

Prevention of Diet-Induced Fatty Liver in Experimental Animals By the Oral Administration of a Fatty Acid Bile Acid Conjugate (FABAC)

Tuvia Gilat,^{1,2} Alicia Leikin-Frenkel,³ Ilana Goldiner,^{1,3} Christine Juhel,⁴ Huguette Lafont,⁴ Diana Gobbi,^{1,3} and Fred M. Konikoff^{1,3}

Fatty acid bile acid conjugates (FABACs) are a new family of synthetic molecules designed to solubilize biliary cholesterol. They were shown to prevent and dissolve cholesterol gallstones in inbred C57L/J mice fed a lithogenic, high-fat diet (HFD). In these mice, fatty liver was observed in the controls but not in the FABAC-treated ones. The present study was designed to study the effect of FABAC (arachidyl-amido-cholanoic acid) on diet-induced fatty liver in rats, hamsters, and mice. The fatty liver score (on a scale of 0-4 by light microscopy) was 4.0 in control hamsters and 0.3 in the FABAC-fed hamsters ($P < .001$). In mice it was 1.5 and 0.4, respectively ($P < .01$). The lipid/protein ratio in the liver was 1.3 ± 0.44 (mg lipid/mg protein) in control rats and 0.66 ± 0.04 in the FABAC group ($P = .001$) after 14 days. In hamsters it was 1.41 ± 0.27 and 1.11 ± 0.20 , respectively ($P = .03$), after 21 days. In Imperial Charles River (ICR) mice the ratio was 0.34 ± 0.10 and 0.17 ± 0.07 ($P = .03$), respectively, after 24 days. Liver fat concentration, measured as mg lipid/g liver tissue, decreased similarly by FABAC feeding. The decrease in liver fat affected mainly the triglyceride levels. FABAC-fed animals gained weight similarly to the controls. Triglyceride absorption was unaffected by FABAC supplementation. In conclusion, oral FABAC therapy prevents/reduces the development of fatty liver in animals consuming a HFD. (HEPATOLOGY 2003;38:436-442.)

Fatty acid bile acid conjugates (FABACs) are new synthetic molecules designed to solubilize biliary cholesterol.¹ When given orally they prevented the formation of cholesterol gallstones,² dissolved cholesterol crystals in bile,¹ and dissolved cholesterol gallstones in experimental animals.³

The diets used to induce the formation of cholesterol crystals and gallstones were high-fat diets (HFDs) containing between 200% to 500% of the amount of fat

present in the regular chow diet. Not unexpectedly, most of the experimental animals developed fatty livers. It was then noted that the FABAC-supplemented animals, receiving an identical HFD, did not develop a fatty liver, or had only small amounts of excess fat in the liver. When these findings were confirmed, the present studies were initiated to test prospectively whether the oral administration of FABACs prevents and/or reduces fatty liver in several animal species. Initial experiments related to the possible mechanisms of this effect also were performed. The present study is a report on the effects of arachidyl-amido-cholanoic-acid² on diet-induced fatty liver in rats, hamsters, and mice.

Material and Methods

Animals. Three animal species: rats, hamsters, and mice, were used in the studies. Rats studied were 4-week-old male Wistar rats (80-120 g), and hamsters were 4-week-old male Golden Syrian hamsters (90-100 g). Four different mouse strains were studied: male ICR mice, 4 weeks old, approximately 20 g; male C57BL/6 mice, 4 weeks old, approximately 20 g; male gallstone-

Abbreviations: FABAC, fatty acid bile acid conjugate; HFD, high-fat diet; ICR, Imperial Charles River; NAFLD, nonalcohol-induced fatty liver disease.

From the ¹Department of Gastroenterology, Tel Aviv Sourasky Medical Center; ²Galmed Medical Research, Ltd; ³Minerva Center for Cholesterol Gallstones and Lipid Metabolism in the Liver, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel; and ⁴INSERM, Unite 476, Marseille, France.

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Address reprint requests to: Fred M. Konikoff, M.D., Department of Gastroenterology, Tel Aviv Medical Center, 6 Weizman St., Tel Aviv, 64239, Israel. E-mail: konikoff@post.tau.ac.il; fax: (972) 3-697 4469.

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susceptible inbred mice C57L/J (Jackson Laboratories, Bar Harbor, ME), 4 to 5 weeks old, approximately 25 g; and female C57Bl/6 mice (Animal House, Sheba Hospital, Israel), 12 weeks old, weighing approximately 20 g. The study was approved by the animal experimentation committees of our institutions.

Diets. All control animals were fed a regular rodent chow diet containing a maximum of 4 g% fat. The test animals were given simultaneously the same chow diet supplemented with various amounts and types of fat as follows: (1) In hamsters: lithogenic diet, cholesterol 1%, palmitic acid 1.2%, and corn oil 2% (wt/wt),⁴ or a HFD of cholesterol 1%, palmitic acid 1.2%, corn oil 2%, butter 6%, and lard 10% (wt/wt). For triglyceride absorption studies hamsters were fed a commercial diet containing 10% olive oil (see later). (2) In mice, a HFD containing butter 6%, lard 10%, cholesterol 1%, palmitic acid 1.2%, and corn oil 2% (wt/wt) was given to ICR and C57BL/6 mice. The HFD given to the female C57BL/6 mice contained 17% fat, simulating a Western diet.⁵ A lithogenic diet was given to the C57L/J mice.⁶ It contained 15% butter, 1% cholesterol, 0.5% cholic acid, and 2% corn oil (wt/wt). (3) The HFD of rats consisted of 10% lard, 2.5% cholesterol, and 0.5% cholic acid (wt/wt).

Experimental Design. All animals were held in standard cages at an animal facility at room temperature (22°C), under a 12-hour light/dark cycle. Water was given *ad libitum* and animals were weighed weekly. FABAC supplementation (150 mg/kg/d) was given by gavage as a suspension in saline (0.1 mL for mice and 0.5-1 mL for hamsters and rats), whereas control animals received the same amounts of saline only. After predetermined time periods, animals were killed after ketamine anesthesia. The livers were excised, weighed, cut into small pieces of approximately 100 to 200 mg, frozen immediately in liquid nitrogen, and kept in -70°C for later analysis. Separate samples were placed in 4% formalin for histologic examination. The principal FABAC used in the present experiments (aramchol) was an amide conjugate of arachidic (C20) with cholic acid at position 3 of the bile acid. FABACs conjugated with palmitic (C16) and behenic (C22) acids were used as stated in the Results section. The FABACs were prepared as previously reported¹ and were a generous gift from Galmed Medical Research Ltd.

Analytic Methods. The liver samples were weighed and homogenized with saline at a ratio of 1:3 or 1:5 (wt/vol) in cryo vials on ice. Lipids were extracted from an aliquot of the liver homogenate according to the procedure of Folch et al.⁷ The total amount was calculated after aliquot evaporation to constant weight. Neutral lipids were separated by thin-layer chromatography on silica gel

plates (Merck, Rehovot, Israel). They were developed with a solvent system constituted by petroleum ether, ethyl ether, acetic acid, 80:20:2 (vol:vol:vol).⁸ The lipids were identified by the distance relative to the front (Rf) according to known standards. They were detected by iodine vapors or phosphomolibdate reagent (Supelco, Rehovot, Israel) and quantified by densitometry (BIS 202D; Rhenium, Jerusalem, Israel), and compared with calibration curves of the appropriate standards.

Protein determination was performed using the Bradford reagent (Sigma, Rehovot, Israel).⁹

Light Microscopic Analysis of Fat Infiltration in the Liver. Formalin-fixed liver samples were stained with hematoxylin-eosin. Coded histologic slides of animal livers were examined and scored by an experienced pathologist, blinded for the treatment. The scores were as follows: no visible fat: score 0; <5% of liver surface infiltrated by fat: score 1; 5% to 25% fat: score 2; 25% to 50% fat: score 3; and >50% fat: score 4. Fatty infiltration was classified as microvesicular, macrovesicular, or mixed. Additional findings, such as cellular infiltration and fibrosis, also were recorded.

Measurement of Triglyceride Absorption in Hamsters. Fourteen male Golden Syrian hamsters (Charles River, L'Arbresle France) weighing approximately 100 g each, were studied. They had free access to food and water and were kept at 22°C, and a 12-hour light/dark cycle. They were fed a commercial diet containing olive oil 10 g, cholesterol 0.2 g, and β sitostanol 0.1 g per 100 g (U.A.-Usine d'Alimentation Rationnelle, Villemoisin sur Orge, France).

The β sitostanol (which served as an unabsorbable marker) was labeled with 0.6 μ Ci of ³H β sitostanol and the fat was labeled with 0.6 μ Ci of ³H Triolein. The labeled and unlabeled lipids were dissolved in chloroform methanol, and the solvents were evaporated to dryness before addition to the whole diet. Seven hamsters received the (labeled) diet, the other 7 received 150 mg/kg/d of FABAC added to the diet. Hamsters were deprived of food for 24 hours before starting the test diets. They were then fed for 7 days and stool and diet collections were performed daily on days 2 through 8. The animals were kept in individual metabolic cages and were weighed daily. The stools were homogenized and lyophilized, and 1 g of homogenate was used for lipid extraction with chloroform/methanol 2/1 (vol/vol) and NaCl 0.15 mol/L, pH 3, by the method of Folch et al.⁷

Triglycerides, diglycerides, monoglycerides, and free fatty acids were separated by thin-layer chromatography on silica gel (Merck) using the Bitman and Woods method.¹⁰ The same procedure was used for β sitostanol extraction. The plates were exposed to iodine to visualize

the different spots that were scraped and placed in counting vials.

The radioactivity was measured by scintillation counting with a Packard 1600 TR instrument (Packard, Meriden, CT) with an external standard for quench correction. The percentage of triglyceride absorption was calculated as follows:

$$\% \text{ Absorption} = 100 \times$$

$$\left[1 - \frac{\text{Fecal } (^3\text{H oleic acids}/^3\text{H}\beta \text{ sitostanol})}{\text{Dietary } (^3\text{H oleic acids}/^3\text{H}\beta \text{ sitostanol})} \right]$$

Statistical Analysis. Each sample was tested in duplicate. Values are expressed as mean \pm SD. The differences between the 2 groups were analyzed by Student's *t* test for comparison of fatty liver constituents and by ANOVA for triglyceride absorption (using a Stat View II software package; SAS Institute, Cary, NC). A *P* value of less than .05 was considered significant.

Results

Animal Weight. The weights of the animals at the end of the various trials are shown in Table 1. There were no significant differences in the weight of animals with and without FABAC supplementation.

Fatty Liver Scores. The microscopically determined scores of fatty infiltration decreased markedly in both types of animals during FABAC supplementation (Table 2). In mice the score decreased from a mean of 1.5 ± 0.7 in the controls to 0.4 ± 1.1 in the FABAC-treated group ($P = .008$). In hamsters the score decreased from 4.0 ± 0.0 to 0.3 ± 0.8 , respectively ($P < .001$). Fig. 1 shows photomicrographs of liver histology from hamsters after 10 weeks on a lithogenic HFD with and without Aramchol supplementation. The fat was microvesicular in the hamsters and mixed (macro- and microvesicular) in the mice. There was no evidence of inflammation or fibrosis.

Fat Measurements in the Liver. The amount of fat per gram of liver tissue and the lipid protein ratio in the liver at the end of trials in animals on regular diets and

Table 2. Histological Fatty Liver Scores of Mice and Hamsters Fed Lithogenic Diets With and Without FABAC

Score	Mice(C57L/J), 4w		Hamsters, 10w	
	Controls (n = 10)	FABAC (n = 14)	Controls (n = 7)	FABAC (n = 7)
0	1	12	-	6
1	3	1	-	-
2	6	-	-	1
3	-	-	-	-
4	-	1	7	-
Mean \pm SD	1.5 ± 0.7	0.4 ± 1.1	4.0 ± 0.0	0.3 ± 0.8

HFDs, with and without FABAC supplementation, are shown in Figs. 2 through 6.

In animals receiving a regular diet, FABAC supplementation did not change significantly the liver lipid concentration or the lipid/protein ratio (Figs. 2 and 6). However, in all groups receiving a HFD, FABAC supplementation reduced fat concentration in the liver and the lipid/protein ratio.

In rats fed a HFD for 14 days ($n = 7$ in the FABAC group), FABAC supplementation reduced the lipid/protein ratio from 1.3 ± 0.44 to 0.66 ± 0.04 ($P = .001$). The fat concentration in liver tissue decreased from 296 ± 41 to 255 ± 30 mg/g liver ($P = .071$) (Fig. 2). In hamsters on a HFD for 21 days ($n = 4$ in the FABAC group), the lipid/protein ratio decreased from 1.41 ± 0.27 to 1.11 ± 0.20 ($P = .031$), whereas the lipid concentration in the liver decreased from 119 ± 30 to 57 ± 12 mg/g liver ($P = .009$) (Fig. 3). In ICR mice given a HFD for 24 days ($n = 5$ in the FABAC group) the lipid/protein ratio decreased from 0.34 ± 0.13 to 0.17 ± 0.07 ($P = .034$), whereas the fat concentration in the liver decreased from 73 ± 23 to 37 ± 5 mg/g liver ($P = .043$) (Fig. 4). In female C57BL/6 mice fed a Western diet⁵ for 16 weeks ($n = 14$ in the FABAC group) the lipid protein ratio decreased from 0.24 ± 0.04 to 0.16 ± 0.02 ($P < .001$), whereas the lipid concentration in the liver decreased from 134 ± 19 to 115 ± 17 mg/g liver ($P < .003$) (Fig. 5). In male C57BL/6 mice fed a HFD for 22 days ($n = 7$ in the FABAC group) the lipid/protein ratio decreased from 0.48 ± 0.1 to 0.27 ± 0.02 ($P = .001$), whereas the fat concentration in liver tissue decreased from 67 ± 19 to 51 ± 16 mg/g liver ($P = .014$) (Fig. 6). The decrease in liver lipids affected mostly the triglyceride levels (Fig. 7). In hamsters, the triglyceride concentration was 11.8 ± 2.5 mg/g liver tissue in FABAC-supplemented animals, compared with 47.3 ± 6.9 mg/g in controls. In C57BL/6 FABAC-treated mice the triglyceride concentration was 8.4 ± 1.5 mg/g liver tissue, as compared

Table 1. Weight of Animals on HFDs at End of Trials

Animal Species	Duration (wk)	Weight (Mean \pm SD), g			
		n	Controls	n	FABAC
Hamsters	3	4	118 ± 8	4	107 ± 6
Hamsters	10	7	164 ± 18	7	174 ± 4
Mice (ICR)	3.5	7	38 ± 7	5	31 ± 5
Mice (C57BL/6)	3	14	24 ± 2	7	22 ± 2
Mice (C57L/J)	4	3	28 ± 1	11	28 ± 2
Mice (C57BL/6)*	16	15	26 ± 3	14	26 ± 2
Rats	2	9	167 ± 18	7	175 ± 13

*Female.

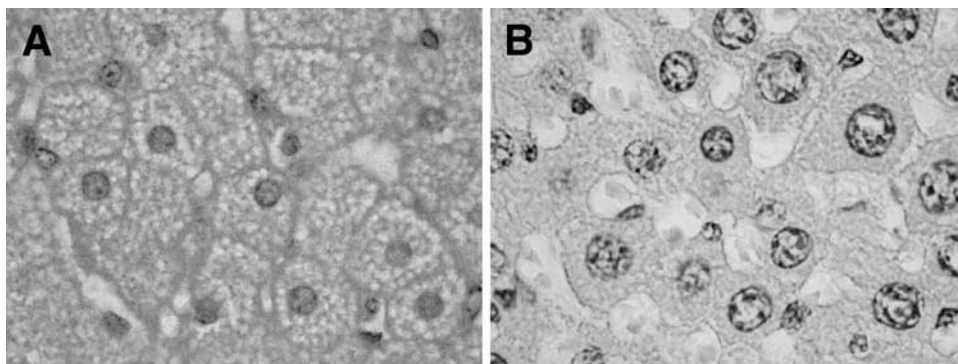


Fig. 1. Photomicrographs of livers from hamsters after 10 weeks on a lithogenic diet (A) without, and (B) with FABAC supplementation. Hepatocytes of (A) control animals are filled with microvesicular fat deposition, whereas (B) FABAC-supplemented animals show only occasional small fat droplets (hematoxylin-eosin, $\times 40$).

with 24.2 ± 2.7 mg/g in the controls. FABAC supplementation also decreased the phospholipid and cholesterol concentrations in both animal species, whereas the effects on fatty acids, diglycerides, and cholesterol esters were less consistent.

The effect of C20-FABAC also was compared with that of C16-FABAC and C22-FABAC in C57L/J mice fed a HFD for 5 weeks. All 3 FABACs reduced the liver lipid content as compared with control animals. The lipid content was 0.11 ± 0.06 ($P = .001$), 0.25 ± 0.10 ($P = .005$), and 0.34 ± 0.16 ($P =$ not significant) mg lipid/mg protein in C16-, C20-, and C22-FABAC-supplemented animals, respectively, compared with 0.70 ± 0.25 mg lipid/mg protein in control animals without FABAC supplementation.

The Effect of FABACs on ^3H -Triolein Absorption in Hamsters. Triglyceride absorption (%) in hamsters during the 7 days of the trial is shown in Fig. 8. Absorption was marginally higher in the FABAC group. However, the differences were not statistically significant. The mea-

sured amount of FABAC ingestion in the absorption experiments was 115.1 ± 15.4 mg/kg/d.

Discussion

These data show that FABACs prevent/reduce diet-induced fatty liver. They produce this effect even though the animals continue to eat a HFD. This fat reduction in the liver was confirmed by light microscopy, by chemical analysis of liver fat, and by measurement of the lipid/protein ratio in the liver. It was shown in 3 species of animals (rats, hamsters, and mice) and in 4 different strains of mice. It was shown under differing experimental conditions (*i.e.*, various HFDs given to different experimental animals for different periods of time).

We initially evaluated the degree of fatty infiltration with the commonly used light microscopy method (Table 2). However, it became quickly apparent that at high degrees of fat deposition in the liver, this method quantitatively was unreliable, even in the hands of experienced pathologists. We therefore now use quantitative chemical

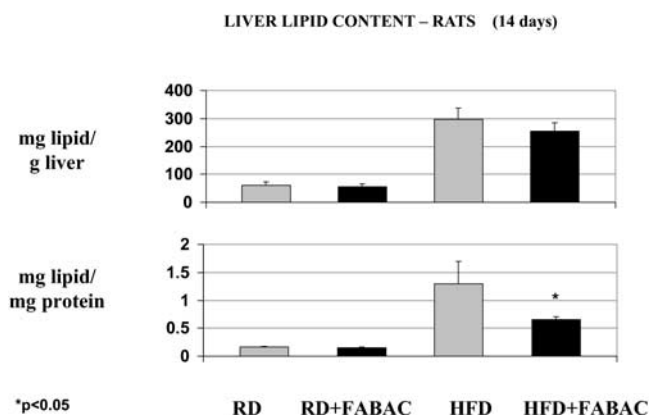


Fig. 2. Liver lipid content of rats after 2 weeks on a regular diet (RD) as well as a HFD, without (□) and with FABAC supplementation (■). There was a significant reduction in liver lipid levels in FABAC-supplemented animals, expressed as mg lipids/mg protein (lower panel). Liver lipid content was unaffected by FABAC supplementation in animals on a regular diet.

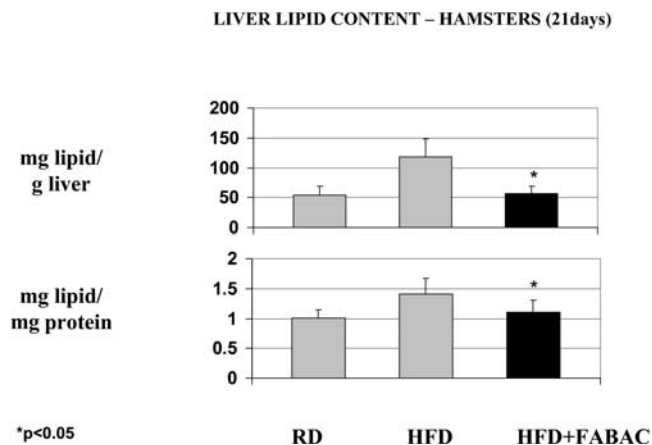


Fig. 3. Liver lipid content of hamsters after 21 days on a regular diet (RD) as well as a HFD without (□) and with FABAC supplementation (■). There was a significant reduction in liver lipid levels in FABAC-supplemented animals, to levels comparable with those in hamsters fed a regular diet.

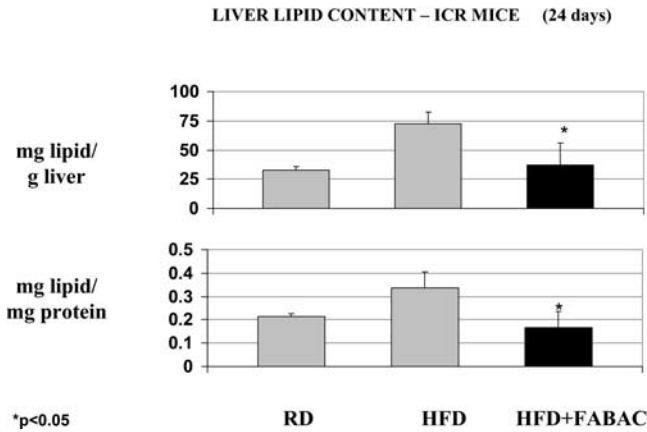


Fig. 4. Liver lipid content of ICR mice after 24 days on a regular diet (RD) as well as a HFD without (□) and with FABAC supplementation (■). There was a significant reduction in liver lipid levels in FABAC-supplemented animals, to levels comparable with those in mice fed a regular diet.

methods (mg lipid/g liver tissue and the lipid/protein ratio), which provide precise and reproducible measurements. In animals given a regular diet, FABACs did not significantly alter liver lipid concentration or the lipid/protein ratio. Thus, FABACs do not reduce liver fat in the normal liver and the effect is confined to the fatty liver.

The mechanism of this effect is not malabsorption of fat in the intestines. ³H-triolein absorption was not reduced. The FABAC-treated animals gained weight throughout the test periods to a similar degree as the controls, given the same diet *ad libitum*. Because the diet, fat absorption, and weight gain were similar in the control and test groups, this would suggest a redistribution of fat deposition in the body. Less fat is deposited in the liver and more fat is deposited elsewhere in the body. The adipose tissue is a likely, but unproven, location. Studies

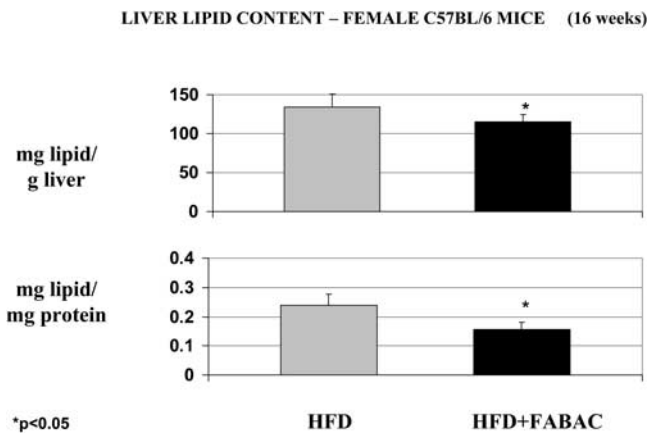


Fig. 5. Liver lipid content of female C57BL/6 mice after 16 weeks on a HFD without (□) and with FABAC supplementation (■). There was a significant reduction in liver lipid levels in FABAC-supplemented mice.

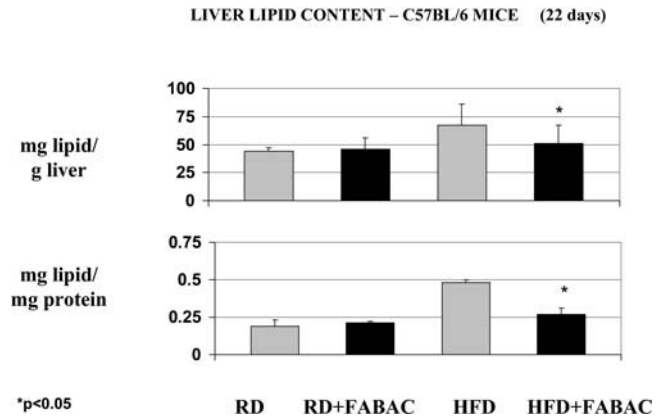


Fig. 6. Liver lipid content of C57BL/6 mice after 22 days on a regular diet (RD) as well as a HFD without (□) and with FABAC supplementation (■). There was a significant reduction in liver lipid levels in FABAC-supplemented animals, to levels comparable with those in mice fed a regular diet. Liver lipid content was unaffected by FABAC supplementation in animals on a regular diet.

are now ongoing to measure fat deposition in the adipose tissue during FABAC therapy. In the liver itself, lipid and, in particular, triglyceride metabolism obviously was altered. Export and catabolism clearly exceeded import and synthesis.

The FABACs, which are exogenous synthetic molecules, recently were shown to have a variety of specific metabolic effects. They increase cholesterol efflux from fibroblasts, possibly via an effect on the ABCA1 transporter.¹¹ They increase the activity of CYP7A and reduce the activity of HMGCoA reductase in C57L/J.¹² They inhibit atherosclerosis in C57BL/6 mice.¹³ Although the mechanisms of the hepatic effects of FABACs remain to be elucidated, several recent data allow the elaboration of

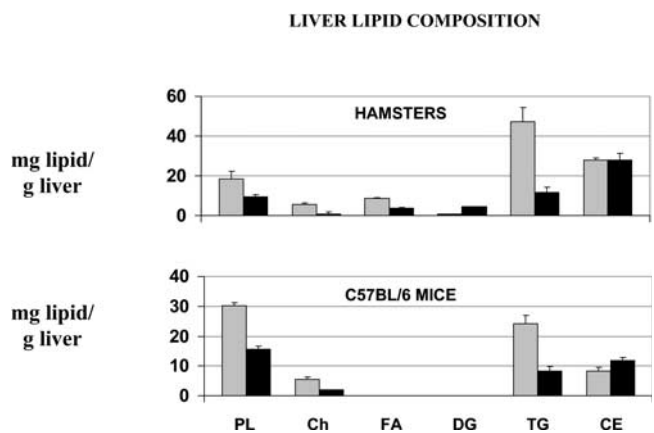


Fig. 7. Liver lipid composition of hamsters (upper panel) and C57BL/6 mice (lower panel) on a HFD after 3 weeks. FABAC supplementation (■) decreased primarily the amount of triglycerides (TG), but also phospholipids (PL) and cholesterol (Ch), as compared with control animals (□). The effects on fatty acids (FA), diglycerides (DG), and cholesterol esters (CE) were less consistent.

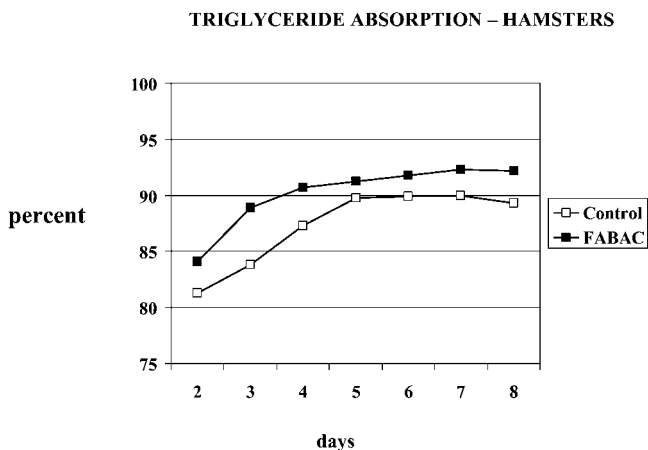


Fig. 8. Percent absorption of triglycerides in hamsters during the 7 days of the trial. There was no significant difference in triglyceride absorption between FABAC-fed animals (■) and control animals (□).

hypotheses for further testing. The import of dietary fat seems largely unaffected. Liver uptake remains to be studied. Export of fat from the liver, however, is not increased and actually has been found to be reduced. In C57L/J mice on a lithogenic diet FABACs were found to reduce triglyceride concentration in the very low density lipoprotein fraction.¹² Thus, the main processes to be studied seem to be fatty acid and triglyceride synthesis and degradation in the liver. Preliminary data indicate that arachidyl-amido-cholanoic acid acts as a peroxisome proliferator-activated receptor (PPAR) α agonist, at micromolar concentrations (J. Bar Tana, personal communication). A PPAR agonist effect would indeed reduce fatty liver by increasing fatty acid catabolism. This has to be confirmed and the synthetic pathway has to be investigated.

The results reported in this study were achieved with a FABAC dose of 150 mg/kg/d. This is our usual screening dose. Once an effect is established we usually try the dose of 25 mg/kg/d, which is physiologic, as evaluated in gallstone disease.^{2,3} This remains to be performed in non-alcohol-induced fatty liver disease (NAFLD). The present study was performed primarily with a FABAC containing a fatty acid of 20 carbon atoms (arachidic acid). However, FABACs containing other long-chain fatty acids (*e.g.*, palmitic and behenic) also seem to be effective.

Diet-induced NAFLD is now a widespread condition in affluent societies, in which up to 24% of the general population has been estimated to have NAFLD.¹⁴ This is due to the epidemic increase in the prevalence of obesity in affluent societies. Although NAFLD mostly remains asymptomatic, up to 20% of affected subjects may progress to cirrhosis and some of them require liver

transplantation. NAFLD often is associated with type II diabetes, hyperlipidemia, and other metabolic abnormalities.¹⁴ Although prolonged and consistent reduction of caloric intake may alleviate NAFLD, this rarely is achieved in real life. Under these circumstances an effective medical therapy would be useful. In this study, we have shown that arachidyl-amido-cholanoic acid is effective in preventing dietary fatty liver in animal models. Further studies of treating pre-established fatty liver are in progress. At present, there is no unanimity and not enough experimental data in relation to a medical therapy for NAFLD. Based on presently available data the FABACs would seem to offer a potentially nontoxic therapy.

We have considerable experience in the use of FABACs in experimental animals for the prevention and dissolution of cholesterol gallstones.^{2,3} In hundreds of animals tested to date, no evidence of toxicity has been noted. Rarely, minor aminotransferase elevations were found, but only in animals on HFDs.¹⁵ Formal toxicity tests as well as an Investigational New Drug permit are, however, required before human trials.

In summary, our studies show that C20-FABAC reduces and prevents diet-induced fatty liver in all animal species tested. This effect was shown by light microscopy and chemical analysis of liver fat. The presumed mechanism of action is a metabolic one. FABACs may have potential for therapy of human fatty liver due to dietary and other causes.

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