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Pharmacological profile of F 12511, (*S*)-2′,3′,5′-trimethyl-4′-hydroxy-α-dodecylthioacetanilide a powerful and systemic acylcoenzyme A: cholesterol acyltransferase inhibitor

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Abstract

The pharmacological profile of F 12511 (*S*)-2′,3′,5′-trimethyl-4′-hydroxy-α-dodecylthio-phenylacetanilide, a new inhibitor of acyl-CoA: cholesterol acyltransferase (EC 2.3.1.26; ACAT), was evaluated by using different *in vitro* and *in vivo* models. *In vitro*, F 12511 was shown to be a highly potent inhibitor of ACAT activity in microsomal preparations from various animal species as well as of cholesterol esterification in relevant human cell lines in culture. The concentrations of F 12511 required to produce 50% inhibition of ACAT activity (IC_{50} values) in microsomal preparations ranged from 41nM for hypercholesterolemic rabbit intestine to 223 nM for normocholesterolemic hamster liver. In whole cell assays using hepatic (Hep G2), intestinal (CaCo-2) and macrophagic (THP-1) cell lines, F 12511 inhibited ACAT activity with IC_{50} values of 3, 7, and 71 nM, respectively. *In vivo*, orally administered F 12511 displayed high potency and efficacy as an antihypercholesterolemic compound in different cholesterol-fed animals (rat, guinea-pig, rabbit). For instance, in guinea-pigs the dose required to reduce plasma cholesterol levels by 30% (ED_{30} value) was 0.008 $mg.kg^{-1.}$ In rabbits, an animal species prone to atherosclerosis, the hypocholesterolemic effect was accompanied by a dose-related reduction in the incidence of aortic fatty streaks that reached asymptote at 2.5 $mg.kg^{-1}$ and by an improvement of the impaired endothelial function. When given orally to chow-fed hamsters, F 12511 elicited a dose-related decrease in plasma cholesterol from 9% at 0.63 $mg.kg^{-1}$ up to 31% at 40 $mg.kg^{-1}$ associated with a preferential reduction in atherogenic lipoproteins, very low density lipoproteins (VLDL) and low density lipoproteins (LDL). Moreover, in the same dose range, F 12511 decreased hepatic cholesteryl ester concentrations and reduced liver *ex vivo* ACAT activity. By using a bioassay, ACAT inhibitory activity was present in plasma of treated hamsters 1 hr after oral administration of F 12511. Hence, the results in chow-fed hamsters are consistent with systemic and direct hepatic effects of F 12511. In guinea-pigs, an adreno-sensitive species, F 12511 did not impair the adrenal function (adrenocorticotrophic hormone challenge) at doses up to 2.5 $mg.kg^{-1,}$ far higher than those eliciting hypocholesterolemic effects in the same species. In conclusion, the results suggest that F 12511, a powerful and systemic ACAT inhibitor, constitutes an appropriate tool to determine whether the inhibition of ACAT constitutes an effective therapy for the treatment of hypercholesterolemia and of atherosclerosis in man.

Keywords: Acyl-CoA, Cholesterol acyltransferase; F 12511; Systemic and hepatic effects; Cholesterol; Atherosclerosis; Adrenal gland

1. Introduction

Atherosclerosis remains one of the major areas for new cardiovascular therapies despite the fact that hypercholesterolemia, one of the leading risk factors for the development of the disease, can now be treated successfully, for example by statins [1]. Indeed, despite effective LDL cholesterol lowering, many patients still present clinical symptoms and are considered to be at risk for cardiac ischemia. Hence, in addition to the lipid-lowering strategy, interven-

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tions based on the events that take place in the arterial wall during atherogenesis could provide enhanced clinical benefit in atherosclerosis-related cardiovascular diseases [2].

One of the recent developments in atherosclerosis research has been the identification of the enzyme ACAT (EC 2.3.1.26). ACAT is an intracellular enzyme that catalyzes the formation of cholesteryl esters from cholesterol and long-chain fatty acylcoenzyme A. The enzyme is believed to play significant roles in intracellular cholesterol storage, lipoprotein assembly, steroid hormone production, and dietary cholesterol absorption. Under pathological conditions, accumulation of cholesteryl esters as cytoplasmic lipid droplets within macrophages and smooth muscle cells is a typical feature of early lesions of human atherosclerotic plaques [3].

ACAT is an attractive target, since its inhibition in the intestine and liver may lower serum cholesterol concentration by reducing intestinal absorption of dietary cholesterol and hepatic secretion of VLDL. Moreover, inhibition of arterial ACAT, leading to depletion of cholesteryl esters in the vascular wall, may provide a direct beneficial anti-atherosclerotic effect at the site of lesion formation. Recent clinical and anatomo-pathological observations indicate that plaque type rather than plaque size is more important for the development of thrombus-mediated acute coronary syndromes. Moreover, lipid-rich and soft plaques are more threatening than collagen-rich and hard plaques because of increased vulnerability and thrombogenicity. With respect to plaque stability, lipids in the form of cholesteryl esters soften plaque, whereas crystalline cholesterol has the opposite effect, notwithstanding the fact that the cholesterol crystals are likely proinflammatory [4]. Hence, an overall reduction in cholesteryl esters is theoretically expected to result in a more rigid and more stable atheromatous lesion [5].

Several laboratories have developed specific inhibitors of ACAT because of the potential therapeutic value of such compounds in hypercholesterolemia and atherosclerosis. However, non-absorbable agents, whose main mechanism of action involves inhibition of dietary cholesterol absorption in the intestine, failed to show hypocholesterolemic efficacy in humans [6]. Indeed, the lack of inhibition of liver and arterial wall enzymes by so-called "intestinal" ACAT inhibitors constitutes a major disadvantage, limiting their potential therapeutic benefit. Thus, there has been a concerted effort to design bioavailable ACAT inhibitors capable of reaching the latter targets. However, some ACAT inhibitors have shown adrenal toxicity [7, 8], thus underscoring the challenges faced in the design and development of bioavailable compounds.

The purpose of our drug discovery effort was to identify systemic ACAT inhibitors able to decrease enzymatic activity in liver and macrophages with a high degree of potency while presenting a favorable separation between desirable pharmacological properties and undesirable adrenal side effects.

The outcome of this effort is F 12511, a powerful and systemic ACAT inhibitor exerting hypocholesterolemic and anti-atherosclerotic actions.

2. Materials and methods

2.1. Materials

CI-976, DuP-128, (±) CP-113818, PD132301–2, CI-1011, and F 12511 were synthesized by a Medicinal Chemistry Division of Pierre Fabre Research Center. Radiochemicals were purchased from DuPont New England Nuclear. Cell lines for tissue culture were obtained from ATCC. All culture media were purchased from GIBCO-BRL-Life Technologies. All plastic ware was provided by Nunc and Falcon. Specific monoclonal antibodies RAM 11 (macrophages) and 1A4 anti α-actin (smooth muscle cells) were from Dako. Other biochemicals were purchased from Sigma. All chemicals were of analytical grade quality. Sep-Pak cartridges were obtained from Millipore.

2.2. Animals and diets

Male OFA rats (Iffa Credo) weighing 160–180 g upon arrival, male Hartley guinea-pigs (Crl: (HA) BR; Charles River) weighing 300 - 350 g upon arrival, male New Zealand White rabbits (ESD) weighing 1.8–2.0 kg upon arrival, and male Syrian hamsters (Rj:AURA (IOPS Han); Elevage Janvier) weighing 80–100 g upon arrival were used. During the experiments, rats were fed a hypercholesterolemic diet (1% cholesterol, 1% cholic acid) (Diet C 1061, Altromin). Both guinea-pigs and rabbits were either fed a standard diet (guinea-pigs: 114, UAR, rabbits: Diet 2023, Altromin) or the standard diet 2023 supplemented with 0.5% cholesterol, 3% peanut oil, and 3% coconut oil (Diet 31G, Altromin). Hamsters were fed a standard hamster diet containing 0.02% cholesterol (105, UAR). Rats, guinea-pigs, and hamsters had food available *ad lib*., whereas rabbits were given a daily ration of 150 g/animal. Suspensions of drugs in 2% Tween 80 in water were administered to animals by gavage at 4 p.m.; vehicle-treated animals received 2% Tween 80 in water. The volume administered was 10 $mL.kg^{-1}$ for rats,

Abbreviations: LDL, low-density lipoprotein; ACAT, acylcoenzyme A: cholesterol acyltransferase; VLDL, very low-density lipoprotein; CI-976, 2,2-dimethyl-*N*-(2,4,6-trimethoxyphenyl) dodecanamide; DuP-128, *N*′-(2,4-difluorophenyl)-*N*-[5-(4,5-diphenyl-1*H*-imidazol-2-ylthio)pentyl]-*N*-heptylurea; (±)CP-113818, *N*-[2,4-bis(methylthio)-6-methylpyridin-3-yl] 2- (hexylthio) decanamide; PD132301-2, *N*[2,6-bis (1-methyl-ethyl) phenyl]-*N*′-[[1-[4-(dimethylamino) phenyl] cyclopentyl] methyl] urea, hydrochloride; CI-1011, ([2,4,6 tris-(1-ethylethyl) phenyl] acetyl) sulfamic acid, 2,6-bis (1-methyl-ethyl) phenyl ester; F 12511, (*S*)-2′,3′,5′-trimethyl-4′-hydroxy-α-dodecylthio-phenylacetanilide; HDL, high-density lipoprotein; and ACTH, adrenocorticotrophic hormone.

guinea-pigs, and hamsters, and 2 mL.kg^{-1} for rabbits. A minimum five-day acclimatization period was allowed before animals were used in experiments. Animals were housed in environmentally controlled rooms (21 ± 1°; humidity 55 ± 5%) on a 12-hr:12-hr light:dark cycle (lights on at 7 a.m.), with food and filtered water continuously available. Animals were handled and cared for in accordance with the Guide for the Care and Use of Laboratory Animals (NRC, 1996) and European Directive 86/609, and the protocols were carried out in compliance with French regulations and with local Ethical Committee guidelines for animal research.

2.3. Microsomal ACAT assays

Microsomes were prepared from liver or intestine of animals that had been fed standard or cholesterol-supplemented diets to saturate the substrate pool for ACAT. ACAT activity was determined according to Chautan et al. [9] with slight modifications, using [1-^{14}C] oleoyl-CoA and endogenous cholesterol as substrates. Each assay, performed in duplicate, contained microsomal protein and defatted BSA (70 μM) in a potassium phosphate buffer (0.1 M, 2mM dithiothreitol, pH 7.4). Drugs dilutions were made in DMSO (0.8% v/v final concentration) and preincubated with microsomes for 5 min at 37°. The reaction was started by the addition of oleoyl-CoA (40 μM final concentration). After 5 min at 37°, the reaction was stopped by the addition of hexane: isopropanol (3:2 v/v). The organic phase was evaporated under nitrogen and taken up by petroleum ether: diethyl ether (98:2 v/v). Cholesteryl oleate was partitioned by chromatography on silica gel columns and counted by liquid scintillation [9]. The concentrations of protein in the microsomal preparations were determined by the method of Peterson [10]. For each inhibitor, results were expressed as IC_{50} values (nM) calculated according to Tallarida and Murray [11], from means derived from at least three distinct experiments (generally performed over a three log unit range of concentrations).

This microsomal assay was adapted to determine the systemic bioactivity of F 12511 by measuring the ACAT inhibitory activity of plasma prepared from the blood of treated hamsters. Aliquots of plasma (30 μL) were preincubated for 20 min with hepatic microsomes from hypercholesterolemic rats. Radiolabeled oleoyl-CoA was added and ACAT activity determined as described above. To validate each measurement, concentrations of F 12511 dissolved in DMSO in the presence of 30 μL of plasma from vehicle-treated hamsters were incubated with hepatic microsomes under the conditions described above.

2.4. ACAT assays in cultured human cell lines

Cholesterol esterification was performed as previously described [12] on subconfluent Hep G_2 and CaCo-2 cells; culture medium was exchanged just before the start of the incubation of cells in Dulbecco's modified Eagle's medium + fetal bovine serum with different compounds (or DMSO 0.1% as their vehicle) for 4 hr at 37° prior to the addition of [1 - ^{14}C] oleic acid complexed to BSA for a further 2 hr. Then, the incubation medium was discarded and cells were washed before the intracellular lipids were extracted by hexane: isopropanol (3:2 v/v). Cholesteryl oleate was partitioned by chromatography on silica gel column and counted by liquid scintillation. THP-1 cells, suspended RPMI-1640 + Ultroser HY, were treated with phorbol 12-myristate 13-acetate (PMA) (400 ng.mL^{-1}) to induce monocyte differentiation into macrophages. Cells were incubated for 2–3 days in this medium containing PMA, and acetylated LDL (50 μg.mL^{-1}) were further added to induce intracellular cholesterol loading. Cholesterol esterification and cell protein concentrations were measured as previously described.

2.5. Specificity of inhibition

The ability of F 12511 to inhibit CEH- and lecithin cholesterol acyltransferase (LCAT)-mediated cholesterol esterification was evaluated by previously described methods using bovine pancreatic cholesterol ester hydrolase [13, 14] (Sigma) and rat plasma LCAT [15].

2.6. In vivo *potency in cholesterol-fed rats and guinea-pigs*

The hypocholesterolemic effect of F 12511 was compared to those of reference ACAT inhibitors in cholesterol-fed rats. Rats were fed the C1061 diet for 4 days. At the same time, they received the different drugs or the vehicle (control group) once daily by gavage. At the end of the study (day 5), non-fasted animals were anesthetized by pentobarbital (90 mg.kg^{-1} intraperitoneally), and blood samples were collected on EDTA (1 mg.mL^{-1}) from the abdominal aorta for plasma cholesterol determination.

Guinea-pigs were given the hypercholesterolemic 31G diet for 4 days. At the same time, F12511 was administered once daily for 4 days at doses ranging from 0.63 μg.kg^{-1} to 2.5 mg.kg^{-1}. On day 5, non-fasted animals were anesthetized with pentobarbital (90 mg.kg^{-1} intraperitoneally), and blood samples were collected on EDTA (1 mg.mL^{-1}) from the abdominal aorta. Livers were dissected out and weighed. Plasma lipoproteins were separated by sequential ultracentrifugation with a fixed angle rotor (TLA 100.3, Beckman), using density (d) cuts of $d < 1.006$, 1.019–1.074, and 1.074–1.21 for VLDL, LDL, and HDL, respectively. Plasma cholesterol and triglyceride levels were measured enzymatically with commercial kits (CHOD-PAP, GPO-PAP Tests, Boehringer Mannheim). Following the homogenization of liver samples in physiological serum, hepatic lipids were extracted by chloroform-methanol (2:1 v/v), the organic phase was evaporated under nitrogen flux, and the dried extracts were dissolved into hexane. Cholesteryl esters

and free cholesterol were separated by Sep-Pak cartridges and eluted by hexane: ethyl ether (96:4 v/v) and ether, respectively [16]. Spectrophotometric determination of cholesterol was carried out as described above.

2.7. Hypolipidemic activity

In advanced studies the hypolipidemic activity of F 12511 was investigated in cholesterol-fed rabbits and normocholesterolemic hamsters. New Zealand White rabbits were given the hypercholesterolemic diet (31G) for 4 weeks. F 12511 (at doses from 2.5 $\mu g.kg^{-1}$ up to 10 $mg.kg^{-1}$) or vehicle was administered daily throughout the 4-week experiment. Blood samples collected on EDTA were obtained from the marginal ear vein of non-fasted animals at days 0, 7, 14, 21, and 28 for cholesterol determination. At the end of the experiments, rabbits were killed after one injection of sodium pentobarbital (90 $mg.kg^{-1,}$ intravenously), and tissues were immediately dissected out: thoracic aorta was placed in cold Krebs-Henseleit solution gassed with 95% 0_2 - 5% CO_2 for vascular reactivity determination according to Junquero et al. [17]. Previously fixed and paraffin-embedded serial sections of the aortic arches were stained routinely with hematoxylin and eosin. Macrophages and smooth muscle cells were detected on additional sections by using the specific monoclonal antibodies RAM 11 and 1A4 anti-α-actin respectively.

Hamsters, while maintained on a standard diet, were treated once daily for 10 days with F 12511 (0.16, 0.63, 2.5, 10, and 40 $mg.kg^{-1,}$ dose-action study), (10 and 40 $mg.kg^{-1,}$ lipoprotein study) or its vehicle. VLDL ($d < 1.006$), LDL (d 1.019–1.063), and HDL (d 1.063–1.21) were separated by sequential ultracentrifugation, and liver samples were homogenized in physiological serum. When needed, tissular lipids were extracted by chloroform: methanol (2:1 v/v). Cholesterol, triglycerides, and phospholipids were measured enzymatically; free and esterified cholesterol were separated by using Sep-Pak cartridges; cholesteryl ester species in lipoproteins were separated and quantified by HPLC according to Kim and Chung [18] with slight modifications. In brief, aliquots of lipoproteins were added to propanol-2: NaOH 0.75 M (33:17 v/v) and mixed; after 25 min, each sample was extracted twice by *n* - octane (5 mL), and the octane layer was dried under a nitrogen stream. Dried extracts were dissolved in propanol-2 and analyzed in an ODS ultrasphere column (Beckman) following an isocratic elution with acetonitrile: propanol-2 (45:55 v/v). The absorbance was monitored at 210 nm; free and esterified cholesterol were quantified by computer integration (System Gold Nouveau, Beckman) and cholesteryl oleate/cholesteryl linoleate ratios were calculated. Assays for *ex vivo* hepatic ACAT activity were performed using liver homogenates according to Burrier et al. [19].

2.8. Adrenal gland function in guinea-pigs

For these experiments, guinea-pigs were individually housed in an access-restricted area and given the standard diet and water *ad lib.* F 12511 was administered once daily for 4 days at doses ranging from 0.16 to 160 $mg.kg^{-1}$; vehicle-treated animals received 2% Tween 80 in water. On the morning of the fifth day, all animals received an intraperitoneal ACTH challenge (25 μg Synacthen Immediat ®, Ciba-Geigy). After 30 min, animals were decapitated and blood was collected on EDTA. Plasma cortisol levels were determined using an iodinated radioimmunoassay kit (CORT-CT2) purchased from CIS-BIO. Adrenal glands were excised and adrenal free and esterified cholesterol contents were measured by HPLC as previously described.

2.9. Statistical analysis

Statistical analyses were performed using one-way or two-way analysis of variance followed by Student's t-test or Mann-Whitney test to determine the degree of significance. The IC_{50} values were estimated using $\log_{10}$ of concentration versus inhibition. Confidence intervals of 95% were provided as well as a conservative test based on the minimal significant difference between F 12511 versus every other product.

3. Results

3.1. In vitro *potency and selectivity*

Since cholesterol concentration in the endoplasmic reticulum may be a key determinant for regulating ACAT activities in cells [20], the major part of the *in vitro* evaluation of the activity of ACAT inhibitors was carried out on microsomes from cholesterol-fed animals. F 12511 appeared to be a highly potent ACAT inhibitor, with IC_{50} values ranging from 41 nM (hypercholesterolemic rabbit intestine) to 223 nM (normocholesterolemic hamster liver) (Table 1). In order to determine the potency of F 12511 against human ACAT, cholesterol esterification in whole cells was evaluated by using hepatic (Hep G_2), intestinal (CaCo-2), and macrophagic (THP-1) cell lines. Whereas Hep G_2 and CaCo-2 cells were investigated without cholesterol loading, THP-1 were differentiated into macrophage foam cells by phorbol ester followed by acetyl LDL treatment. F 12511 at nanomolar concentrations inhibited the esterification of cholesterol in the different human cell lines (IC_{50} values ranging from 3 nM [Hep G_2] to 71 nM [THP-1]) (Table 1). F 12511 compared favorably (particularly on the human ACAT) with CI-976, DuP-128, PD 132301–2, and CI-1011, and appeared to be of the same order of potency as (±) CP-113818. F 12511 was also examined for its ability to modulate two other enzymes involved in cholesterol metabolism: F 12511 up to 10^{-5}M had no effect on

Table 1
Inhibitory potencies of F 12511 and of different well-documented ACAT inhibitors against ACAT activity in microsomal preparations and in human whole cell lines

Species	Tissues	IC_{50} values [nM]					
		F 12511	CI-976	DuP-128	±CP-113818	PD132301-2	CI-1011
HC Rat	Liver	59	492	25	73	94	41385*
		[13–262]	*[82–2958]*	*[5–127]*	*[18–296]*	*[15–613]*	*[10670–160515]*
NC Hamster	Liver	223	995	89	70	376	71339*
		[19–2606]	*[92–10729]*	*[9–924]*	*[6–846]*	*[52–2689]*	*[15125–336469]*
HC Rabbit	Intestine	41	389	66	37	151	22029*
		[10–167]	*[69–2184]*	*[14–301]*	*[10–141]*	*[46–499]*	*[4700–103236]*
Human	Hep G_2	3	697*	59*	6	183*	391*
		[0.7–14]	*[131–3708]*	*[13–276]*	*[1–34]*	*[40–848]*	*[80–1910]*
	CaCo-2	7	270*	272*	9	145	1252*
		[1–38]	*[52–1409]*	*[46–1625]*	*[1–63]*	*[36–589]*	*[367–4263]*
	THP-1	71	1244	239	63	196	664
		[10–483]	*[261–5925]*	*[36–1598]*	*[6–611]*	*[35–1096]*	*[117–3756]*

ACAT activity was determined by measuring the incorporation of [^{14}C]oleoyl-CoA into cholesterol esters. Results are expressed as IC_{50} values (nM) calculated from means derived from at least three distinct experiments (each in duplicate) generally performed over a three-log unit range of concentrations for each compound. Figures in brackets represent 95% confidence interval. Asterisks denote values significantly different from F 12511. NC = normocholesterolemic, HC = hypercholesterolemic.

* $P < 0.05$.

CEH from bovine pancreas, while a slight decrease (35%) in plasma lecithin cholesterol acyltransferase from rat was observed with F 12511 at 10^{-5}M.

3.2. In vivo *potency in cholesterol-fed rats and guinea-pigs*

As a preliminary evaluation of its *in vivo* potency prior to more detailed investigations, the hypocholesterolemic efficacy of F 12511 was evaluated in cholesterol-fed rats and guinea-pigs. In rats, the cholesterol enriched diet elicited a 6-fold increase in the mean plasma total cholesterol level (up to 500–600 mg.dL^{-1} vs 90 mg.dL^{-1} in chow-fed rats). F 12511 significantly decreased plasma cholesterol levels with an ED_{50} of 0.12 mg.kg^{-1}. In this model, F 12511 compared favorably with DuP-128, PD 132301–2, and CI-976 (respective ED_{50} values of 1.08, 5.29, and 10.6 mg.kg^{-1}), ($P < 0.05$), whereas it appeared to be equipotent to (±) CP-113818 and CI-1011 (respective ED_{50} values of 0.28 and 0.09 mg.kg^{-1}). In contrast to its *in vitro* profile and in agreement with published data [21], CI-1011 exerted a potent hypocholesterolemic effect in this *in vivo* model.

When fed a chow diet supplemented with 0.5% cholesterol plus 3% peanut oil plus 3% coconut oil for 4 days, guinea-pigs developed a marked hypercholesterolemia (204 mg.dL^{-1} vs 35 mg.dL^{-1} in chow-fed animals); 60% of cholesterol was transported in the LDL fraction. When administered by gavage concurrently with the hypercholesterolemic diet throughout the experiment, F 12511 (Fig. 1) dose-dependently decreased plasma cholesterol levels with an ED_{30} of 0.008 mg.kg^{-1} and a maximal efficacy of around 60% at 0.04 mg.kg^{-1}. F 12511 treatment resulted in a dose-dependent cholesterol reduction in all classes of lipoproteins, up to 78%, 54%, and 52% in VLDL, LDL and HDL, respectively. Plasma triglycerides rose up to 80 ± 8 mg.dL^{-1} in control group fed the hyperlipidemic diet, whereas this parameter was previously measured at around 50 mg.dL^{-1} in chow-fed guinea pigs. F 12511 prevented the increase in plasma triglycerides with a maximal inhibition (about 45%), which plateaued at doses of 0.04 mg.kg^{-1} and higher, thus allowing a return to normal triglyceride values (data not shown).

Total cholesterol content in the liver rose from 2.1 (chow-fed) up to 6.6 mg.g^{-1} wet tissue weight relative to dietary intervention. Cholesteryl esters accounted for around 30% of liver total cholesterol concentrations. F 12511 prevented, in a dose-dependent manner, the build-up in total liver cholesterol essentially by impairing the formation of esterified cholesterol with an ED_{50} of 0.077 mg.kg^{-1}, while moderately decreasing unesterified cholesterol, ED_{50} > 2.5 mg.kg^{-1}. Thus, in no case did this substrate for ACAT accumulate in the livers from treated animals.

3.3. Effect on adrenal gland function in guinea-pigs

Since the impairment of adrenal gland functioning in guinea-pigs has already been described for some ACAT inhibitors [22], this issue was addressed for F 12511. When administered by gavage for 4 days to normocholesterolemic guinea-pigs, 12511 did not significantly impair, up to 2.5 mg.kg^{-1}, the acute increase in plasma cortisol resulting from ACTH injection. At higher doses, F 12511 inhibited the ACTH response in a dose-related manner, with an ED_{50} of 16 mg.kg^{-1} (Fig. 2A). This effect was associated with a parallel decrease in the adrenal cholesteryl ester pool from 10 mg.kg^{-1} as well as with a moderate increase in free cholesterol levels at the two highest doses (Fig. 2B).

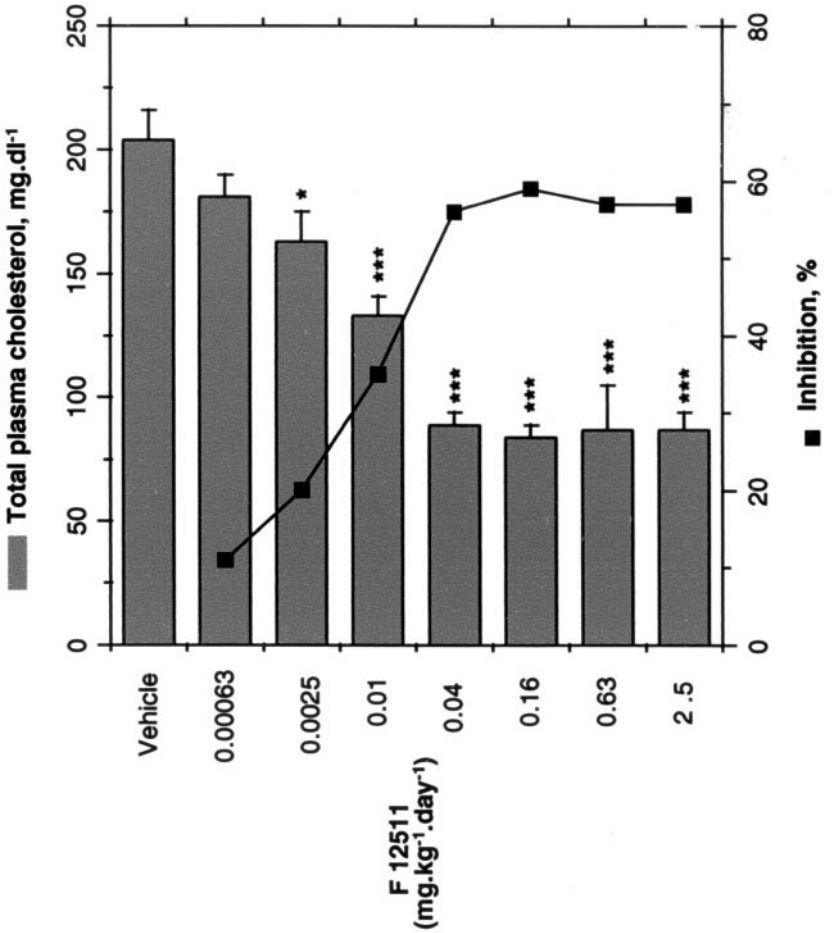

Fig. 1. The effects of F 12511 at different dose levels for 4 days on total plasma cholesterol concentrations in guinea-pigs fed a hypercholesterolemic diet: the results are represented as means (± SEM) based on N= 4–16 animals/group (bar graphs). Asterisks denote values significantly different from vehicle: $P < 0.05$,***: $P < 0.001$, using Student's *t*-test or Mann-Whitney test.

3.4. Hypocholesterolemic and anti-atherosclerotic activity in cholesterol-fed rabbits

New Zealand cholesterol-fed rabbits that develop a rapid and severe hypercholesterolemia have been used for evaluating putative lipid-lowering activity of ACAT inhibitors [23]; they also represent a useful model to investigate experimental atherosclerosis [24]. When fed a chow diet supplemented with 0.5% cholesterol plus 3% peanut oil and 3% coconut oil for 4 weeks, New Zealand White rabbits developed a major hypercholesterolemia (2894 mg.dL^{-1} vs 67 mg.dL^{-1} in chow-fed animals) in a time-dependent manner, reaching a steady state at the third week of the hypercholesterolemic diet. The majority (about 70%) of cholesterol was transported in the VLDL fraction. When given by gavage concurrently with the hypercholesterolemic diet throughout the experiment, F 12511 prevented the increase in plasma total cholesterol in a dose-dependent manner without affecting body weight and food consumption at any dose tested (mean cumulative food consumption: 3330 grams at 10 mg.kg^{-1}, the highest dose tested, vs 3014 grams in the vehicle; mean body weight variation throughout the study: + 725 grams at 10 mg.kg^{-1} vs + 719 grams in the vehicle). The lowest dose of F 12511 producing a statistically significant inhibition was 0.04 mg.kg^{-1}.day^{-1} (Fig. 3). An ED_{50} value of 0.37 mg.kg^{-1} was calculated from the areas under the curves of total plasma cholesterol levels throughout the study.

As a consequence of their severe hypercholesterolemia, cholesterol-fed rabbits developed pre-atherosclerotic lesions. According to the classification of the arterial lesions [25, 26], the general histological appearance of aortic segments consisted of fibrofoamy lesions with a predominance of monocyte-macrophage foam cells. The incidence of aortic fatty streaks was reduced when rabbits received F 12511 at 2.5 and 10 mg.kg^{-1}.day^{-1}. A downward trend in lesion surface was also found in F 12511-treated animals in comparison with the vehicle group. Endothelium-dependent relaxations to acetylcholine were impaired in hypercholesterolemic rabbits compared with normocholesterolemic controls ($P < 0.05$). F 12511 at 2.5 mg.kg^{-1}.day^{-1} prevented the impairment of endothelial function (Fig. 4).

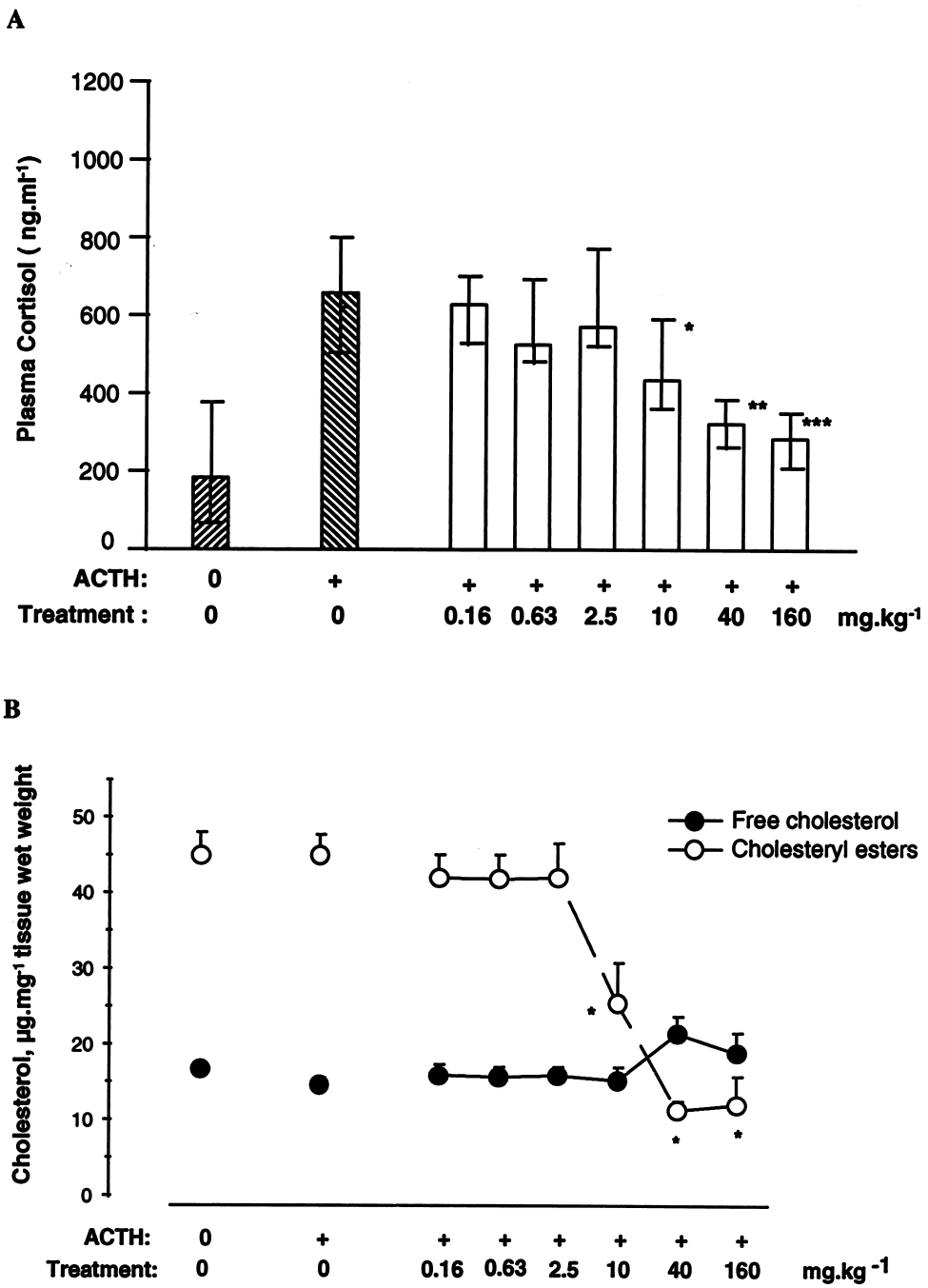

Fig. 2. The effects of oral administration of F 12511 on ACTH-stimulated cortisol release (A) and on adrenal cholesterol contents (B) in normolipemic guinea-pigs. Animals were treated with drug or vehicle for 4 days and ACTH (+: 25 μg ACTH/animal) was administered on the fifth day by intraperitoneal route 30 min before blood sampling and excision of adrenal glands. Data are expressed as medians with interquartiles (25 - 75%) for plasma cortisol and means (± SEM) for adrenal cholesterol contents, based on N=18 per vehicle groups and N=9 per dose. Asterisks denote values significantly different from the corresponding ACTH control, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

3.5. Hypolipidemic efficacy in chow-fed hamsters

The hamster has recently become an important animal model for studying nutritional factors and drugs affecting lipoprotein metabolism. In contrast to the rat, the hamster has a significant amount of LDL in plasma and its hepatic cholesterol synthesis is similar to that in humans [27]. When given orally for ten days to chow-fed hamsters, F 12511 elicited a dose-related decrease in plasma cholesterol from 9% at 0.63 mg.kg^{-1} up to 31% at 40 mg.kg^{-1}. F 12511 preferentially reduced cholesterol concentrations in the atherogenic lipoproteins VLDL and LDL. After treatment with F 12511 at 10 and 40 mg.kg^{-1}, significant decreases of 29 and 47%, respectively, in VLDL mass were observed

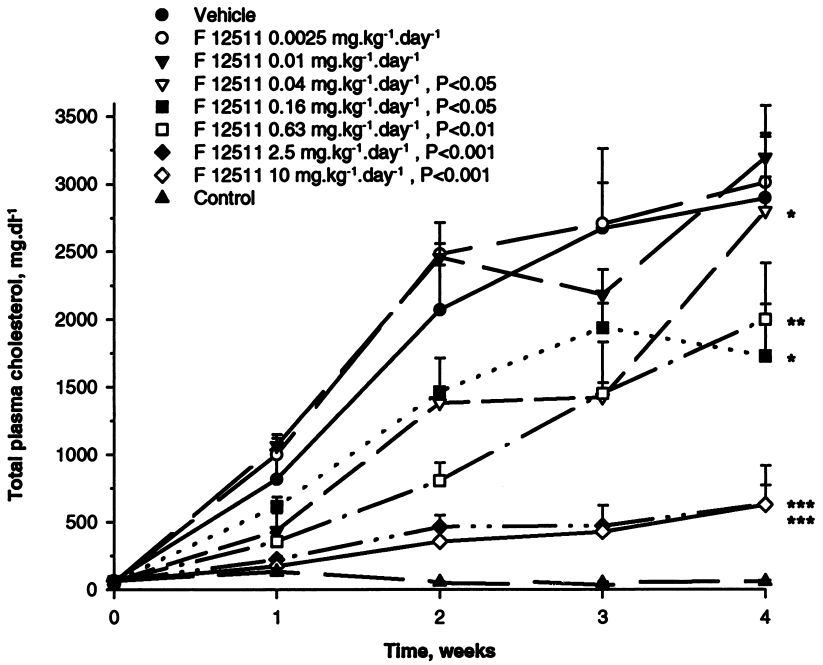

Fig. 3. The effects of F 12511 at different dose levels for 4 weeks on total plasma cholesterol concentration in New Zealand rabbits fed a hypercholesterolemic diet: the results are represented as means (± SEM) based on N= 3–6 animals/group. Asterisks denote significant differences from vehicle, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, using two-way (time, treatment) analysis of variance (ANOVA) performed on values recorded throughout the four-week treatment period. Also represented is a group of (control) animals fed a normocholesterolemic diet.

(Table 2). They were related to reductions in the concentrations of all VLDL components. LDL and HDL concentrations were also reduced (by 29 and 27%, respectively) at 40 mg.kg^{-1} of F 12511. Lipid compositions determined for VLDL and LDL demonstrated that the percent mass of cholesteryl esters decreased at the expense of triglycerides in F 12511 treated groups. Analysis of the cholesteryl esters present in VLDL revealed a marked difference in the cholesteryl oleate/cholesteryl linoleate ratios (Table 2). Treatment by F 12511 significantly reduced this ratio by 45 and 57% at 10 and 40 mg.kg^{-1}, respectively. Cholesteryl esters accounted for 72% of liver total cholesterol content in control animals. F 12511 decreased, in a dose-dependent manner, hepatic cholesteryl ester concentrations with an ED_{50} of 1.40 mg.kg^{-1}, while moderately reducing free cholesterol contents from 13% at 0.16 mg.kg^{-1} up to 33% at 40 mg.kg^{-1}. Finally, a significant decrease of 19 and 30% in liver ex vivo ACAT activity was elicited by F 12511 at 10 and 40 mg.kg^{-1}, respectively, when compared to control animals.

3.6. Systemic bioactivity

Systemic bioactivity of F 12511 was determined by using a bioassay that provided information on the level of ACAT inhibitory activity present in the plasma following a single oral administration of the compound. Plasma samples from treated hamsters were incubated with hepatic microsomes and the degree of ACAT inhibition measured. Under these experimental conditions, maximal ACAT inhibitory activity was present in plasma approximately 1 hr after oral administration of F 12511. Increasing the dose of F 12511 from 0.63 to 40 mg.kg^{-1} resulted in a dose-related increase in the metabolic pool exerting ACAT inhibitory activity (Table 3).

4. Discussion

The hypolipidemic and anti-atherosclerotic efficacy of ACAT inhibitors in humans is likely to depend on their bioavailability, regardless of the potency that they may otherwise possess. Recent developments concerning the potential role of cholesteryl esters in the hepatic overproduction of atherogenic lipoproteins [28, 29] and in the increased vulnerability and thrombogenicity of the atherosclerotic plaque [30] explain the interest in designing ACAT inhibitors able to reach liver and artery wall enzymes. ACAT inhibitors must also display favorable ratios between desirable pharmacological properties and the undesirable adrenal side effects that can result from ACAT inhibition in the adrenal glands, as cholesteryl ester stores represent a reservoir for steroid hormone synthesis [31].

The studies described in this report demonstrate that F 12511 is a highly potent ACAT inhibitor. At nanomolar concentrations, it inhibits the esterification of cholesterol in different human cell lines. Under our experimental conditions, it appears to be more potent than CI-1011, a weak *in vitro* ACAT inhibitor [21] currently in clinical develop-

Table 2
Effects of F 12511 on plasma lipoprotein mass and composition in chow-fed hamsters

Lipoprotein fraction	Concentration ($mg \cdot dL^{-1}$)				
	Controls		F12511: 10 $mg \cdot kg^{-1}$		F 12511: 40 $mg \cdot kg^{-1}$
VLDL component		A		B	
Total cholesterol	27.4 ± 2.6	**	14.7 ± 1.5 (53.6)	***	11.9 ± 1.4 (43.4)
Esterified cholesterol	18.3 ± 1.4	**	9.2 ± 1.1 (50.3)	***	7.6 ± 1.1 (41.5)
Ratio (18:1/18:2)	0.87 ± 0.06	***	0.48 ± 0.05 (55)	***	0.37 ± 0.03 (42.5)
Free cholesterol	9.1 ± 1.3	*	5.4 ± 0.7 59.3)	*	4.4 ± 0.5 (48.3)
Triglycerides	165 ± 26		128 ± 17 (77.6)	*	96.3 ± 10 (58.4)
Phospholipids	22.8 ± 3.8		14.8 ± 2.1 (64.9)	*	11.8 ± 2 (51.8)
Proteins	28.5 ± 1.4	***	15.3 ± 1.6 (53.7)	**	7.6 ± 1.7 (26.7)
Lipoprotein (mass)	*243 ± 32*	***	*173 ± 22 (71.2)*	***	*128 ± 13 (52.7)*
LDL component					
Total cholesterol	43.4 ± 2.1	***	30.1 ± 2.0 (69.3)	***	24 ± 1.8 (55.3)
Esterified cholesterol	33.6 ± 1.9	**	23.4 ± 1.7 (69.6)	***	18.1 ± 1.0 (53.9)
Free cholesterol	9.8 ± 0.8	*	6.7 ± 0.9 (68.4)	*	5.9 ± 1.0 (60.2)
Triglycerides	69.2 ± 3.2		74.9 ± 9.9 (108.2)		62.6 ± 9.9 (90.5)
Phospholipids	40.7 ± 3.1		34.8 ± 2.9 (85.5)	*	28.9 ± 3.1 (71)
Proteins	36.3 ± 2.8	**	23.3 ± 2.0 (64.2)	***	18.4 ± 1.4 (50.7)
Lipoprotein (mass)	*190 ± 6*		*163 ± 12 (85.8)*	**	*134 ± 16 (70.5)*
HDL component					
Total cholesterol	83.6 ± 5.1		70.2 ± 4.7 (84)	**	59 ± 2.7 (70.6)
Esterified cholesterol	69.6 ± 4.2		60.5 ± 4.5 (86.9)	**	51.2 ± 2.3 (73.6)
Free cholesterol	14 ± 2.1		9.7 ± 1.6 (69.3)	*	7.8 ± 1.7 (55.7)
Triglycerides	62.2 ± 8.5		48.7 ± 4.5 (78.3)	*	36.4 ± 4.5 (58.5)
Phospholipids	138 ± 12		118 ± 9 (85.5)	*	94.6 ± 10.8 (68.5)
Proteins	134 ± 10		144 ± 16 (107.5)		114 ± 10 (85.1)
Lipoprotein (mass)	*418 ± 23*		*381 ± 28 (91.1)*	**	*304 ± 20 (72.7)*

F 12511 was administered for 10 days in chow-fed hamsters. The results are expressed as means (±SEM) based on N = 6 animals/group. A, B, statistical comparison between control and treated animals. Asterisks denote values significantly different from controls using Student's *t*-test or Mann–Whitney test. Figures in parentheses in columns 2 and 3 represent % of control values.

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

ment, and comparable to (±) CP-113818, one of the most potent ACAT inhibitors described to date [32]. The ability of F 12511 to inhibit, at very low concentrations, cholesterol esterification in the human intestinal CaCo-2 cell line and in intestinal microsomal preparations probably explains its remarkable potency and efficacy as an antihypercholesterolemic compound in different species of cholesterol-fed animals (rat, guinea-pig, rabbit). For instance, in guinea-pig, F 12511 dose-dependently decreases plasma cholesterol levels with an ED_{30} as low as 0.008 $mg.kg^{-1}$. Even though the promising hypocholesterolemic effects of intestinal ACAT inhibitors in cholesterol-fed animal models have not always been reproduced in man [33], evidence [34] that human postprandial LDL induce cellular cholesteryl ester accumulation in macrophages offers new perspectives for evaluating the ability of potent ACAT inhibitors to reduce atherogenicity of these lipoproteins in man.

Table 3
Determination of plasma-dependent ACAT inhibitory activity of non-fasted hamsters dosed with F 12511

F 12511 ($mg \cdot kg^{-1}$)	ACAT inhibitory activity in plasma (%)
0.63	9 ± 1
2.50	45 ± 11
10	51 ± 2
40	78 ± 3

Blood samples were obtained 60 min after the acute oral administration of F 12511 in aqueous Tween 80, 2%. Each plasma sample was assayed for ACAT inhibitory activity, which is expressed as % inhibition of plasma-dependent ACAT activity from vehicle treated animals. Results are means ± SEM (N = 3–4 animals/group).

It has been reported that in chow-fed hamster the liver is responsible for 92% of the cholesterol flux into plasma [12]. Hence, it seems likely that, among ACAT inhibitors, only those having a hepatic impact could exert hypocholesterolemic properties in this non-cholesterol-fed animal model. When given orally to chow-fed hamsters, F 12511 elicits hypocholesterolemic properties at a dose as low as 0.63 $mg.kg^{-1}day^{-1}$. Besides reducing circulating apo B-100 containing lipoproteins, F 12511 also decreases the cholesteryl ester/triglyceride ratio and hepatic ACAT-derived cholesteryl oleate, as described previously with CI-976 in a non-cholesterol-fed rabbit model [35]. F 12511 also de-

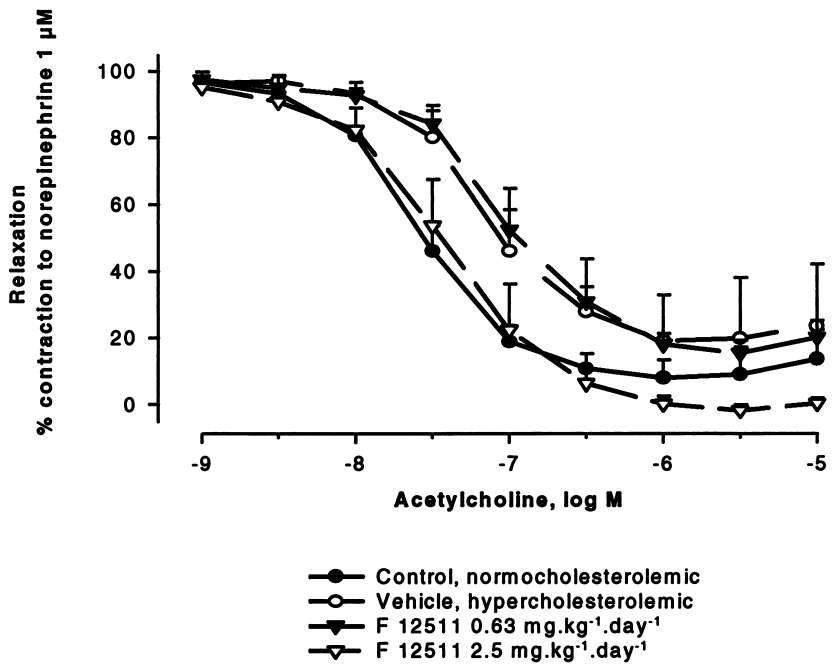

Fig. 4. Relaxation evoked by acetylcholine on thoracic aortic rings from New Zealand rabbits fed a hypercholesterolemic diet. Effects of F 12511 administered at different dose levels for 4 weeks. The results are represented as means (± SEM) based on N=3–5 animals/group. Also represented is a group of (control) animals fed a normocholesterolemic diet.

creases liver *ex vivo* ACAT activity and hepatic cholesteryl ester concentrations.

Even if the direct effect on VLDL triglycerides, cholesteryl esters and apo B production has not been measured, these data are in accordance with reports demonstrating the relationship between hepatic cholesteryl esters, apo B secretion, and ACAT activity both *in vitro* [36, 37] and *in vivo* [38]. Moreover, by utilizing a hepatic microsomal ACAT assay whose activity can be modulated by plasma samples from F 12511-treated hamsters, a putative transient exposure of target tissues to this compound was demonstrated. Hence, besides its ability to inhibit *in vitro* ACAT activity at very low concentrations in hepatic microsomal preparations from different animal species (including hamster) and in intact Hep G_2 cells (IC_{50} = 3 nM), results obtained *in vivo* with F 12511 in chow-fed hamsters are consistent with systemic and direct hepatic effects of this compound.

Accumulation of cholesteryl esters as cytoplasmic lipid droplets within macrophages is a characteristic of early atherosclerotic lesions. By inhibiting cholesterol esterification at nanomolar concentrations in the macrophagic (THP-1) human cell line, F 12511 could exert beneficial anti-atherosclerotic effects at the site of lesion formation provided the drug reaches the arterial wall in effective concentrations. A downward trend in fibrofoamy lesion surface, accompanied by a recovery of the impaired endothelial functionality, was found in cholesterol-fed rabbits under oral treatment by low doses of F 12511. However, since in this model F 12511 reveals strong antihypercholesterolemic properties, it remains to be elucidated whether it may also act directly at the vessel wall (this question can be addressed by using Watanabe rabbits [39] and transgenic mice [40]).

Adrenal toxicity observed with some ACAT inhibitors [41, 8] represents a major concern. If the toxicity is mechanism-related, the design of a compound that reaches only desirable peripheral targets (i.e. liver, artery wall) may be difficult to achieve. Indeed, the recent discovery of ACAT 2, an additional ACAT localized to the endoplasmic reticulum of liver and intestine in non-human primates and mice [42], and the observation that ACAT 1, found ubiquitously throughout the body, plays a major catalytic role in adult human liver, adrenal gland, and macrophages, but not in intestines [43], illustrate the complexity of this approach. However, Meiner et al. [44] recently reported that complete deletion of the ACAT gene cloned from macrophages in mice had no discernible effect on adrenal steroidogenesis in response to an acute ACTH stimulus, in spite of dramatic cholesteryl ester depletion in adrenal glands. In mice, whose blood cholesterol, unlike that of humans, is mainly transported in HDL, the provision of cholesterol through the HDL:cholesteryl ester-selective uptake pathway [45] may be more critical for steroidogenesis than cytosolic cholesteryl ester pools during acute stress. In guinea-pig, an animal species transporting the majority of blood cholesterol in LDL like humans [46] and pertinent to reveal hypolipidemic activities as well as possible adrenocortical side effects of ACAT inhibitors, F 12511 does not impair the ACTH response at doses up to 2.5 $mg.kg^{-1}$. Since F 12511 reduces plasma cholesterol levels in cholesterol-fed guinea-pigs with an ED_{30} of 0.008 $mg.kg^{-1}$, a high safety

ratio of 312.5 can be calculated. Further studies addressing the eventual selectivity of F 12511 against ACAT subtypes and its tissue distribution could help to explain this result. The effects of F 12511 on the functionality and viability of a pertinent human adrenal cell line (NCI-H295R) [47, 48] have recently been evaluated. It was demonstrated that, at concentrations far higher than those inhibiting ACAT, F 12511 impairs neither steroid production nor transcription of genes involved in steroidogenesis and lipoprotein uptake in this human adrenal cell line. Cell viability is also unaffected [49].

In summary, F 12511 appears as a highly potent and systemic ACAT inhibitor that displays strong hypocholesterolemic and anti-atherosclerotic properties in different animal models. Even if these results must be supported by long-term regulatory toxicological studies, the high safety ratio presented by F 12511 in an animal species known to be sensitive to adrenal side effects of ACAT inhibitors and the absence of any deleterious effects in the pluripotent human adrenal cell line NCI-H295R suggest that F 12511 constitutes a suitable tool for examining whether the inhibition of ACAT can constitute an effective therapy for the treatment of hypercholesterolemia and of atherosclerosis in man.

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