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Biochemical Pharmacology 61 (2001) 387–398

Biochemical Pharmacology

Lack of toxic effects of F 12511, a novel potent inhibitor of acyl-coenzyme A: cholesterol *O*-acyltransferase, on human adrenocortical cells in culture

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Received 31 January 2000; accepted 19 June 2000

Parts of this work were presented at the 71st Congress of the European Atherosclerosis Society (May 26–29, 1999), Athens, Greece.

Abstract

Inhibition of acyl-coenzyme A: cholesterol *O*-acyltransferase (EC 2.3.1.26; ACAT) reduces intracellular cholesteryl esters that are substrates for steroidogenesis in adrenal cells. The adrenal side effects of ACAT inhibitors remain a key point for their development as antiatherosclerotic agents. The aim of this study was to characterize the effects of a novel and powerful ACAT inhibitor, F 12511 (*S*)-2′,3′,5′-trimethyl-4′-hydroxy-α-dodecylthio-phenylacetanilide, on the NCI-H295R cell line, which has functional properties comparable to those of normal human adrenal cells. F 12511 incubated with cultured cells for 4–72 hr strongly inhibited cholesteryl oleate formation. The concentrations required to produce 50% inhibition (IC_{50} values) ranged from 20 to 50 nM; in the presence of low-density lipoproteins (LDL), this effect was paralleled by a decrease in cholesteryl ester mass and an increase in intracellular free cholesterol. At concentrations 100-fold larger than the IC_{50} value for up to 48 hr, F 12511 reduced neither the basal release of cortisol and aldosterone nor the production of cortisol stimulated by forskolin. F 12511 did not modify the mRNA levels of the steroidogenic enzyme genes cytochrome P450 cholesterol side-chain cleavage (P450scc), cytochrome P450 17α-hydroxylase (P450c17), or cytochrome P450 21-hydroxylase (P450c21) or those of the LDL receptor and high-density lipoprotein scavenger receptor class B, type I (SR-BI) genes, either in the presence or absence of adenosine 3′,5′-cyclic monophosphate stimulation for 24 hr. Exposure to F 12511 at up to 3 μM for 24 or 48 hr did not result in significant change in morphological and ultrastructural characteristics; the cytoplasm contained large numbers of mitochondria with intact crystae, and the same typical features of secretory activity were observed in NCI-H295R control cells. Exposure to 3 μM of F 12511 for 96 hr also did not affect cell viability. These data demonstrate that reduction of the substrate for steroidogenesis by the ACAT inhibitor F 12511 impairs neither steroid production nor transcription of genes involved in steroidogenesis and lipoprotein uptake in the pluripotent human adrenal cell line NCI-H295R.

Keywords: ACAT; Adrenal cells; F 12511; Steroidogenesis; Lipid metabolism; Adrenal ultrastructure

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Abbreviations: ACAT, acyl-coenzyme A: cholesterol *O*-acyltransferase; F 12511, (*S*)-2′,3′,5′-trimethyl-4′-hydroxy-α-dodecylthio-phenylacetanilide; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; LDL, low-density lipoprotein; HDL, high-density lipoprotein; SR-BI, scavenger receptor class B, type I; P450scc, cytochrome P450 cholesterol side-chain cleavage; P450c17, cytochrome P450 17 α-hydroxylase; P450c21, cytochrome P450 21-hydroxylase; PMA, phorbol 12-myristate 13-acetate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; cAMP, adenosine 3′5′-cyclic monophosphate; ITS, insulin-transferrin-linoleic acid; and MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

0006-2952/01/$ – see front matter
PII: S0006-2952(00)00555-4

1. Introduction

Dangerous atherosclerotic lesions do not necessarily appear highly stenotic but give rise to occlusive thrombus. Thrombus results from the disruption of the cap of a lipid-rich thin plaque that promotes the contact of blood cells with the thrombogenic core [1,2]. Many current pharmacological strategies are focused on plaque stabilization, aiming at rendering it more fibrous by reducing inflammatory as well as infectious events and by modulating lipid and lipoprotein metabolism [3,4]. The recent data showing that a decrease in cholesteryl esters from the vascular wall reduces matrix metalloproteinase-1 activity and increases collagen content in rabbit atherosclerotic plaques [5,6] elucidate one of the putative mechanisms whereby cholesterol lowering in the arterial wall contributes to stabilization of atheromatous lesions.

ACAT (EC 2.3.1.26) is an intracellular enzyme that catalyzes the formation of cholesteryl esters from cholesterol and fatty acyl-coenzyme A [7]. The ACAT enzyme plays key roles in intracellular cholesterol storage, in liver for lipoprotein assembly, in intestine for dietary cholesterol absorption, in adrenal for steroid hormone production, and in arteries for fatty streak development. Cholesterol esterification also plays a prominent part in cellular protective mechanism(s) against toxicity due to excess of free cholesterol in many tissues [8–10], including adrenocortical cells where cholesterol used as substrate for steroidogenesis is stored as cytosolic cholesteryl esters [11]. Hence, the inhibition of ACAT may represent an attractive mechanism for inducing both hypolipidemic and antiatherosclerotic effects, although the modulation of intracellular pools of free and esterified cholesterol needs to be tightly controlled to avoid major adverse effects. Various ACAT inhibitors [12–14] have been described to cause adrenal toxicity in dogs, rabbits, and guinea pigs, raising the major question as to whether these adrenal side effects are mechanism-based or compound-specific. Thus far, most of the available data concerns PD 132301-2, which was also shown to impair mitochondrial respiration leading to ATP depletion and cytotoxicity in primary cultures of adrenocortical cells isolated from guinea pigs [15]; however, these effects appear to be species-dependent, since the ACAT inhibitor SAH-58.035 modulates lipid homeostasis in primary culture of bovine adrenocortical cells without impairing either cortisol secretion or cellular integrity [16].

To our knowledge, no ACAT inhibitors have been characterized in a cell culture system of human origin relevant for studying both cholesterol metabolism and steroidogenesis. A potentially suitable model may be the NCI-H295R cell line, which was adapted [17] from the NCI-H295 pluripotent adrenocortical carcinoma cell line established by Gazdar *et al*. [18]; in addition to fulfilling the above-mentioned criteria, it has functional properties that in many respects are similar to those of normal human adrenal cells. The aims of the present study were: (i) to address the activity of F 12511, a powerful ACAT inhibitor [19], on cholesterol metabolism; (ii) to evaluate its potential effects on both steroidogenic enzyme genes and lipoprotein receptor genes; and (iii) to determine whether steroid production can be affected by depletion of cholesteryl ester pools in cultured human NCI-H295R cells. Because of the high efficacy of F 12511 at inhibiting cholesterol esterification, this study should contribute to explore the consequences of ACAT inhibition for human adrenal physiology and provide insight into the putative adrenal tolerance, at least *in vitro*, of this particular ACAT inhibitor.

2. Materials and methods

2.1. Materials

[1-^{14}C]Oleic acid sodium salt, [1-^{14}C]acetic acid sodium salt, and [α-^{32}P]dCTP were obtained from DuPont NEN. [^{3}H]Cholesterol was from Amersham. DMEM and Ham's F-12 medium (DMEM–F12 1:1 mixture containing HEPES 15 mM, pyridoxine–HCl), insulin (10 μg/mL)-transferrin (5.5 μg/mL)-selenium (5 ng/mL)-linoleic acid (4.7 μg/mL) (ITS+), PBS, trypsin–EDTA, gentamicin, L-glutamine, amphothericin B, BSA, and FBS were purchased from GIBCO BRL Life Technologies. DMSO, MTT tetrazolium salt, PMA, forskolin, and aminogluthetimide were purchased from Sigma-Aldrich. Taxol was from Alexis Corporation; Ultroser SF was obtained from Biosepra. All plasticware was provided by Nunc and Falcon, and all chemicals were of analytical grade quality. The analysis of steroid hormones (cortisol, aldosterone, and androstenedione) was measured in cell culture media with radioimmunoassays (CORT-CT2, CIS Bio International; Coat-a Count®-Aldosterone, Behring Diagnostic; Δ4-Androstenedione, Immunotech).

The synthesis of F 12511 was performed by a medicinal chemistry division of the Pierre Fabre Research Center; it was dissolved in DMSO and successive dilutions were made to obtain 0.1% DMSO final concentration; concentrations were expressed in molarities (M).

2.2. Cell culture

Human adrenocortical carcinoma NCI-H295R cells (2128-CRL) were obtained from ATCC and were propagated routinely in DMEM–F12 containing ITS+, hydrocortisone and 17-β estradiol (both at 10^{-8} M), 4 mM L-glutamine, 50 μg/mL gentamicin, 2.5 μg/mL amphothericin B, and 2% Ultroser SF or 10% FBS in a humidified incubator at 37°; medium was changed every 2 days and cells were subcultured once a week using trypsin–EDTA (0.5%–0.2%) in a split ratio 1:3. For experiments concerning lipid metabolism, cells were seeded in 24-well multiwell plates in medium containing ultroser SF until they became subconfluent 4 to 5 days later. For RNA analysis, cells were incubated for 24 hr before the start of the experiment in

medium with 10% FBS but without hydroxycortisone, 17-β estradiol, and ITS and were subsequently treated for 24 hr with the indicated concentrations of F 12511 and forskolin.

2.3. Cholesterol metabolism

Cholesterol esterification was performed as previously described [20]. Medium was changed just before the start of the incubation of NCI-H295R cells with increasing concentrations of F 12511 (10^{-8}–10^{-6} M) or its vehicle for 4, 16, 48, and 72 hr at 37° prior to the addition of [1-^{14}C]oleic acid (1 μCi/mL) complexed to BSA (0.2 mg/mL final concentration) for a further 2 hr. Then, cells were washed with PBS, and the intracellular lipids were extracted by hexane: isopropanol (3:2) for 1 hr, dried under a nitrogen flux, and dissolved in petroleum ether:diethyl ether (98:2). Cholesteryl oleate was separated by chromatography on silica gel column [21] and counted by liquid scintillation. All incubations with cultured cells and blank assays were performed in duplicate. By comparing the dpm of cholesteryl oleate formed in treated cells with those in control cells, the activity of F 12511 (% inhibition) was calculated. Mean IC_{50} values (concentration of drug required to inhibit cholesteryl oleate formation by 50%) were further calculated.

Cholesterol synthesis was performed as previously described [22], with slight modifications. Subconfluent cells were cultured in Ultroser SF-free medium containing BSA 0.01% for the final 24 hr before the start of incubation with F 12511 (or its vehicle) for 1 hr at 37° prior to the addition of [1-^{14}C]acetate. Four hours later, cells were washed with cold PBS and lysed in 15% aqueous KOH; lipids were saponified for 1 hr at 75° in 30% KOH in methanol 40%. Then, the non-saponifiable lipids were extracted with petroleum ether, dried under a nitrogen flux, and dissolved into isopropyl alcohol before being separated by TLC in a solvent system containing hexane:diethyl ether:acetic acid (85: 15:4). Radioactive metabolites were quantified with a linear analyzer.

For measurement of intracellular cholesterol mass, medium was changed just before the start of the incubation of NCI-H295R cells with human LDL or HDL for 24 hr, then increasing concentrations of F 12511 (10^{-9}–10^{-6} M) or its vehicle were added for another 24 hr. At the end of incubation, cells were washed with PBS, lysed with aqueous KOH (15%). The intracellular lipids were extracted by chloroform:methanol (1:2) and the chloroform layer dried under a nitrogen stream. The dried extracts were dissolved in hexane and analyzed by HPLC in a Zorbax SIL column (4.6 cm $\times$ 25 cm, Interchim) following an isocratic elution with hexane:isopropanol-2 (99:1, v/v). The absorbance was monitored at 210 nm and free and esterified cholesterol were quantified by computer integration (System Gold Nouveau, Beckman Instruments Inc.).

2.4. Cell RNA extraction and analysis

Total RNA was prepared by the acid guanidinium thiocyanate/phenol/chloroform method [23,24]. Northern blot hybridization of total cellular RNA was performed as described previously [25] using human LDL receptor [26], CLA-1 (human homologue of SR-BI) [27], human P450scc [28], P450c17 [29], and P450c21 [30] cDNA clones; a GAPDH probe was used as control. All cDNA probes were labeled by random-primed labeling. Filters were hybridized to 1×10^6 cpm/mL of each probe as described [25]. They were washed twice in 0.5 X NaCl/Cit and 0.1% SDS for 5 min at 42° and twice for 30 min at 65° and subsequently exposed to x-ray film (BioMax MS, Kodak).

2.5. Production of steroid hormones

Confluent NCI-H295R cells were rinsed, placed in Ultroser SF-free medium (without hydrocortisone and 17-β estradiol) containing BSA 0.01%, and treated with F 12511 (10^{-8}–3×10^{-6} M) or vehicle in the absence or presence of forskolin (10^{-5} M) or PMA (30 ng/mL) for up to 48 hr. The cortisol, aldosterone, and androstenedione contents of the culture medium were determined by specific radioimmunoassays (cross-reactivity of antiserum for cortisol measurement versus aldosterone and androstenedione: less than 0.1%; of antiserum for aldosterone measurement versus cortisol and androstenedione: less than 0.06%; of antiserum for androstenedione measurement versus cortisol and aldosterone: less than 0.04%) when the cell viability was not affected by more than 30% following drug addition (MTT assay), thus validating the notion that any major modification of steroid synthesis did not result from a significant loss of viability. The effects of F 12511 were expressed as % variation versus vehicle-treated cells under basal conditions or as % variation of the stimulation evoked by either forskolin or PMA. Both intra- and interassay coefficients of variation for steroid measurements were not larger than 10%; cell protein content was subsequently determined.

2.6. Lipoprotein isolation and labeling

LDL (1.030 g/mL $<$ d $<$ 1.053 g/mL) and free of apo E HDL fraction 3 (HDL_3, 1.12 g/mL $<$ d $<$ 1.21 g/mL) were prepared from human plasma by sequential ultracentrifugation [31]. The major proteins in HDL_3 were apo AI and apo AII, whereas apo E was undetectable. HDL_3 and LDL apolipoproteins were radiolabeled with [^{125}I]iodine as described by Bilheimer *et al.* [32]. The specific radioactivity usually obtained was 300–600 cpm/ng of protein. HDL_3 and LDL were also labeled using [^{3}H]cholesterol by incubation overnight at 37° with a plasma fraction enriched with lecithin cholesterol acyltransferase (d $>$ 1.21 g/mL) [33]. Labeled LDL and HDL_3 were re-isolated at density 1.063 and 1.21 g/mL, respectively. The specific radioactivity usually obtained was approximately 28 dpm/ng of cholesterol.

Table 1
Cholesteryl oleate formation and inhibitory potencies (IC_{50}) of F 12511 in NCI-H295R cells: effect of time of incubation

	4 hours	16 hours	48 hours	72 hours
Specific activity	1013 ± 267	379 ± 69	250 ± 27	190 ± 25
IC_{50}, nM	49 ± 18	18 ± 4	24 ± 2	32 ± 5

Cholesteryl oleate formation. Cells in the presence of DMSO 0.1% were cultured for the indicated period of time in the presence of medium containing lipid-free serum substitute before the addition of radiolabeled oleic acid–BSA complex, as indicated in Materials and Methods. After a 2-hr incubation period, cholesteryl oleate was measured (Specific activity: pmol/hr/mg cell protein). Results are expressed as means ± SEM (N = 7 for 4 hr, N = 6 for all other times, each point performed in duplicate and obtained from separate experiments). Inhibitory potencies. Cells in the presence of F 12511 were cultured for the indicated period of time in medium containing lipid-free serum substitute before the addition of radiolabeled oleic–acid-BSA complex, as indicated in Materials and Methods. After a 2-hr incubation period, cholesteryl oleate was measured. Percent inhibition and IC_{50} values (expressed as means ± standard deviation) were calculated from 6 separate experiments for each time.

The [^{3}H]cholesterol esterification was controlled by TLC and was always more than 95%.

2.7. Binding of ^{125}I-labeled lipoproteins to NCI-H295R cells

NCI-H295R cells were cultured in 12-well dishes. Twenty-four hours before analysis, cells were changed to medium containing Ultroser 2% (without hormones) and incubated with or without the appropriate concentrations of forskolin and/or ACAT inhibitor. To determine the specific binding of ^{125}I-HDL_3 to cells, the 12-well plates were washed with serum-free culture medium and incubated for 1 hr at 37°. Cells were then incubated for 2 hr at 4° with serum-free medium containing 100 μg/mL of ^{125}I-HDL_3 or ^{125}I-LDL with or without a 50-fold excess of unlabeled HDL_3 or LDL. Dishes were washed twice with ice-cold 2% (w/v) albumin–Tris buffer (10 mM Tris/HCl, pH 7.4, containing 0.150 M NaCl) and twice again with cold Tris buffer. Cells were then solubilized in 0.1 M NaOH and the radioactivity determined. Specific-bound ^{125}I-lipoprotein is calculated by subtracting the binding of ^{125}I-lipoprotein in the absence and presence of an excess of unlabeled lipoprotein. Results are the means of triplicate assays.

2.8. Selective uptake of cholesteryl esters by NCI-H295R cells

NCI-H295R cells were cultured in 12-well dishes. Twenty-four hours before analysis, cells were changed to medium with Ultroser 2% (without hormones) and incubated with or without the appropriate concentrations of forskolin and/or ACAT inhibitor. The cells were washed with serum-free culture medium and incubated for 1 hr at 37°. Cells were then incubated for 5 hr at 37° with serum-free medium containing 100 μg/mL protein of [^{3}H]cholesteryl ester-labeled HDL_3 or 15 μg/mL protein of [^{3}H]cholesteryl ester-labeled LDL. The non-specific uptake obtained with a 50-fold excess of unlabeled lipoprotein was <20%. Cells were washed and then solubilized in 0.1 M NaOH before the radioactivity of [^{3}H]cholesteryl esters incorporated into cells was determined. Results are the means of triplicate assays.

2.9. Determination of cell viability

In another set of experiments, cells in exponential phase were grown in the presence of drugs or vehicle for up to 96 hr. Cell viability was evaluated by the MTT test: the tetrazolium salt MTT (0.5 mg/mL) was reduced for 4 hr to blue MTT formazan by mitochondrial succinate dehydrogenases from living cells. MTT formazan was further measured by colorimetry after solubilization overnight in SDS (10% in HCl 0.01 M).

2.10. Electron microscopy

NCI-H295R cells were cultured in 12-well dishes in FBS in the presence or absence of F 12511 (10^{-7}, 10^{-6}, and 3×10^{-6} M) for 24 or 48 hr. At the end of incubation, cells were washed with PBS and fixed in pellet in 2% glutaraldehyde in a cacodylate buffer at 4°. For *in situ* observation following perpendicular sections to the monolayer culture cells, NCI-H295R cells grown on collagen-coated Millicell CM inserts were fixed for conventional electron microscopy. Aldehyde fixation was followed by osmium tetroxide post-fixation and cells were treated with a 0.5% aqueous solution of uranyl acetate. After dehydration in ethanol, the cells were embedded in epon. Ultrathin sections contrasted with lead hydroxide were examined with a Philips EM420 electron microscope.

3. Results

3.1. Effects of F 12511 on lipid metabolism

The rate of [1-^{14}C]oleic acid incorporation by NCI-H295R cells treated by DMSO 0.1% for 4 hr corresponded to the formation of 1013 ± 267 pmol cholesteryl oleate/hr/mg protein. The decrease in ACAT-specific activity in confluent NCI-H295R cells treated by DMSO 0.1% for up to 72 hr (Table 1) was likely related to the time-dependent depletion of ACAT substrate(s), since these cells were cultured in lipid-free serum substitute (Ultroser SF). Following a 4-hr incubation period, F 12511 inhibited the incorpora-

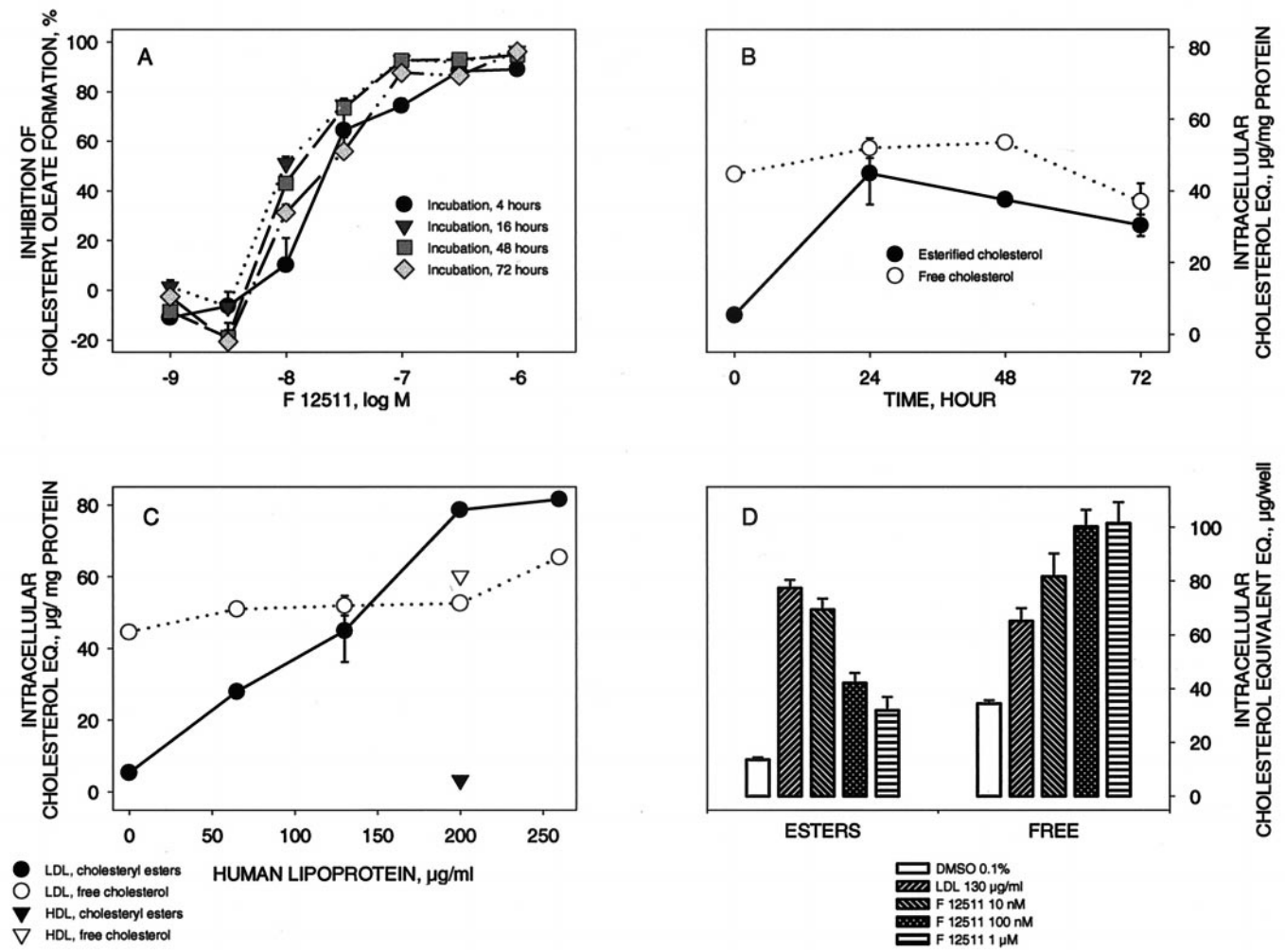

Fig. 1. Pharmacological characterization of the lipid-modulating effects of F 12511 in cultured NCI-H295R cells. (A) Effect of F 12511 on incorporation of the [^{3}H]oleic acid–BSA complex into cholesteryl oleate in NCI-H295R cells: influence of the duration of the incubation time. Each point represents the mean ± SEM of duplicate determinations from 3 separate experiments. (B) Effect of human LDL (130 μg/mL) loading on intracellular cholesterol stores from NCI-H295R cells: time–course study. Chloroform–methanol extracts were analyzed for cholesterol mass by HPLC. Each point represents the mean ± SEM from 3 separate experiments. (C) Effects of the addition of human lipoproteins to NCI-H295R cells on their intracellular cholesterol stores: concentration–response, LDL and HDL were added to cells for 24 hr; chloroform–methanol extracts were analyzed for cholesterol mass by HPLC. (D) Effect of F 12511 on intracellular cholesterol in cultured cells. NCI-H295R cells were enriched in free and esterified cholesterol for 48 hr with LDL (130 μg/mL) and treated with F 12511 (10^{-8}–10^{-6} M) for the last 24 hr. Each point represents the average cholesterol mass ± SEM from 3 separate experiments.

tion of radiolabeled oleic acid into cholesteryl oleate in a concentration-dependent manner (Fig. 1) with an IC_{50} of 49 nM; the maximal inhibition (90–95%) was reached at 10^{-7} M. Whatever the duration of the incubation, F 12511 suppressed the formation of cholesteryl oleate, and no significant modification (ANOVA, one-way) of either its potency (Table 1) or its maximal inhibition of cholesteryl oleate production was obtained (Fig. 1A). In order to check the putative cytotoxic effects of the ACAT inhibitor in these rather long-term experiments carried out in the absence of extracellular acceptors of free cholesterol, cell viability was evaluated in parallel by the MTT assay; concentrations of F 12511 close to the IC_{50} values (10^{-8} M) as well as those eliciting submaximal pharmacological effects (10^{-6} M) were selected. The marginal decrease (at most 10%) in the MTT index for the very high concentrations tested and for the longest incubation period (72 hr, data not shown) could not account for the pronounced inhibition of ACAT activity evoked by F 12511 in human adrenocortical NCI-H295R cells. As assessed by radiolabeled acetate incorporation into non-saponifiable intracellular lipids, F 12511 did not significantly modify the rate cholesteryl synthesis (-3 ± 7% and $+28 \pm 8$% at 10^{-7} and 10^{-5} M, respectively), despite an upward trend observed at the highest concentration.

Treatment of NCI-H295R cells with human LDL resulted in a time- and concentration-dependent increase in intracellular esterified cholesterol without any significant modifications of free cholesterol content (Fig. 1, B and C), thus suggesting that intracellular free cholesterol pools were tightly controlled even during a massive extracellular uptake of lipoproteins. The elevation of the intracellular cholesteryl ester level was likely related to the activation of

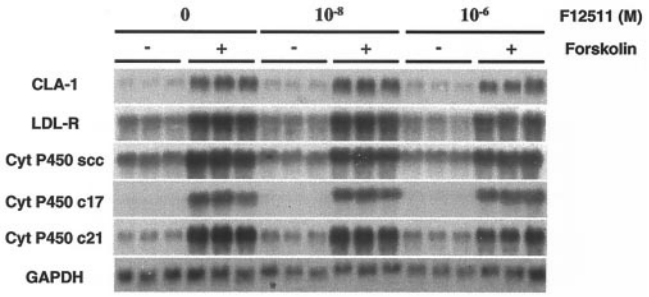

Fig. 2. Effect of ACAT inhibitor F 12511 on expression and regulation by forskolin of the SR-BI, LDL receptor, Cyt P450scc, Cyt P450c17, and Cyt P450c21 genes. Cells were incubated for 24 hr in FBS 10% with increasing concentrations of F 12511 and with or without forskolin 50 μM. RNA was extracted and SR-BI, LDL receptor, Cyt P450scc, Cyt P450c17, Cyt P450c21, and GAPDH mRNA levels were measured by Northern blot analysis. This experiment was performed in triplicate.

ACAT by free cholesterol, one of its substrates; in the absence of LDL, the low intracellular content of esterified cholesterol was due to propagating cells in a lipid-free medium. The addition of human HDL at the same protein concentration as LDL did not significantly affect the intracellular mass of cholesterol. Following intracellular cholesterol loading via the LDL receptor pathway for 24 hr, a treatment with F 12511 for a further 24 hr evoked a concentration-dependent decrease in cholesteryl ester mass associated with an increase in free cholesterol (Fig. 1D), due to the inhibition of ACAT. Hence, the pharmacological effects of F 12511 were not affected in cells previously loaded by LDL cholesterol, as confirmed by its potency for inhibiting oleic acid incorporation into cholesteryl oleate (IC_{50} = 22 nM).

In this human adrenocortical cell line, free cholesterol increased in the absence of extracellular acceptor of cholesterol and in relation to ACAT inhibition; the ability of these cells to manage free cholesterol, given that it appeared to be finely regulated even during cholesterol loading and that free cholesterol may be toxic for cells, remained a crucial issue. Since cholesterol is the precursor of steroid hormones, its influence on basal and stimulated steroid secretion as well as on the regulation of the rate-limiting steps of these metabolic pathways, in the absence or presence of F 12511, was addressed.

3.2. Effect of F 12511 on the expression and regulation of steroidogenic enzyme genes and lipoprotein receptor genes

To determine whether steroidogenic enzyme and lipoprotein receptor gene expression and regulation were modified by treatment with F 12511, cells were treated for 24 hr with 10^{-8} and 10^{-6} M of F 12511 or vehicle and stimulated with 50 μM of forskolin. This effect was tested in 10% FBS medium (Fig. 2) and in free cholesterol medium (containing Ultroser SF) in the absence of or after supplementation with purified LDL or HDL (not shown). Stimulation was higher in FBS than in lipid-free medium, but under these two conditions F 12511 did not impair the expression level or cAMP-dependent regulation of the SR-BI, LDL-R, P450scc, P450c17, and P450c21 genes. Forskolin stimulated the expression of these different genes (Fig. 2), with a smaller effect at the highest concentration of F 12511 (10^{-6} M), but this stimulation was not significantly different when corrected for the GAPDH expression level.

3.3. Effects of F 12511 on LDL- and HDL-specific binding and uptake of cholesteryl esters in lipid-free medium

Since LDL receptor and SR-BI gene expression and regulation by forskolin were not modified by F 12511, the effect of F 12511 on the binding of LDL and HDL to NCI-H295R cells was addressed. Cells cultured in lipid-free medium (complemented with Ultroser SF) were treated with 10^{-6} M of F 12511 or vehicle and with 50 μM of forskolin. Then, cells were incubated for 2 hr at 4° with 100 μg/mL of ^{125}I-LDL or ^{125}I-HDL. cAMP signaling pathway stimulation by forskolin increased LDL and HDL binding to cells by 190 ± 11 and 151 ± 13%, respectively, compared to control cells (N = 2, in triplicate); both under basal and forskolin-stimulated conditions, F 12511 had no effect on the binding of these lipoproteins to cells. Similarly, forskolin-stimulation of [^{3}H]cholesteryl ester uptake from LDL or HDL was 133 ± 14 and 140 ± 25%, respectively (N = 2, in triplicate), with no effect of F 12511 on basal or stimulated uptake, suggesting that ACAT inhibition interferes neither with the binding nor the uptake of lipoproteins in NCI-H295R cells.

3.4. Effects of F 12511 on the synthesis of steroid hormones

In lipid-free medium, NCI-H295R cells secreted cortisol, aldosterone, and androstenedione both under basal and stimulated (forskolin 10 μM for cortisol and androstenedione, PMA 30 ng/mL for aldosterone) conditions after a treatment for 48 hr (Table 2). The production of cortisol increased in a time-dependent manner (Fig. 3), and this was also the case for aldosterone and androstenedione (data not shown), since substrate availability was thought to be the rate-limiting step for steroidogenesis under these experimental conditions. The level of cortisol produced by NCI-H295R cells was about 1000-fold that of aldosterone; forskolin (10 μM) and PMA (30 ng/mL) elicited a 3- to 4-fold increase in cortisol and aldosterone, respectively. Regarding androstenedione, the stimulation evoked by forskolin (10 μM) was inferior (1- to 2-fold increase); this might be related either to the low specificity of the agonist or to the assessment of the C19 androgen pathway located downstream of the steroidogenic metabolism when compared to glucocorticoid or mineralocorticoid pathways, or both. The

Table 2
Basal and stimulated production of cortisol, aldosterone, and androstenedione by cultured NCI-H295R cells

	Cortisol (ng/mg protein)	Aldosterone (pg/mg protein)	Androstenedione (ng/mg protein)
Basal	696 ± 95(N = 11)	321 ± 33(N = 4)	400 ± 43(N = 4)
Stimulated	2254 ± 322(N = 11)	1433 ± 88(N = 4)	659 ± 116(N = 4)

Cortisol and androstenedione syntheses were stimulated by forskolin (10 μM) for 48 hr, and aldosterone by PMA (30 ng/mL) for 48 hr as described in Materials and Methods. Results are expressed as means ± SEM.

addition of human LDL during the treatment of the cells by forskolin or PMA or vehicle for 48 hr induced an increase of cortisol and aldosterone secretion (50–70% and 250–300% vs control, respectively) both under basal and stimulated conditions. By contrast, in lipid-free medium, aminogluthetimide (10^{-7}–10^{-4} M), known to be an inhibitor of both cytochrome P450scc (the first rate-limiting enzyme of steroidogenesis) as well as aromatases (playing a role downstream) concentration-dependently reduced and even suppressed the synthesis of all steroids under both stimulated and basal situations (Fig. 4).

The ACAT inhibitor F 12511, at concentrations ranging from 10^{-8} M (threshold for inhibiting ACAT enzyme) up to 3×10^{-6} M (30-fold greater than concentrations evoking submaximal inhibition of ACAT activity), did not decrease but rather increased cortisol production at each point of the time–course study, whatever the conditions (forskolin- or vehicle-treated cells) (Figs. 3 and 5). Neither aldosterone nor androstenedione production was impaired after a treatment with F 12511 for 48 hr at any concentration tested under basal conditions; since the efficiency of forskolin (10 μM) in stimulating the synthesis of androstenedione was rather weak (1.5-fold increase) and substantial variations in the response were observed, the reduction caused by F 12511 (10^{-6} M) did not appear to be dramatic. Combined treatments with F 12511 and FBS resulted in a comparable pharmacological profile on steroid production as in the absence of an exogenous supply of cholesterol, thus demonstrating that modifying intracellular pools of cholesterol

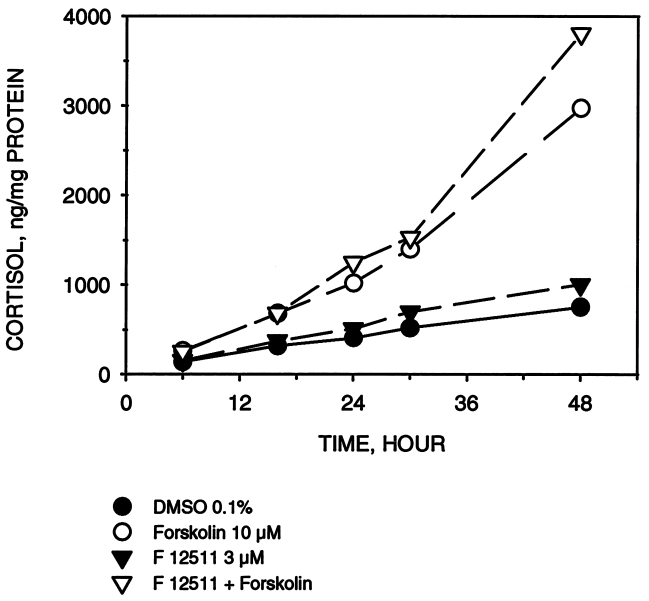

Fig. 3. Effects of forskolin (10 μM) and F 12511 (3 μM) on secretion of cortisol by cells: kinetic experiment. Each point represents the mean ± SEM from at least 3 separate experiments.

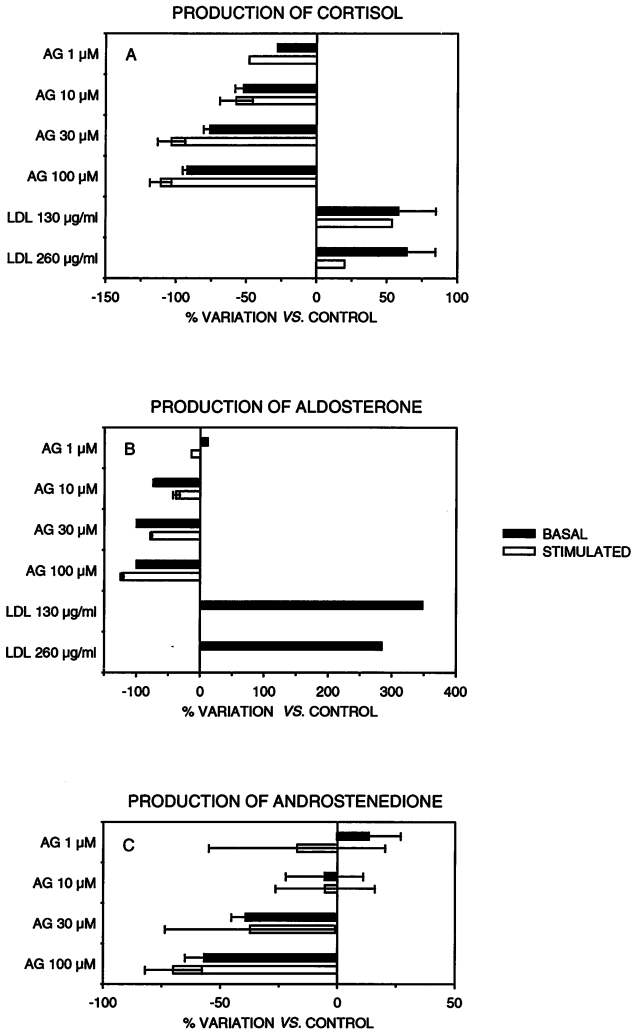

Fig. 4. Basal and stimulated production of cortisol (A), aldosterone (B), and androstenedione (C) by cultured NCI-H295R cells following a 48-hr incubation period. The effects of human LDL (130–260 μg/mL), and aminogluthetimide (10^{-7}–10^{-4} M) were evaluated; each point represents the mean ± SEM from 3 separate experiments.

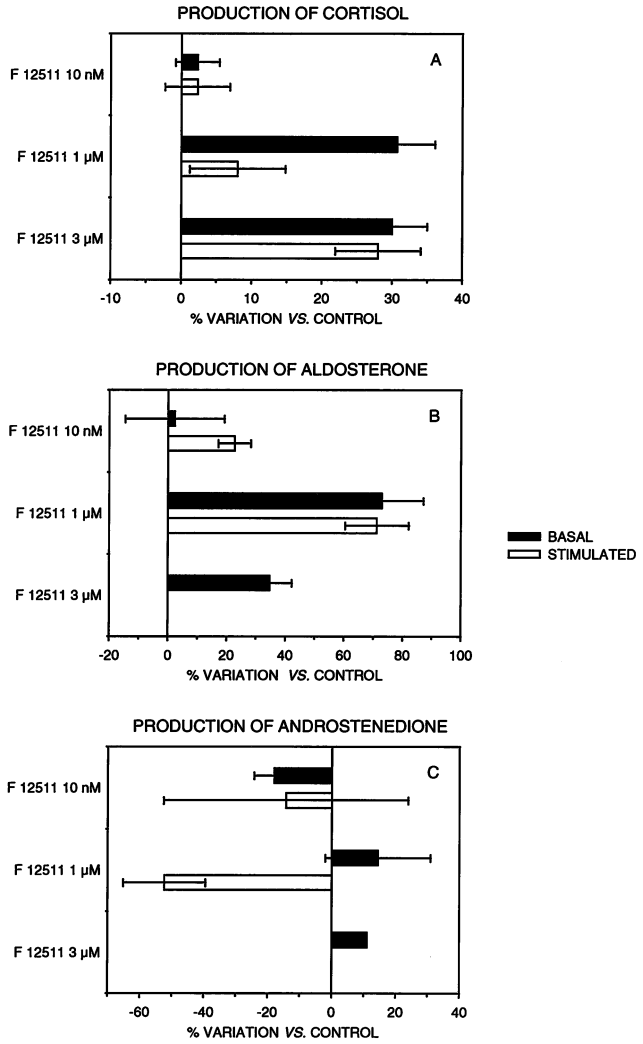

Fig. 5. Effects of F 12511 (10^{-8}–3×10^{-6} M) on basal and stimulated production of cortisol, aldosterone, and androstenedione by cultured NCI-H295R cells after a 48-hr incubation period. Each point represents the mean ± SEM from 3 separate experiments.

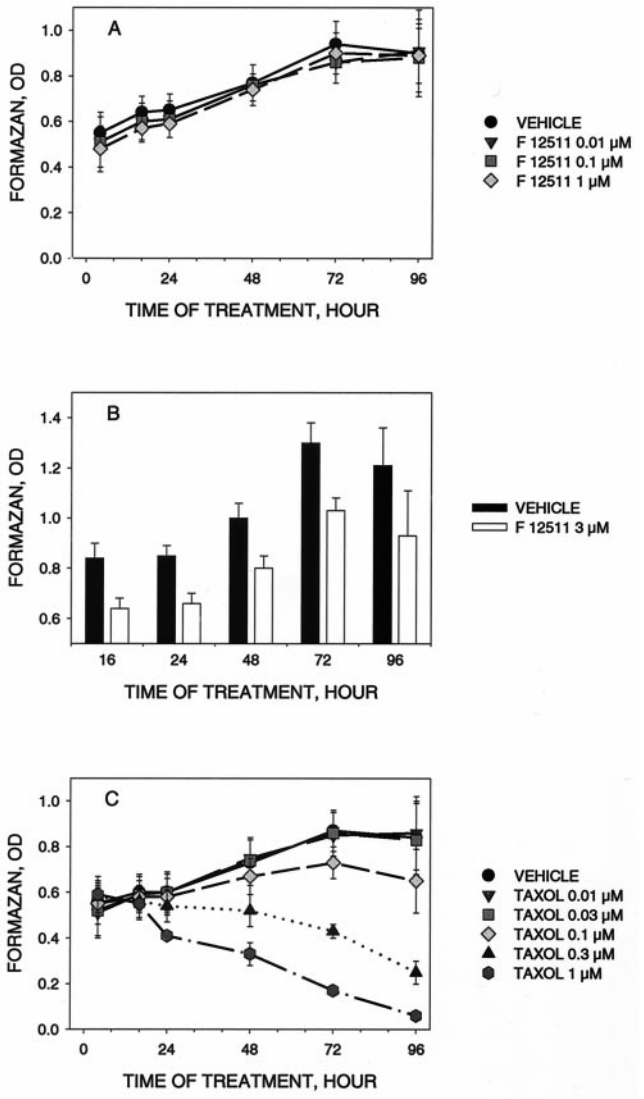

Fig. 6. Direct effects of F 12511 (10^{-8}–3×10^{-6} M) and Taxol (10^{-8}–3×10^{-6} M) on cultured NCI-H295R cell viability after a 96-hr incubation period. Cells in exponential growth were treated once with compounds in Ultroser SF-free and hormone-free medium. Cell viability was addressed by the MTT assay, and each point represents the mean ± SEM from 3 separate measurements of formazan.

with this ACAT inhibitor did not blunt free cholesterol availability from either *de novo* synthesis or cholesteryl ester hydrolysis for steroidogenesis.

3.5. Effects of F 12511 on cell viability

All experiments were performed in exponentially growing cells seeded in the presence of Ultroser SF 2% but cultured after 3 days in Ultroser SF-free, hormone-free medium; cells were incubated in the absence or presence of various concentrations of F 12511 and Taxol. At any point of the time–course study, F 12511 up to 10^{-6} M did not impair the proliferation of NCI-H295R (Fig. 6A), whereas at 3×10^{-6} M F 12511 slightly delayed proliferation without causing cell death (Fig. 6B); by contrast, Taxol promoted time- and concentration-dependent cytotoxic effects on NCI-H295R cells (Fig. 6C).

3.6. Effects of F 12511 on NCI-H295R cells

The effects of F 12511 (from 10^{-7} to 3×10^{-6} M) on NCI-H295R ultrastructure were evaluated for 24 or 48 hr in comparison to cells treated by the vehicle (DMSO 0.1%). No alteration was observed in DMSO-treated cells (Fig. 7A). Electron microscopic observations from *in situ* sections confirmed the epithelioid character of the cultured cells. At the sites of close contact of the bottom cell membrane with the collagen substrate, typical dense plaques of fibrillar material were present. Their basal location and their orientation suggested a certain degree of polarity. Mi-

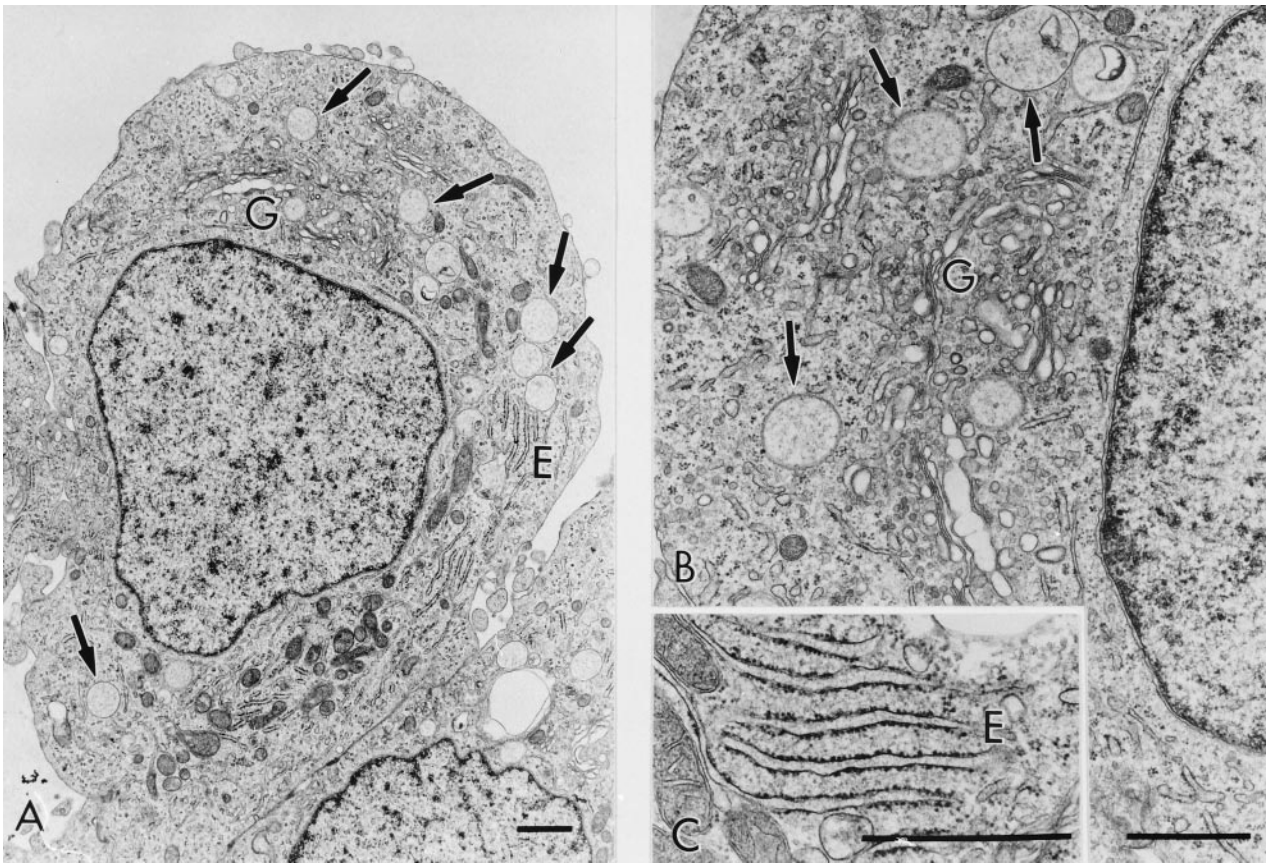

Fig. 7. Ultrastructural aspect of a typical NCI-H295R cell (A) and one incubated with F 12511 ACAT inhibitor (B and C). The control cell (A) remained epithelioid with a large nucleus. The cytoplasm contained large numbers of mitochondria, a moderate rough endoplasmic reticulum (E), and a prominent Golgi apparatus (G) with vesicles and granules (arrows), features typical of secretory activity. No modification of the ultrastructural characteristics of NCI-H295R cells was observed following incubation for 24 hr with F 12511 (10^{-6} M) (B and C). The cytoplasm contained a prominent Golgi apparatus (G) with vesicles and granules (arrows) (B) and a typical rough endoplasmic reticulum (C), features typical of secretory activity. Bars = 1 μm.

crovilli, which are characteristic of cultured epithelioid cells with large nuclei, were quite prominent. The cells revealed a complex pattern of cytoplasmic interdigitations with a certain loss of cell contact inhibition. Cytoplasmic extensions frequently enclosed a space with the adjacent cells. The cytoplasm contained large numbers of mitochondria, a moderate rough endoplasmic reticulum, and a prominent Golgi apparatus with vesicles and granules, features typical of secretory activity. After cell trypsin detachment and fixation in pellet, the cultured human adrenocortical carcinoma cells, even from different passages over a three-month period, clearly contained many of the same intracellular features noted above (Fig. 7A). F 12511, whatever the concentration tested, did not induce any significant change in morphological and ultrastructural characteristics of cells observed 24 and 48 hr after incubation. The cytoplasm contained large numbers of mitochondria with intact crystae and the same typical features of secretory activity observed in the non-treated cells (Fig. 7, B and C). Hence, the ACAT inhibitor F 12511 had no influence either on cellular morphology or on ultrastructural characteristics of cell components of the human adrenal cell line NCI-H295R.

4. Discussion

This report confirms earlier work demonstrating the potency of the novel lipid regulator F 12511 in inhibiting ACAT activity in various cultured human cell lines [23] with IC_{50} values in the 10-nM range. F 12511 suppresses the rate of cholesterol esterification with submaximal inhibition at 10^{-7} M. These data establish the efficacy of F 12511 or its metabolites, if any, under conditions of long-term exposure in the NCI-H295R human adrenocortical cell line, resulting in a decrease in esterified cholesterol and an increase in intracellular free cholesterol mass.

One of the key points for the development of ACAT inhibitors are the possible consequences that modifications of intracellular lipid homeostasis may have on the physiology of target tissues, among them steroidogenic organs such as the adrenals. To date, various studies have demonstrated species-dependent differences in the adrenotoxic effects of ACAT inhibitors: adrenal glands from guinea pigs, rabbits, and dogs are sensitive to this pharmacological class and/or to compounds that deplete their cholesterol pools, whereas mice, rats, and monkeys appear to be adreno-resistant to

ACAT inhibitors. Moreover, whether this adrenotoxicity of ACAT inhibitors is related to their mechanism(s) of action or to some specific pharmacophores remains to be established. The study by Meiner *et al*. [34] demonstrates that the disruption of the ACAT gene in mouse induces a severe depletion of esterified cholesterol in $Acact^{-/-}$ adrenocortical cells without any significant modification of steroidogenesis in response to an acute adrenocorticotrophic hormone (ACTH) challenge. These data are in stark contrast to those obtained in apo AI-deficient mice [35] in which steroid hormone production is blunted in response to different degrees of stress or stimulation, suggesting that provision of cholesterol may be more critical for steroidogenesis than cytosolic cholesteryl ester pools. Moreover, caution should be exercised in extrapolating an interpretation from one species (mouse) to another (e.g. rabbit, human) because of the well-known differences in both lipid metabolism and adrenal sensitivity. Beyond *de novo* cholesterol synthesis, a selective pathway for cellular uptake of lipoprotein-derived cholesterol is able to efficiently supply cells with substrate for hormone or product synthesis [36]; the scavenger receptor SR-BI, described as the HDL receptor, and the classical endocytic B/E LDL receptor pathway are among the factors that may contribute to lipid uptake. Although apo AI levels and SR-BI functionality are crucial for cholesteryl ester delivery and adrenal physiology in mouse models [35,37], this may not be the case in other species (e.g. humans) in which plasma LDL levels are higher than those of HDL and where LDL receptors may also provide substantial amounts of cholesterol to the adrenal gland [38].

In human adrenocortical carcinoma NCI-H295R cells, both SR-BI and LDL receptor gene expression are induced by activators of the cAMP pathway; this induction of SR-BI and LDL receptor mRNA parallels the stimulation of most steroidogenic enzymes such as P450scc, P450c17, and P450c21. Hence, NCI-H295R cells meet their increased need of cholesterol for steroidogenesis by up-regulating both the HDL and LDL cholesterol uptake pathways. At concentrations up to 30-fold larger than those eliciting maximal inhibition of ACAT, F 12511 impairs neither the regulation of genes involved in steroidogenesis and lipoprotein uptake pathways nor lipoprotein binding and uptake. These results are in accordance with the lack of alteration of steroid hormone production by F 12511 and parallel those obtained with another ACAT inhibitor (SAH-58.035) in bovine adrenocortical cells [16]. The synthesis of cortisol and aldosterone by NCI-H295R cells is dependent on intracellular cholesterol provision because LDL uptake increases cholesteryl ester mass as well as cortisol and aldosterone release. Despite the presence of SR-BI, the cellular uptake of HDL-derived cholesterol does not appear to be predominant in this human cell model, as compared to mouse adrenocortical cells [36,38]. Since the experiments with F 12511 were carried out in the absence of an exogenous source of cholesterol, basal and stimulated steroid production rely only on *de novo* cholesterol synthesis; F 12511 did not significantly modify the rate of cholesterol synthesis, but minute movements of free and esterified cholesterol might explain the upward trend of steroid production evoked by high concentrations of F 12511. Therefore, even during extreme pharmacological modification of the intracellular lipid balance in response to F 12511, NCI-H295R cells still retained the ability to take up extracellular lipids and adapt their physiological responses.

Whatever the experimental conditions, F 12511 added either to confluent or to proliferating cells did not significantly affect cell proliferation and viability. These data are confirmed by morphological and ultrastructural analyses that demonstrate intact mitochondria and features of secretory activity after a treatment for 48 hr with F 12511 at 3×10^{-6} M.

Even if the experiments reported herein are performed with a permanent cell line that is probably less sensitive to an excess of intracellular free cholesterol than cells in primary culture [8], NCI-H295R cells exert a tight regulation over free cholesterol in response to lipoproteins and F 12511. It is tempting to speculate that this occurs through modulations of the production of steroid hormones. The apparent discrepancy in the cellular viability data obtained in this human adrenal cell line versus guinea pig adrenocortical primary culture in response to PD 132301-2 [15] may illustrate either differences between F 12511 and PD 132301-2 in terms of their putative intrinsic adrenotoxic effects or *in vitro* species differences in adrenal sensitivity as previously demonstrated *in vivo* for ACAT inhibitors. To date, no ACAT inhibitor has been characterized in the NCI-H295R cell model which is relevant for steroid metabolism, as demonstrated by an LDL-evoked increase and aminogluthetimide-mediated decrease in production of steroids, the latter effect being related in most part to cytochrome P450scc inhibition [39]. This model is also pertinent for evaluating the activity of cytotoxic agents such as Taxol [40]. Regarding lipid and lipoprotein metabolism, this human cell line is characterized by a very active classical B/E LDL receptor pathway, which may be reconciled with the amount of plasma LDL in humans; this indicates major differences in adrenal and lipoprotein physiology when compared to cells from mouse or rat [36,41]. Whether the physiological responses of this cell line to F 12511 reflect the human response warrants further investigation; the acute response to a steroidogenic signal that should immediately mobilize free cholesterol substrate for P450scc via steroidogenic acute regulating protein (STAR) was not addressed [42].

The concept of ACAT inhibition for the stabilization of human atherosclerosis has recently been reinforced by both the role of esterified cholesterol content in experimental atheroma [5,6] and the demonstration that the ACAT-1 protein is very abundant in human atherosclerotic plaques, essentially overexpressed in monocyte-derived macrophages [43]. ACAT-1 protein appears to be the enzyme involved in cholesterol metabolism in human hepatocytes,

macrophages, and adrenal glands [44]. Therefore, the possibility of uncoupling hepatic from adrenal effects of an ACAT inhibitor in human appears to be rather complicated, in contrast with the situation in mice [34]. Nevertheless, the present results obtained with the novel potent ACAT inhibitor F 12511 on the human adrenocortical NCI-H295R cell line are in agreement with the lack of adrenotoxicity in $ACAT^{-/-}$ mice despite a marked decrease in cholesteryl ester content. These data underline the redundancy in functional pathways for providing cholesterol for steroidogenesis, allowing cells to respond normally to external stimuli in the presence of F 12511.

Acknowledgment

The authors wish to thank L. Puech, V. Barthès, N. Beneteau, and A. Rouquette for expert technical assistance, and C. Aussenac for help in typing the manuscript.

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