezetimibe (0.0003–0.01 mg/kg/day). Data shown are the LDL and HDL cholesterol values for all rhesus monkeys before the onset of the experiment ($n = 28$; indicated by "basal" on the x-axis) and the LDL and HDL cholesterol concentrations 3 weeks after the treatments indicated. Values are means ± S.E.M. ($n = 5$–10/group). [a]$P <$ at least 0.05. Statistical analysis was done by comparing monkeys on high fat, cholesterol diet treated with ezetimibe to monkeys on the same diet without ezetimibe.

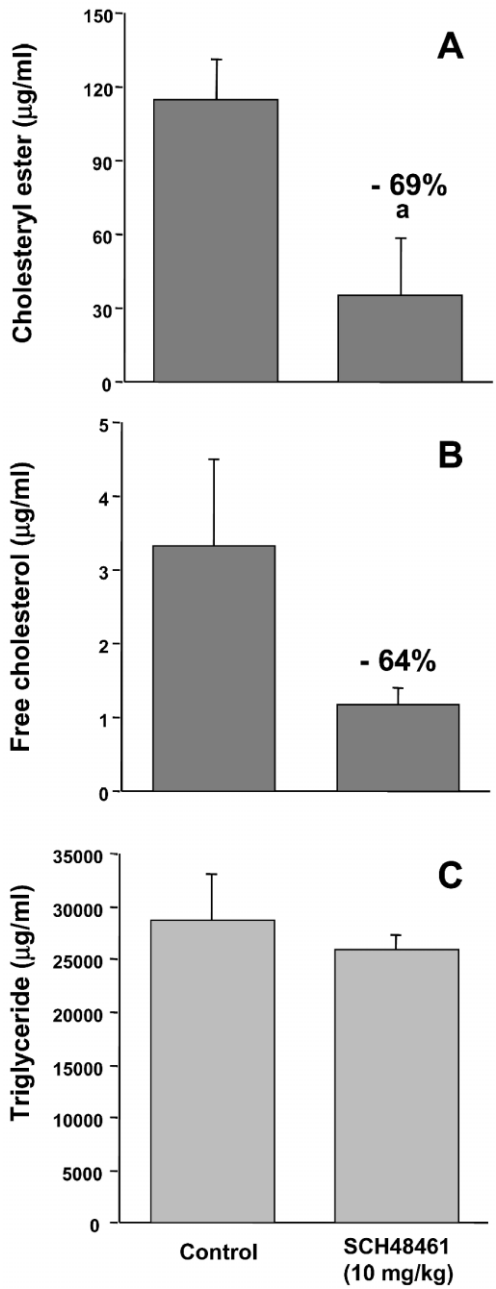


Fig. 6. Effect of a single dose of the ezetimibe analog, SCH48461, on cholesteryl-ester (A), free cholesterol (B) and triglyceride (C) content of postprandial chylomicrons in cynomolgus monkeys. Cynomolgus monkeys were fed a single meal containing 375 mg cholesterol/d and 22.5% fat (w/w) with or without SCH48461 (10 mg/kg). Chylomicrons were isolated from plasma 5 h into the postprandial period. Values are means ± S.E.M. ($n = 3$/group). [a]$P < 0.05$ comparing control to SCH48461-treated groups.

mained at baseline for 3 days after ezetimibe had been withdrawn, despite the high fat, cholesterol diet, but then rose rapidly for the duration of the study. These changes were reflected in LDL cholesterol only (Fig. 3); HDL cholesterol did not differ between groups at any of the timepoints (data not shown).

A full dose–response (0.0003–0.01 mg/kg) of ezetimibe in cholesterol-fed rhesus monkeys was conducted to determine an ED_{50} (Fig. 4). At all the doses tested, plasma cholesterol was significantly lower compared to untreated, cholesterol-fed monkeys. Complete inhibition of the rise in cholesterol was achieved at 0.003 mg/kg. The ED_{50} for ezetimibe was calculated to be 0.0005 mg/kg. Ezetimibe dose-dependently decreased LDL cholesterol (Fig. 5) without affecting HDL cholesterol. Under cholesterol-fed conditions without ezetimibe, LDL/HDL rose from 0.75 ± 0.04 to 1.75 ± 0.32, which was dose-dependently reversed with increasing doses of ezetimibe. Cholesterol content in the VLDL + IDL lipoproteins was very low in all groups and did not differ between groups (data not shown).

An acute study in cynomolgus monkeys was conducted to determine the effect of a single dose of the ezetimibe analog SCH48461 (10 mg/kg) on the cholesterol and triglyceride content of chylomicrons during the postprandial period. Cholesteryl ester and free cholesterol were reduced 69% ($P < 0.05$) and 64% (not significant), respectively, while triglyceride remained unchanged between groups 5 h after the meal (Fig. 6). In a separate study, rhesus monkeys were treated with SCH48461 (10 mg/kg) for 19 days. Chylomicrons, VLDL + IDL, and LDL were isolated from a plasma sample taken 4 h into the postprandial period. Apo B_{48} in the chylomicron fraction did not differ between groups, but the mass of apo B_{100} was significantly decreased in the rhesus monkeys treated with SCH48461 (-41%, $P < 0.05$; Table 1). VLDL + IDL had an insufficient protein content for quantification. These data indicate that this class of cholesterol absorption inhibitors reduces LDL cholesterol and LDL particle number, and reduces the cholesterol content of apo B_{48} containing particles without affecting particle number.

Table 1
Mass of apo B_{48} and apo B_{100} in chylomicrons and LDL from rhesus monkeys fed a high fat, cholesterol containing diet with or without SCH48461 (10 mg/kg/day) for 19 days

Lipoprotein	Apo B_{48} (μg/fraction)	Apo B_{100} (μg/fraction)
Chylomicron		
Control	144 ± 84	0
SCH48461	144 ± 76	0
LDL		
Control	0	449 ± 51
SCH48461	0	265 ± 53[a]

Values = means ± S.E.M. ($n = 4$/group).
[a] Significantly different from control, $P < 0.05$.

4. Discussion

The present studies demonstrate that ezetimibe can dose-dependently prevent the rise in plasma cholesterol induced by a high fat, cholesterol diet, as well as rapidly reverse pre-established hypercholesterolemia in rhesus monkeys. This decrease in plasma cholesterol was observed in the LDL fraction, but not in HDL. Further studies were conducted to specifically determine the cholesterol, triglyceride and apo B content of lipoproteins during the postprandial period. It is important to note here that the samples in these experiments were obtained 4–5 h into the postprandial period; thus, the chylomicron fraction isolated would be a mixture of chylomicons and their remnants. For simplicity, this mixed fraction is referred to for the present experiments as "postprandial chylomicrons". In cynomolgus monkeys, the ezetimibe analog, SCH48461, significantly reduced the cholesterol content of postprandial chylomicrons without affecting the triglyceride content. Another study in rhesus monkeys indicated that chronic treatment with SCH48461 led to a decrease in apo B_{100} in LDL, but apo B_{48} content in postprandial chylomicrons did not change with SCH48461 treatment. These data indicate that ezetimibe and its analog may reduce chylomicron cholesterol content without affecting chylomicron particle number. The data further suggest that the reduction of chylomicron cholesterol would decrease the amount of exogenous cholesterol delivered to the periphery and the liver, which could ultimately reduce LDL cholesterol, LDL particle number and atherosclerosis.

These data in monkeys may have important implications for the human population, particularly in humans that consume a diet high in fat and cholesterol, which is a growing population of people throughout the world. Although the number of research articles discussing the potential atherogenicity of postprandial lipoproteins in humans is too numerous to detail in this discussion, several recent papers are highlighted. McNamara et al. (1998) has found that remnant-like lipoprotein particle cholesterol (RLP-C), defined as the sum of chylomicron remnants and VLDL remnants, is higher in men than women, higher in postmenopausal women than premenopausal women, and higher in older compared to younger individuals. This is of interest because risk factors for atherosclerosis include male gender, postmenopausal status, and age. Another study in humans, found that, compared to normal controls, serum RLP-C in Type 2 diabetic patients and patients with impaired glucose tolerance was increased fourfold (Watanabe et al., 1999). These populations are at significantly higher risk for combined hyperlipidemia and death from complications of cardiovascular disease. A comparison of data from individuals who died of sudden cardiac death vs. non-cardiac death, indicated that RLP-C levels were more strongly correlated with severity of coronary atherosclerosis than LDL-C levels (Takeichi et al., 1999). Finally, it is well known that a significant number of

patients with atherosclerosis are normolipidemic. It is of importance, therefore, to note that Masuoka et al. (2000) have demonstrated that RPL-C levels are strongly associated with coronary artery disease in patients with normal total cholesterol levels.

In conclusion, this novel class of potent and selective cholesterol absorption inhibitors may provide a new approach for the treatment of hypercholesterolemia by inhibiting the absorption of both dietary and biliary cholesterol in the intestine. The present data in nonhuman primates indicate that these cholesterol absorption inhibitors may have an impact on both LDL cholesterol, an established risk factor for atherosclerosis, and chylomicron cholesterol. In addition, ezetimibe may provide a direct pharmacological tool to specifically address questions about the potential atherogenecity of cholesterol in postprandial lipoproteins.

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