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Fatty Acid Bile Acid Conjugate Inhibits Hepatic Stearoyl Coenzyme A Desaturase and Is Non-atherogenic

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Background and Aims. Suppression of stearoyl-coenzyme A desaturase (SCD) activity leads to reduction of obesity, fatty liver as well as of insulin resistance. It was, however, recently reported to enhance atherogenesis. The aim of the present study was to investigate whether inhibition of SCD by Aramchol, a fatty acid bile conjugate with known hypocholesterolemic effects, will affect atherogenesis and how.

Methods. Aramchol was tested *in vitro* in cultured cells and *in vivo* in rodents.

Results. Aramchol, at very low concentrations, reduced SCD activity in liver microsomes of mice. Aramchol enhanced cholesterol efflux from macrophages more than twofold. *In vivo* it increased fecal sterol output and decreased markedly plasma cholesterol levels in mice. In ApoE$^{-/-}$, LDRL$^{-/-}$ and C57Bl6 mice, the effects of Aramchol on atherogenesis were non-atherogenic.

Conclusions. Aramchol reduces SCD activity and is non-atherogenic. It may offer a means to obtain the desirable hepatic metabolic effects of SCD inhibition without the deleterious atherogenic effect.

Key Words: Fatty acid bile acid conjugate, SCD, Atherosclerosis, Plasma lipids, Metabolic syndrome.

Introduction

Stearoyl-coenzyme A desaturase (SCD) catalyzes the delta 9 monounsaturation of palmitic and stearic acids and plays an important role in lipid metabolism (1). It functions as a key lipogenic gene. Thus, its suppression/inhibition was found to have beneficial effects, including reduction of liver fat in nonalcoholic fatty liver disease (NAFLD) and protection against insulin resistance, as well as protection against obesity (1–3). In addition to these well-known, mostly hepatic effects, SCD was reported to have additional influences: protection against lipotoxicity (4), microinflammation and endoplasmic reticulum (ER) stress (5). Recent studies that examined its effect on atherogenesis conflictingly found an enhancement (6,7) or reduction (8) of atherogenesis in mice. This potential pro-atherogenic effect was highly disturbing. Savransky et al. (8) presented human data showing that nocturnal hypoxia a, known proatherogenic factor, was associated with increased hepatic SCD mRNA levels and an atherogenic lipid profile. Aramchol (a conjugate of arachidic and cholic acids) is a member of the fatty acid bile acid conjugate (FABAC) family of molecules. It was found to prevent and reduce NAFLD (2,9) by inhibiting SCD activity (2,10,11). It also prevents and dissolves cholesterol gallstones (12,13) and has beneficial effects on cholesterol metabolism (14–16). It is considered a potential therapeutic agent for NAFLD (2).

If the whole class of SCD inhibitors is found to be pro-atherogenic, this would pose a question in relation to their use in medicine in general and as a treatment for NAFLD in

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particular. Two types of recent results make this problem particularly relevant. A series of recent publications provided strong and concordant evidence that NAFLD is an important risk factor for subsequent cardiovascular disease and its complications (17–19). A recent study (20) has presented data that it is not visceral fat but rather liver fat that is the culprit in the metabolic alterations leading to CVD. Furthermore, the thiaglitazones, the only compounds convincingly shown to reduce liver fat in NAFLD, are known to increase obesity (21,22) and were recently implicated in increasing CVD complications (23). All the above clearly point to the need to determine whether inhibitors of SCD activity in general and Aramchol in particular contribute to the pro-atherogenic effects of NAFLD or reduce them by decreasing the primary cause, namely, increased liver fat. The present article examines the known and new effects of Aramchol in an effort to answer this question.

Materials and Methods

Animals and Diets

Four- to 5-week-old male C57Bl6/J mice were obtained from the animal facility of Tel-Aviv University and used in most experiments. One-month-old male Golden Syrian Hamsters were obtained from a local breeder (Rehovot, Israel) and used as specified. Regular powdered rodent chow diet (4% fat, RD) was purchased from Koffolk (Petach Tikva, Israel). The high-fat diet (HFD 100%) was prepared as previously described (2) and consisted of 15% butter fat, 1% cholesterol, 0.5% cholic acid and 2% corn oil added (w/w) to 81.5 g of RD to produce the final 100% HFD. The high-fat maintenance diets (HFD 25 and 50%) were prepared by adding 25% or 50% of the ingredients added to RD for HFD 100%.

Animals were kept on a 12-h day/night cycle and had free access to water and food. The studies were approved by the institutional committee for animal experiments at Tel-Aviv University, Tel-Aviv, Israel.

Experimental Procedure

The FABAC used in the present experiments was 3β-arachidyl amido, 7α,12 α-dihydroxy, 5β-cholan, 24-oic acid, which is an amide conjugate (at position 3 of the bile acid) of arachidic and cholic acids (Aramchol; C-20 FABAC). Animals were fed RD or HFD. Aramchol was given by gavage at a dose of 25 mg/kg/day or 150 mg/kg/day (FABAC 25 or FABAC 150) suspended in saline, as specified in each experiment. Control animals received the same volume of saline by gavage. After the experimental period, all animals were given ketamine anesthesia and sacrificed. Blood was drawn from the abdominal aorta and collected in tubes containing 0.01% EDTA. Plasma was separated by centrifugation and kept frozen at −20°C for further determinations. The liver was excised and immediately frozen in liquid nitrogen for further analyses.

Chemical Analysis of Plasma

Cholesterol was measured by an enzymatic method using an Advia 1650 autoanalyzer (Bayer, Japan).

SCD Activity

Enzyme activity was measured in liver microsomes. (1-^{14}C) Palmitic acid was used as substrate, followed by conversion to (1-^{14}C)palmitoleic acid. Assays were run for 20 min in a buffered solution with ATP, CoA and NADH as cofactors. The reaction was stopped with 0.5 mL KOH 10% in ethanol. Fatty acids were transformed into methyl esters and separated in 10% $AgNO_3$-impregnated TLC plates. After identification under iodine, fatty acid methyl esters were scraped and the radioactivity counted using a scintillation cocktail (Opti-Fluor, Perkin Elmer, Waltham, MA). The activity of the enzyme was expressed as pmol palmitoleic acid/min/mg microsomal protein (24).

Cholesterol Efflux Experiments

Cholesterol efflux from cells (ABCA1 mediated) was measured as previously described (14). In brief, cells were preincubated with ^{3}H cholesterol, washed four times and then placed in the incubation medium for 20 h. The incubation medium always contained bovine serum albumin (BSA), with or without the addition of Aramchol 30 μg/mL or ApoA1 5 μg/mL (optimal concentrations). After 20 h of incubation the radioactivity was counted separately in the medium and the cells. Cholesterol efflux % was calculated as radioactivity in medium/total radioactivity (medium + cells). Peritoneal macrophages were isolated from Balb/c mice 3 days following the IP injection of 2 mL of 3% thioglycolate.

Fecal Sterol Analyses

Feces were collected during the last 96 h of the experimental period. Fecal lipids were analyzed by GLC according to Batta et al. (25). In brief, 75 mg of lyophylized feces were butylated by addition of 1 mL n-butyl alcohol and 0.1 mL 6 N HCL. Nor-cholic acid and 5-α-cholestane (0.1 mg of each) were added as internal standards. The mixture was heated at 60°C for 4 h. The solvents were evaporated at 60°C under air and the sample was silylated with 1 mL Sil-Prep kit (Alltech, Deerfield, IL) at 55°C for 0.5 h. After solvent evaporation, the sample was resuspended in 1 mL hexane, centrifuged to eliminate particles, transferred to a new vial and brought to a final volume of 50 μl. One μl of sample was applied to an SGE 30 m × 0.25 mm 0.25 μm capillary GLC column. The lipid components were separated under a temperature cycle of 150°C for 1 min, increasing from 150–272°C for 7 min and

keeping this final temperature until the end of the run. The results were analyzed with a Clarity program (Data Apex Ltd., Czech Republic).

Atherosclerosis Studies

Animals. Female, 12-week-old, LDLR$^{-/-}$ mice (C57BL6 background, Jackson Laboratories, Bar Harbor, ME) and female apoE$^{-/-}$ mice (C57BL6 background, Jackson Laboratories) as well as wild-type female C57Bl6 mice were used. The mice were housed in plastic cages on a 12:12 light/dark cycle with free access to water and diet. The mice were distributed evenly into the treatment groups according to their plasma cholesterol and TG levels. All atherosclerosis studies were performed at The Bert W. Strassburger Lipid Center, Sheba Medical Center. The Animal Care and Use Committee of Sheba Medical Center, Tel-Hashomer, approved all animal protocols.

Study Design

ApoE$^{-/-}$ mice. The effects of FABACs on atherogenesis in the aortic sinus area were studied in young (12 week) female (study #3) apoE$^{-/-}$ mice (n = 30) and the effect on prevention of atherosclerosis progression was studied in old (5 month), female apoE$^{-/-}$ mice (study #2). The mice were randomly divided into four groups: 1) control group young (n = 15); 2) FABAC group young (n = 15); 3) control group old (n = 15); and 4) FABAC group old (n = 15). The experiments lasted 12 weeks. In a preliminary (study #1), 14 mice (seven control, seven Aramchol) were studied for 1 month. All control groups were treated with DDH_2O and all FABAC groups were daily treated with Aramchol (150 mg/kg body weight) throughout the experiment. Atherosclerosis was measured as described (26).

LDLR$^{-/-}$ mice. To study the effect of FABAC on the aortic sinus lesion area in LDLR$^{-/-}$ mice, 60 female, 8-week-old mice were randomly divided into two groups (study #5). Control group (n = 30) was treated with DDH_2O, and FABAC (Aramchol) group (n = 30) was daily treated with FABAC (150 mg/kg body weight). During the first 3 weeks of the experiment the animals were fed chow diet (Harlan, Teklad certified global 18% protein rodent diet, Israel) (RD) followed by 6 weeks of a Western-type (HFD) diet (Harlan, 42% fat. TD88137). At the final stage the animals were fed chow diet for an additional 4 weeks.

To study the effect of FABAC on prevention of atherosclerosis progression in LDLR$^{-/-}$ mice, 45 female, 12-week-old mice were fed a Western diet for 6 weeks to induce atherogenesis followed by a 4-week chow diet to reduce plasma cholesterol levels (study #6). At this time point, 15 mice were killed and aortic sinus lesion area was determined. The mice (n = 30) were randomly divided into two groups. Control group (n = 15) was treated with DDH_2O and FABAC group (n = 15) was treated daily with FABAC for an additional 6 weeks. Only in this study was Aramchol given during part of the time (last 6 weeks). Body weights were measured weekly. At the end of the experiments the lesion area in the aortic sinus was measured in all studies as reported (26). Study #4 in wild-type C57Bl6 mice was previously reported (27).

Data are represented as mean ± standard deviation. Statistical differences were analyzed by paired Student's t-test; $p < 0.05$ was regarded as statistically significant.

Results

SCD Activity

The effects of Aramchol on SCD activity *in vitro* are exemplified in Figure 1. The effect on SCD1 activity was compared to that of the established (28) SCD inhibitor, *trans*-10, *cis*-12-conjugated linoleic acid (CLA) and the non- inhibitory isomer *cis*-9, *trans*-11 CL, at similar effector/substrate proportions. Aramchol reduced SCD activity. The fact that it acted on SCD activity in hepatic microsomes within the very short time span of 20 min suggests that it is a direct effect. Results in Figure 1 indicate that Aramchol is a more potent SCD inhibitor in comparison to CLA t 10c12. The mechanism of action of Aramchol on SCD1 was previously reported (10,11) and the detailed description including its kinetic parameters forms part of a forthcoming separate communication.

Fecal Sterol Output

Figure 2 shows the effects of Aramchol feeding for 12 days in mice and in hamsters eating a regular chow diet. In both

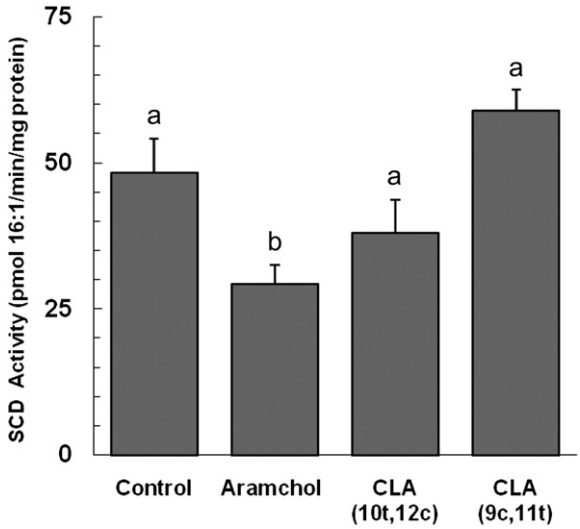

Figure 1. SCD activity in C57Bl6/J mouse liver microsomes. The assay was performed in the absence or presence of Aramchol (7 μM), *trans*-10, *cis*-12 CLA or *cis*-9,*trans*-11 CLA. Values are expressed as mean ± SD. Columns not sharing a common superscript are significantly different ($p < 0.05$) from one another (Student t-test).

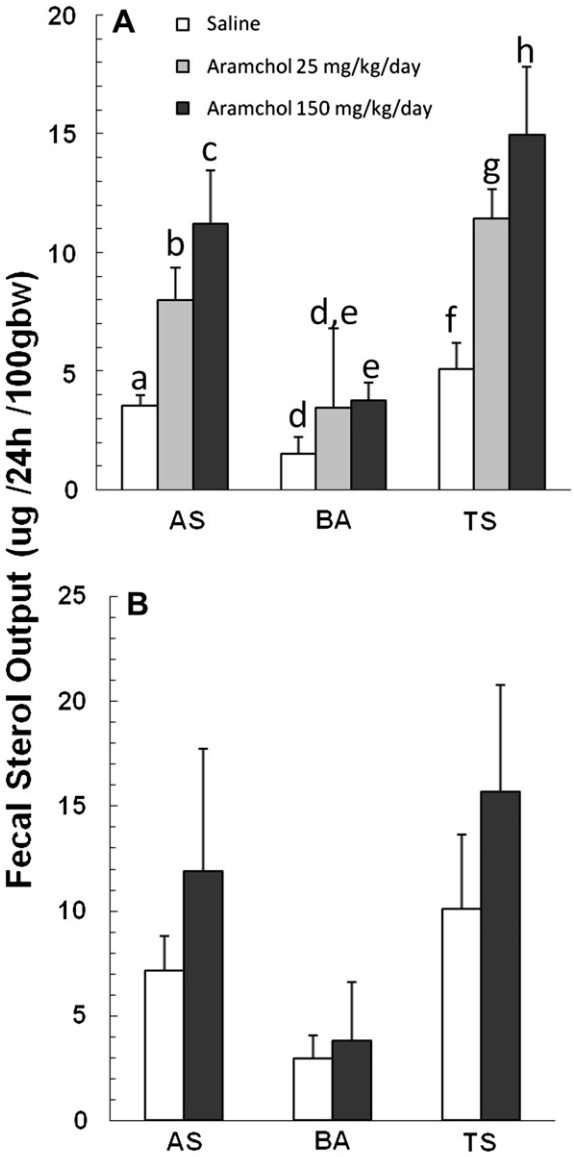

Figure 2. Fecal sterol output. In C57Bl6/J mice (A) and Golden Syrian Hamsters (B). Animal sterols (AS), bile acids (BA) and total sterols (TS) (excluding phytosterols) were measured in stools and collected during the last 4 days of a 12-day period during which they were fed regular chow diet and given either saline or Aramchol at a dose of 25 or 150 mg/kg/day. In (B) differences between Aramchol (150 mg/kg/day) and saline were not significant.

species there was an increase in fecal sterol output (measured during the last 4 days of each study), particularly in the output of neutral sterols (excluding dietary plant sterols). In mice, two dose levels were tested with a suggestion of a dose-response effect (NS). Statistical significance was reached in most mice studies (Figure 2). In hamsters the trend was similar but did not reach statistical significance. This chronic sterol loss contributes to the overall hypocholesterolemic effects of Aramchol.

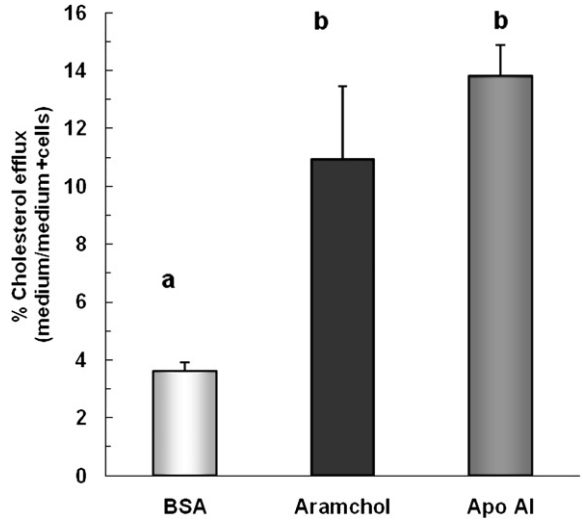

Figure 3. Cholesterol efflux from Balb C mice peritoneal macrophages. Data are from a representative ($n = 4$) experiment. Cells were incubated for 20 h in a medium containing either BSA alone or with the addition of Aramchol (30 μg/mL) or ApoA1 (5 μg/mL), optimal concentrations for these cells.

Cholesterol Efflux

Figure 3 shows the effect of Aramchol on cholesterol efflux from macrophages (mouse peritoneal macrophages) in comparison to efflux to ApoA1. In comparison to the basal efflux to bovine serum albumin (BSA), the Aramchol-induced efflux was several-fold higher although lower than the physiological efflux induced by ApoA1. This effect was previously shown to be ABCA1 mediated (14).

Plasma Cholesterol Levels

Figure 4A shows plasma cholesterol levels in C57Bl6/J mice. High cholesterol levels were induced by feeding the HFD for 55 days and maintained for another 56 days by feeding a maintenance diet containing only 50% of the fat added to the chow in the original induction diet. Cholesterol levels remained relatively stable in the HFD controls on this diet. Aramchol at 150 mg/kg/day reduced plasma cholesterol significantly (but moderately) already after day 28. After 56 days the reduction was very marked to levels lower than even in the RD controls.

A similar experiment is shown in Figure 4B. Induction of hypercholesterolemia was achieved within 28 days. The maintenance diet contained much less fat—only 25% of the amount added during induction. The high cholesterol levels on this 25% diet in the controls were maintained for 28 days and decreased subsequently. On this diet, however, the effect of Aramchol therapy was more marked already after 28 days and was similar for the 25 mg/kg/day and the 150 mg/kg/day doses. At 38 days, despite the decrease of plasma cholesterol in the 25% HFD controls, the decrease in the treated mice was more marked and

statistically significant for both doses. Again, cholesterol levels despite the 25% HFD were lower in the Aramchol-treated mice than even in the RD controls.

Atherosclerosis Studies

Table 1 shows the results of all our atherosclerosis studies performed with Aramchol in various animal models. The basic parameter is the total area (μm^2) of the atherosclerotic lesions in the aortic sinus as measured by computerized morphometry. It is expressed as the % difference in total lesion area between the Aramchol-treated mice and controls receiving the diet only. Minus denotes a decrease in the atherosclerotic area in the treated animals whereas plus denotes an increase in that area. The results in the various models and various treatment schedules are not uniform. The first two studies were performed on old (5 months at start) Apo $E^{-/-}$ mice on a HFD. These animals have well-established, advanced and severe atherosclerotic lesions at the start of study, which are very resistant to therapy. The changes that we found were indeed minor, insignificant and in opposite directions. In the other four studies, three showed a decrease and one an increase in the lesion area. In three studies the differences were statistically significant—two of them showing a decrease and one showing an increase in lesion area. It is noteworthy that the only study showing a major (60%) difference ($p < 0.019$) in favor of the treated group was in wild-type animals (27). All others were genetically modified mice. In study #6, the only one showing a significant increase in lesion area, a paradoxical, unexplained increase in plasma triglycerides was observed. These six studies in >200 animals also show the potential problems involved when relying on single or small studies.

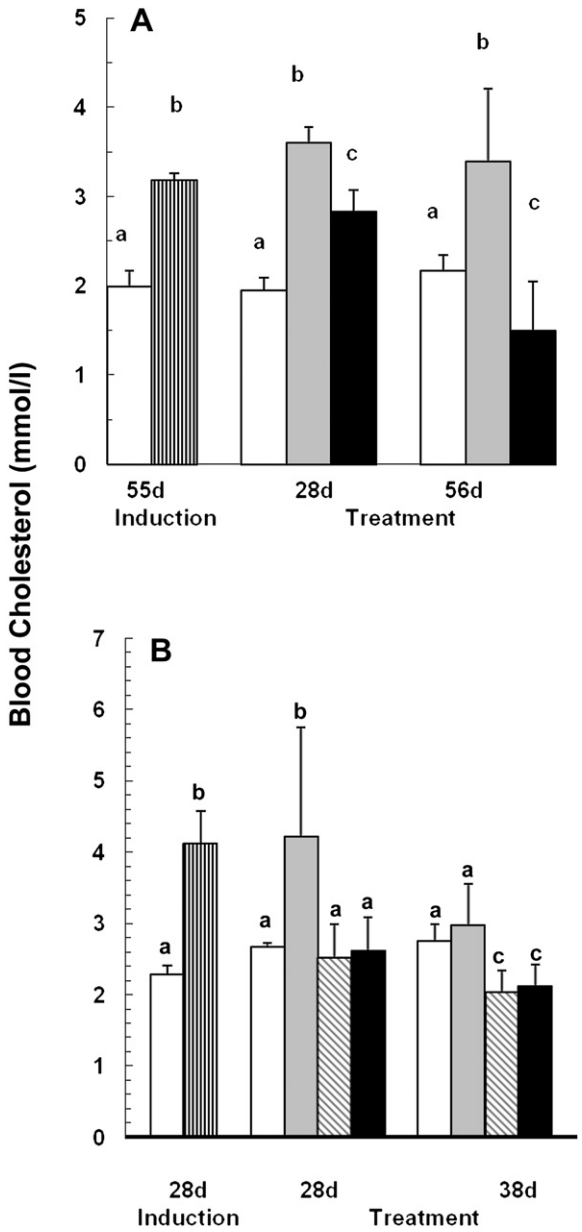

Figure 4. Blood cholesterol levels. (A) C57Bl6/J mice fed a regular chow diet (white bars) or a HFD 100% (vertical stripes) for 55 days. Animals were then fed a 50% HFD (gray bars) without or with Aramchol (150 mg/kg/day) (black bars) supplementation. (B) C57Bl6/J mice fed a regular chow diet (white) or HFD 100% (vertical stripes) for 28 days. Following induction, animals were fed a 25% HFD (gray bars) without or with Aramchol at 25 (diagonal stripes) or 150 mg/kg/day (black bars) supplementation.

Discussion

Our data reconfirm that Aramchol inhibits, probably directly, SCD activity. It was previously shown to reduce *in vivo* a reliable plasma marker of SCD activity, namely, the 16:1/16:0 fatty acid ratio (2). This is supported by the reduction in liver fat in NAFLD (2) as well as by the improvement in carbohydrate tolerance and decrease in insulin resistance found in treated animals (2). Finally, in a direct study of liver SCD activity in mice treated with Aramchol for weeks and months, SCD activity was decreased by 60−84% (10,11).

In relation to atherosclerosis, the effects of Aramchol in our six different studies (Table 1) were on the whole not pro-atherogenic. The results in the literature are variable (6−8) using gene-deleted or -suppressed animals. It should, however, be pointed out that there are major differences between SCD gene KO or gene-suppressed mice and the administration of an exogenous compound that has SCD inhibitory activity. The most basic difference is that the exogenous compound may have, and in our case does have, additional effects on other genes or metabolic processes. These additional effects may outweigh the suggested pro-atherogenic effects reported and the result may well be anti-atherogenic. In view of the major importance of the SCD gene, we shall analyze the various components of the evidence.

Table 1. Atherosclerosis trials

Remarks	*p*	Lesion area, m^2	Experiment	Mice
Old ApoE$^{-/-}$, short trial prevention	0.64	Control 171,250 ± 52,051 Aramchol 157,500 ± 54,461 −8%	#1, HFD 4 weeks	ApoE$^{-/-}$ Age 5 months *n* = 14 F
Old ApoE$^{-/-}$	0.245	Control 724,281 ± 124,281 Aramchol 779,281 ± 121,989 + 8.6	#2, HFD 12 weeks	ApoE$^{-/-}$ Age 5 months *n* = 30 F
Young ApoE$^{-/-}$	0.049	Control 371,279 ± 22,833 Aramchol 314,691+15,945 −15%	#3, HFD 12 weeks	ApoE$^{-/-}$ Age 12 weeks *n* = 30 F
Prevention, published (27)	0.019	Control 11,692 ± 2,765 Aramchol 5,072 ± 956 −60%	#4, HFD 15 weeks	C57Bl6 *n* = 40 Age 3 months F
Treatment of established lesions	0.10	Control 128,439 ± 37,231 Aramchol 108,564 ± 21,300 −15%	#5, RD 3 weeks, HFD 6 weeks, RD 6 weeks	LDLR$^{-/-}$ *n* = 60 Age 8 weeks F
Treatment, worsening paradoxical increase in triglycerides	0.02	Control 121,623 ± 54,445 Aramchol 170,590 ± 54,246 +29%	#6 HFD 6 weeks[a] RD 4 weeks, RD 6 weeks	LDLR$^{-/-}$ *n* = 45 Age 12 weeks F

RD, regular diet, HFD, high-fat diet.
[a]No Aramchol treatment during induction stage (first 6 weeks).

The differences between gene KO, gene-suppressed and enzyme-inhibited animals are, in the case of SCD, apparent even to the naked eye. SCD1$^{-/-}$animals have alopecia and are cachectic and underweight as compared to their wild-type littermates (29). The gene-suppressed animals have no alopecia but do lose weight (30). Aramchol-treated animals had no alopecia and gained weight to the same degree as their controls (2,9,31). This is based on >2000 animals treated over 8 years in several laboratories. The skin lesion and weight loss may be linked. In a recent study by Binczek et al. (32) from Stoffel's laboratory it was demonstrated that the SCD1$^{-/-}$ animals have not only alopecia but also severe skin damage. Water and body heat are lost through the damaged skin causing the animals to increase their metabolic rate, which results in weight loss. Simply covering the skin with an oily ointment reduced the heat loss and hypermetabolism by about 80%. MacDonald et al. (7) also found severe skin disease in their gene-deleted mice. Sampath et al. (36) caused skin-specific deletion of the SCD gene. They also found skin damage, hypermetabolism and weight loss, although their explanation is different.

These findings raise the question whether the much desired weight loss associated with SCD suppression or KO is achieved at the unacceptable price of a severe skin lesion. It is not excluded that some of the hypermetabolism is due to increased beta oxidation of fatty acids. Further studies are needed.

Aramchol Reduces Blood and Body Cholesterol

Aramchol has several effects that are all additive, working in the same direction. All of these effects could, according to current knowledge, contribute to an anti-atherogenic outcome. Aramchol was proven to stimulate (2- to 4-fold) the ABCA1 transporter (14), which is the universal cholesterol export pump present in all cells. This has been proven mainly in human fibroblasts (14) but also tested in murine hepatocytes, liver cell lines of human origin (unpublished) and now in macrophages. The latter are particularly relevant to atherosclerosis. Aramchol enhances bile acid synthesis from cholesterol (15), which is the main cholesterol catabolic pathway in the body. Preliminary evidence indicates that this also occurs in human hepatocytes (Dr. Paolo Parini, personal communication).

Figure 2 shows that Aramchol markedly increases fecal sterol output, mostly neutral sterols. This has also been previously demonstrated in rats (16). The mechanism of this effect is still uncertain. The biliary concentrations of cholesterol and bile acid were not increased (16).

Figures 4A and 4B show that the net effect is indeed a marked lowering of blood cholesterol to levels below those of animals on a regular diet. The results of the six atherosclerosis studies shown in Table 1 can be classified as inconclusive or as showing a slight anti-atherogenic trend. They definitely do not support any pro-atherogenic

effect of Aramchol. Brown et al. recently reported (33) that fish oil counteracted the pro-atherogenic effects of SCD suppression. This is an important study demonstrating that the atherogenicity of SCD suppression can be avoided. On a practical level it is, however, much easier to give one small molecule than to cause gene suppression and simultaneously treat its undesirable effects. Duewell et al. (34) recently reported on the major potential role of cholesterol microcrystals in atherosclerosis. The microcrystals, unlike the the large crystals, may be a cause rather than a result in the early atherogenic process. Aramchol has been shown to prevent and dissolve cholesterol crystals both *in vitro* and *in vivo* (12,13). The concentrations used *in vitro* were too high and thus nonphysiologic; however, the *in vivo* doses used were similar to those used in the present atherosclerosis experiments. Thus, there are several possible mechanisms to explain the overall anti-atherogenic effect of Aramchol.

There is a wealth of SCD-related information from various animal studies. There are scant and fragmented data related to humans (35). It is not even certain that the effects in humans are identical or quite similar. More human data are needed.

Other Effects

Aramchol has additional effects. It was previously proven to prevent (12) and dissolve (13) cholesterol gallstones in animals. It prevents and reduces liver fat in diet-induced NAFLD (2,9), as would be expected from an SCD inhibitor. A Phase IIA multicenter study is imminent to test whether Aramchol reduces liver fat in patients with established NAFLD and NASH (NIH study no. NCT 01094158). In addition, other pathogenetic factors in NAFLD will be investigated. In particular, endothelial dysfunction will be investigated to check whether reduction of liver fat will decrease the atherosclerotic tendency.

For all of these reasons it was important to know whether it has the highly undesirable side effect of being pro-atherogenic. Based on all the above it would seem that the question whether SCD gene deletion or suppression has a pro-atherogenic effect is still open. Inhibition of the enzyme activity by Aramchol is, however, non-atherogenic.

The potential explanations vary. Other studies may not confirm that $SCD1^{-/-}$ or SCD-suppressed animals have an enhancement of atherogenesis or that incomplete inhibition of SCD activity does not produce this effect or that the effects of Aramchol on cholesterol metabolism outweigh its putative pro-atherogenic effect resulting in a net moderate anti-atherogenic effect. The results of the present study are compatible with any of these possibilities. A combination of incomplete inhibition of SCD with the specific hypocholesterolemic effects of Aramchol seems a likely explanation.

In conclusion, Aramchol inhibits hepatic SCD while simultaneously causing hypocholesterolemic effects. In the present work it was found to be non-atherogenic. It thus produces the desirable hepatic metabolic profile of SCD inhibition without the undesirable pro-atherogenic effect.

Acknowledgments

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Competing Interests

T.G. is associated with Galmed Medical Research Ltd. F.M.K. is a member of the scientific advisory board of that company. All other authors have nothing to disclose.

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