Effects of NTE-122, a Novel Acyl-CoA:Cholesterol Acyltransferase Inhibitor, on Cholesterol Esterification and Secretions of Apolipoprotein B-Containing Lipoprotein and Bile Acids in HepG2

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ABSTRACT—We studied the effect of NTE-122 (trans-1,4-bis[[1-cyclohexyl-3-(4-dimethylamino phenyl) ureido)methyl]cyclohexane), a novel acyl-CoA:cholesterol acyltransferase (ACAT) inhibitor, on intracellular cholesterol esterification and the secretion of apolipoprotein B100 (apoB)-containing lipoprotein and bile acids in the human hepatoma cell line HepG2. NTE-122 markedly inhibited [3H]oleate incorporation into cholesteryl esters in HepG2 cells incubated with 5 μg/ml 25-hydroxycholesterol as a stimulus for ACAT (IC50=6.0 nM). On the other hand, NTE-122 did not affect [3H]oleate incorporation into triglycerides and phospholipids and [14C]acetate incorporation into cholesterol. The stimulation of ACAT by 25-hydroxycholesterol caused significant increases in the secretion of radiolabeled cholesteryl esters, radiolabeled triglycerides and apoB mass. NTE-122 pronouncedly inhibited the secretion of radiolabeled cholesteryl esters in proportion to the inhibition of cellular cholesterol esterification, and it significantly reduced the secretion of radiolabeled triglycerides and apoB mass in HepG2 cells incubated with 25-hydroxycholesterol. Furthermore, NTE-122 increased the secretion of bile acids synthesized from [14C]-cholesterol. These results suggest that NTE-122 is capable of exhibiting anti-hyperlipidemic effects by reducing both the cholesterol content and the amount of secreted very low-density lipoprotein and enhancing the excretion of bile acid from the liver.

Keywords: NTE-122, Acyl-CoA:cholesterol acyltransferase (ACAT) inhibitor, HepG2 cell, Apolipoprotein B-containing lipoprotein secretion, Bile acid secretion

Liver is the major tissue for cholesterol metabolism, including biosynthesis of cholesterol and uptake of exogenous cholesterol in the form of remnant lipoproteins and low-density lipoprotein (LDL) mediated by each receptor. These cholesterol molecules are intracellularly esterified by acyl-CoA:cholesterol acyltransferase (ACAT), an enzyme localized in the rough endoplasmic reticulum (ER) (1). The resultant cholesteryl esters are stored in the hepatocytes or packed into the lipid cores of very low-density lipoprotein (VLDL) particles, secreted into blood and transported to other tissues (2, 3). Secretion of VLDL by the liver is an important determinant of plasma LDL level, which is positively correlated with the risk of developing premature coronary heart disease (4). Nascent VLDL contains triglycerides, cholesterol, cholesteryl esters and phospholipids as lipid components, and apolipoprotein B100 (apoB) as a major structural protein that is synthesized only in the liver (1, 5). Hepatic apoB secretion is regulated by the mechanism that determines whether de novo synthesized apoB is secreted or degraded intracellularly (6, 7). Several studies have suggested that triglycerides, as major core lipids, play a key role in protecting de novo synthesized apoB from intracellular degradation within the ER, thereby facilitating the assembly and secretion of VLDL (1, 6, 8). Moreover, there is also evidence to suggest that the formation of intracellular cholesteryl esters may regulate apoB secretion (9, 10) and the lipid composition of the secreted lipoprotein (11, 12). Davis et al. (11) reported that hepatocytes prepared from hypercholesterolemic diet-fed rats secreted VLDL containing less triglycerides and more cholesteryl esters, compared with hepatocytes prepared
from chow-fed rats. There is evidence to suggest a link between inhibition of liver ACAT activity, depletion of liver cholesteryl esters and inhibition of VLDL secretion. The systemically available ACAT inhibitor CI-976 lowered total cholesterol, apoB and LDL cholesterol in the plasma of hamsters fed a cholesterol-free diet (13). Musanti et al. (14) showed that an ACAT inhibitor, FCE27677, suppressed apoB secretion from HepG2 cells, especially in the presence of 25-hydroxycholesterol that stimulates ACAT activity (1) and enhances the secretion of apoB-containing lipoprotein from HepG2 cells (10). On the contrary, Graham et al. (15) reported that an ACAT inhibitor, CL 277,082, decreased apoB secretion from HepG2 cells, but another ACAT inhibitor, 447C88, did not, thus showing that cholesterol esterification would not be essential for lipoprotein secretion from the cells. It was unknown whether direct inhibition of ACAT results in decreased numbers of secreted apoB-containing lipoproteins, changes of the lipid composition of the lipoproteins, or both.

Cholesterol is also excreted into the bile along with phospholipids and bile acids from the liver. Cholesterol is also used to synthesize bile acids in the liver (16). As bile secretion is the only pathway for extrabody cholesterol excretion, the stimulation of bile acid secretion can contribute to the regression of hypercholesterolemia. For example, Nestel and Billington (17) reported that probucol, a clinically used hypocholesterolemic agent, stimulated bile acid secretion. The synthesis of bile acids is stimulated by the increase of hepatic free cholesterol mass. In fact, ACAT inhibitors also caused the increase in the bile acid synthesis and cholesterol secretion via the bile (18–20).

Recently, we reported a novel ACAT inhibitor, NTE-122 (trans-1,4-bis[1-cyclohexyl-3-(4-dimethylamino phenyl) ureido][methyl]cyclohexane), that can strongly inhibit of ACAT from various sources and causes cholesterol lowering in several animal models (21). In the present study, we investigated the effects of NTE-122 on intracellular cholesterol esterification, the secretion of apoB-containing lipoprotein and the excretion of bile acids by HepG2 cells.

MATERIALS AND METHODS

Materials

NTE-122 and two other ACAT inhibitors, E5324 (n-butyl-N\'-2-[3-(5-ethyl-4-phenyl-1H-imidazol-1-yl)propoxy]-6-methyl[phenyl]urea) (Eisai compound) (22) and CI-976 (2,2-dimethyl-N-(2,4,6-trimethoxyphenyl)-dodecanamide) (Warner-Lambert compound) (13, 19), were synthesized at the Central Research Institute, Nissin Food Products, Co., Ltd., Kusatsu. Probucol was purchased from Sigma (St. Louis, MO, USA). [9,10(n)-3H]Oleic acid (370 GBq/mmol) and [1-14C]acetic acid, sodium salt (2.11 GBq/mmol) were obtained from Amersham Corp. (Little Chalfont, UK). [4-14C]Cholesterol (1.9 GBq/mmol) was obtained from New England Nuclear Corp. (Boston, MA, USA). All other chemicals were of reagent grade.

Cells and culture conditions

HepG2, a human hepatoma cell line, was purchased from Dainippon Pharmaceutical Co., Ltd. (Osaka). HepG2 cells were maintained in Eagle’s medium (MEM) (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS), 50 units/ml penicillin (Gibco), 50 µg/ml streptomycin (Gibco), 1% non-essential amino acids (Gibco) and 1 mM pyruvic acid (Gibco) at 37°C in a humid atmosphere containing 95% air and 5% CO2.

Cellular cholesterol esterification and the biosynthesis of triglycerides and phospholipids

Cells were seeded in a 12-well plate (Falcon, Bedford, MA, USA) at the density of 1×10^6 cells/ml/well and cultured in the medium containing 10% FBS for 2 days and then cultured overnight in the medium containing 5% lipoprotein deficient serum (LPDS) (Biomedical Technologies, Inc., Stoughton, MA, USA). The medium was replaced by 900 µl of the medium containing 5% LPDS and 5 µg/ml 25-hydroxycholesterol as a stimulus of ACAT activity, followed by incubation for 2.5 h. Then 100 µl of various concentrations of each test compound dissolved in medium containing 5% LPDS and 1% DMSO was added to the medium. After incubation for 30 min, 20 µl of [3H]oleate-BSA complex ([3H]oleate: 5 mM, specific radioactivity of 296 MBq/mmol; BSA: 120 mg/ml) was added, followed by incubation for 2 h. The lipids were extracted from the cells with hexane / 2-propanol (3:2, v/v), followed by separation by thin layer chromatography (TLC, silica gel G aluminum sheets; Merck Co., Rahway, NJ, USA) using n-hexane / diethyl ether / acetic acid (80:20:1, v/v/v) as a solvent system. The radioactivity of cholesteryl esters, triglycerides and phospholipids were determined by liquid scintillation counting. The remaining cellular protein was dissolved in 0.1 N NaOH for protein determination.

Cholesterol biosynthesis

Cholesterol biosynthesis in HepG2 cells was measured by the method of Nagata et al. (23) with some modifications. Cells were cultured in the medium containing 10% FBS for 3 days in a 12-well plate and then cultured overnight in the medium containing 5% LPDS. The medium was replaced by the medium containing 5% LPDS and various concentrations of test compound. After incuba-
tion for 30 min, 20 μl of [14C]acetic acid ([14C]acetic acid, sodium salt: 50 mM, specific radioactivity of 263.75 MBq/mmol) was added to the medium, followed by incubation for 2 hr. The lipid and the cellular protein were assayed as described above.

**The secretion of cholesteryl ester, triglycerides and apoB**

Cells were cultured in the medium containing 10% FBS for 4 days in a 12-well plate. The medium was replaced by 900 μl of the medium containing 1.5% BSA and 5 μg/ml of 25-hydroxycholesterol, followed by incubation for 2.5 hr. One hundred microliters of various concentrations of each test compound dissolved in medium containing 1.5% BSA and 1% DMSO was added to the culture medium. After incubation for 30 min, 20 μl of [3H]oleate-BSA complex ([3H]oleate: 5 mM, specific radioactivity of 29.6 or 296 MBq/mmol; BSA: 120 mg/ml) was added, followed by incubation for 18 hr. The lipids were extracted from the medium and the cells by the method of Bligh and Dyer (24) and the method described above, respectively. Radiolabeled cholesteryl esters and triglycerides were assayed as described above.

ApoB was quantified by an enzyme immunoassay system. After non-radiolabeled oleate-BSA was added to the culture, the conditioned medium was used for the quantification of apoB mass according to the method of Forte et al. (25). The cellular protein was assayed by the method described above.

**Bile acid secretion**

Bile acid secretion from HepG2 cells was measured by method of Murakami et al. (20) with some modifications. Cells were seeded into a 24-well plastic plate (Falcon) at the density of 2 × 10^5 cells/500 μl/well, followed by culture in the medium containing 10% FBS for 2 days. After [14C]cholesterol dissolved in methanol was added to the medium at the final concentration of 19.5 μM, the cells were cultured for an additional 24 hr. The medium was replaced with 500 μl of fresh medium containing 10% FBS. At the same time, NTE-122 or probucol dissolved in DMSO was added (the final concentration of DMSO was 0.1%). After incubation for 48 hr, the medium was collected and applied to an Amplep™ C18 column (Amerham). After washing the column with 2 ml of distilled water, bile acids were eluted with 2 ml of 50% ethanol. Radioactivity in the eluted bile acids was counted as described above. The cellular protein was assayed by the method described above.

**Determination of protein**

Protein was determined by the method of Lowry et al. (26), using BSA as the standard.

**Statistical analyses**

Values are expressed as the mean ± S.E.M. Statistical significance of differences between the groups was determined by the Dunnett's multiple comparison test. Student's t-test or Aspin-Welch's t-test was also used in the comparison between the groups. A difference was considered to be statistically significant when the P-value was less than 0.05.

**RESULTS**

**Effects on cellular cholesterol esterification and biosynthesis of cholesterol, triglycerides and phospholipids**

The effects of NTE-122, E5324 and CI-976 on cellular cholesterol esterification were evaluated by the incorporation of [3H]oleate into cholesteryl esters (cellular cholesterol esterification) in HepG2 cells incubated with 5 μg/ml 25-hydroxycholesterol (Fig. 1). NTE-122, E5324 and CI-976 inhibited the cholesterol esterification in HepG2 cells in a dose-dependent manner with the IC50 value of 6.0, 960 and 390 nM, respectively. These results were consistent with our previous report, which indicated that NTE-122 was one of the most strong ACAT inhibitors (21).

As shown in Table 1, the biosynthesis of triglycerides and phospholipids, evaluated by the [3H]oleate incorporation, was unaffected by NTE-122 up to 100 nM, a concentration that was enough to inhibit completely ACAT

![Fig. 1](image)

**Fig. 1.** Effects of NTE-122 (○), E5324 (▲) and CI-976 (■) on cellular cholesterol esterification in HepG2 cells. HepG2 cells were cultured in MEM containing 5% LPDS and 5 μg/ml 25-hydroxycholesterol for 2.5 hr at 37°C, followed by addition of drugs and [3H]oleate-BSA complex to the cell culture. After incubation for 2.0 hr, [3H]oleate incorporations into cellular cholesteryl esters were determined. Each value represents the mean ± S.E.M. of triplicate assays. The activity of the control cells was 1.758 ± 0.115 nmol/mg cell protein.
Table 1. Effect of NTE-122 on incorporation of [H]oleate into cellular triglycerides and phospholipids in HepG2 cells in the presence of 25-hydroxycholesterol

<table>
<thead>
<tr>
<th>Product</th>
<th>NTE-122 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>(nmol incorporation/mg cell protein)</td>
<td></td>
</tr>
<tr>
<td>Triglycerides</td>
<td>24.70±1.84</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>2.51±0.39</td>
</tr>
</tbody>
</table>

HepG2 cells were incubated in MEM containing 5% LPDS and 5 μg/ml 25-hydroxycholesterol for 2.5 hr at 37°C, followed by addition of NTE-122, [H]oleate-BSA complex to the cell culture. After a 2-hr incubation, [H]oleate incorporations into cellular triglycerides and phospholipids were determined as described in Materials and Methods. Each value represents the mean±S.E.M. of triplicate assays.

Table 2. Effect of NTE-122 on incorporation of [14C]acetate into cellular cholesterol in HepG2 cells

<table>
<thead>
<tr>
<th>Product</th>
<th>NTE-122 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(nmol incorporation/mg cell protein)</td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>5.99±0.00</td>
</tr>
</tbody>
</table>

HepG2 cells were incubated in MEM containing 5% LPDS and NTE-122 for 0.5 hr at 37°C, followed by addition of [14C]acetate the cell culture. After a 2-hr incubation, [14C]acetate incorporations into cellular cholesterol were determined as described in Materials and Methods. Each value represents the mean±S.E.M. of triplicate assays.

activity in HepG2 cells. Table 2 shows the effect of NTE-122 on the incorporation of [14C]acetate into cellular cholesterol in HepG2 cells. The cholesterol biosynthesis was unaffected by NTE-122 up to 10 μM.

Effects on the secretion of cholesteryl esters and triglycerides

The effects of NTE-122, E5324 and CI-976 on the secretion of cholesteryl esters and triglycerides were evaluated by the incorporation of [H]oleate into cholesteryl esters and triglycerides. The stimulation of ACAT by 5 μg/ml 25-hydroxycholesterol caused the pronounced increases of the cellular content (threefold) and secretion (eightfold) of [H]cholesteryl esters and a significant increase (1.5-fold) of the [H]triglyceride secretion (Figs. 2 and 3). NTE-122 remarkably reduced both the cellular content and the secretion of [H]cholesteryl esters in dose-dependent manners. When the compound was added at the final concentration of 1 μM, the cellular content of [H]cholesteryl esters was decreased by 99.5% and the secretion was decreased by 94% (Fig. 2). In comparison with the values for the non-loaded cells, the cellular content of [H]cholesteryl esters was decreased by 98.8% and the secretion was decreased by 48% (Fig. 3). The secretion of [H]cholesteryl esters by HepG2 cells appeared to follow the intracellular [H]cholesteryl esterification. E5324 and CI-976 also reduced the cellular content and the secretion of [H]cholesteryl esters at doses from 1 to 10 μM (Fig. 2). On the other hand, NTE-122 did not inhibit [H]oleate incorporation into triglycerides in HepG2 cells, agreeing with Table 2. Moreover, the inhibitory effect of NTE-122 on the triglyceride secretion was weaker than that on cholesteryl ester secretion, although it significantly reduced the [H]triglyceride secretion by 31% at 1 μM in comparison with the value for 25-hydroxycholesterol-treated cells (Fig. 3). E5324 also slightly reduced the [H]triglyceride secretion without any influence on the triglyceride synthesis in HepG2 cells at doses from 1 to 10 μM (Fig. 3). CI-976 slightly increased the [H]triglyceride content and had no effect on the [H]triglyceride secretion.

Effect on ApoB secretion

The apoB secretion in HepG2 cells was also significantly enhanced 1.4-fold by 25-hydroxycholesterol. NTE-122 significantly reduced the secretion of apoB by HepG2 cells at doses higher than 10 nM (by 26% at 1 μM) (Fig. 4). E5324 and CI-976 also reduced the secretion of apoB by HepG2 cells at doses higher than 1 μM (Fig. 4). All of ACAT inhibitors appeared to counteract the effect of 25-hydroxycholesterol.

Effect on bile acid secretion

The effects of NTE-122 on bile acid secretion in HepG2
Effect of NTE-122 on HepG2 ACAT

(a) Cellular cholesteryl esters

(b) Secreted cholesteryl esters

Fig. 2. Effects of NTE-122, E5324 and CI-976 on \(^{3}H\)oleate incorporation into cellular (a) and secreted (b) cholesteryl esters in HepG2 cells. HepG2 cells were incubated in MEM containing 1.5% BSA and 5 μg/ml 25-hydroxycholesterol (25(OH)Chol) for 2.5 hr at 37°C, followed by addition of drugs and \(^{3}H\)oleate-BSA complex to the cell culture. After incubation for 18 hr, \(^{3}H\)oleate incorporations into both cellular and secreted cholesteryl esters were determined. Each value represents the mean ± S.E.M. of triplicate assays. \(*\): Significantly different from the non-loaded cells, P < 0.01. \(*\): Significantly different from the 25(OH)Chol-treated control cells, P < 0.01.

(a) Cellular triglycerides

(b) Secreted triglycerides

Fig. 3. Effects of NTE-122, E5324 and CI-976 on \(^{3}H\)oleate incorporation into cellular (a) and secreted (b) triglycerides in HepG2 cells. After performing the procedures indicated in Fig. 2, \(^{3}H\)oleate incorporations into both cellular and secreted triglycerides were determined. Each value represents the mean ± S.E.M. of triplicate assays. \(*\): Significantly different from the non-loaded cells, P < 0.01. \(*\): Significantly different from the 25(OH)Chol-treated control cells, P < 0.05, P < 0.01.

cells was evaluated. Probufol, which was reported to stimulate bile acid secretion in HepG2 cells (20), significantly increased the secretion of bile acid synthesized from \(^{14}C\)cholesterol (Fig. 5). NTE-122 enhanced the bile acid secretion more effectively than probufol did (1.5-fold increase at 1 μM).

DISCUSSION

ACAT plays an important role in cholesterol absorp-

tion from the intestine, VLDL secretion from the liver and vascular cholesterol accumulation. Therefore, systemically available inhibitors of ACAT may have therapeutic potential in the treatment of hyperlipidemia and atherosclerosis (1, 27). The role of cholesterol esterifica-
tion in determining the level of secretion of lipids and apoB by HepG2 cells is an subject of some controversy. Studies with the 58-035, an ACAT inhibitor, have led to conflicting results: decreases (28), increases (non-significant) (29) or no change (8) in apoB secretion being
![Diagram showing the effects of NTE-122, E5324, and CI-976 on the secretion of apolipoprotein B from HepG2 cells.](image)

**Fig. 4.** Effects of NTE-122, E5324 and CI-976 on the secretion of apolipoprotein B from HepG2 cells. After performing the procedures indicated in Fig. 2, apoB mass in the conditioned medium was determined. Each value represents the mean ± S.E.M. of triplicate assays. **: Significantly different from the non-loaded cells, **P < 0.01. **: Significantly different from the 25(OH)Chol-treated control cells, **P < 0.01.

![Diagram showing the effects of NTE-122 and probucol on bile acid secretion from HepG2 cells.](image)

**Fig. 5.** Effects of NTE-122 and probucol on bile acid secretion from HepG2 cells. HepG2 cells were cultured in the presence of [14C]cholesterol for 24 hr at 37°C, followed by addition of drugs. After incubation for 48 hr, the rate of incorporation of [14C]cholesterol into bile acids in the conditioned medium was determined. Each value represents the mean ± S.E.M. of triplicate assays. **: Significantly different from the control cells, **P < 0.05, **P < 0.01.

reported. Wu et al. (8) demonstrated that apoB secretion from HepG2 cells was unaffected by either long- or short-term changes in cholesteryl ester content. Recently, Graham et al. (15) reported that ACAT inhibitors, CL 277,082 and 447C88, could exert differential effects on the secretion of apoB-containing lipoproteins, which would not correlate with their efficacy in inhibiting ACAT, concluding that cholesterol esterification would not be essen-

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tial for apoB-containing lipoproteins. On the other hand, Musani et al. (14) showed that an ACAT inhibitor, FCE27677, suppressed apoB secretion from HepG2 cells, especially in the presence of 25-hydroxycholesterol as a stimulus for ACAT. Meanwhile, changes in triglyceride synthesis are clearly associated with corresponding changes in secretion of apoB-containing lipoproteins by HepG2 cells (8, 29, 30), which can occur independently of changes in cholesterol esterification (8, 30).

In this study, we demonstrated that 25-hydroxycholesterol increased both synthesis and the secretion of cholesteryl esters and both the secretion of triglycerides and apoB in HepG2 cells, which were significantly reduced by NTE-122. However, in the stimulation by 25-hydroxycholesterol, the increases of the secretion of triglycerides and apoB were smaller than that of cholesteryl esters (the secretion of triglycerides and apoB increased 1.5-fold, while that of cholesteryl esters 8-fold). Moreover, NTE-122 appeared to only counteract the effect of 25-hydroxycholesterol on secretion of triglycerides and apoB even under the condition where the secretion of cholesteryl esters pronouncedly decreased below the level of non-loaded cell (other ACAT inhibitors, E5324 and CI-976, had similar effects, although it is necessary to use these compounds at more than ten times the dose of NTE-122, in order to mimic the effect of NTE-122). On the contrary, the secretion of triglycerides and apoB showed superimposable changes. These results suggest that the secretion of apoB-containing lipoprotein is not directly associated with cholesteryl esters but triglycerides. Moreover, these results suggest to us that there may be an intimate relationship between ACAT inhibition and 25-hydroxycholesterol-stimulated increase in the secretion of apoB-containing lipoprotein. The activation mechanism of ACAT by 25-hydroxycholesterol is speculated to involve the interaction of the oxysterol with the binding site of cholesterol for ACAT activation (1), but it is unclear whether 25-hydroxycholesterol really causes direct enzymatic activation of ACAT or secondary activation mediated by another factor. Furthermore, if the increases in the secretion of triglycerides and apoB were due to the activation of ACAT by 25-hydroxycholesterol, it is contradictory that most of the secretion of triglycerides and apoB even is maintained in the presence a sufficient concentration of NTE-122, a compound shown to be the most strong and selective ACAT inhibitor in the previous and present studies (21). At this point, it is possible that 25-hydroxycholesterol also has other effects that can enhance the secretion mechanism of apoB-containing lipoprotein. In fact, 25-hydroxycholesterol causes the down-regulation of HMG-CoA (3-hydroxy-3-methylglutaryl-CoA) reductase and LDL receptor mediated by SREBP (sterol regulatory element-
binding protein) (31). However, if so, this conflicts with the significant reduction of the secretion of triglycerides and apolipoprotein B even in the presence of a sufficient concentration of NTE-122. Finally, we hypothesized that more than one process are involved in the secretion mechanism of apolipoprotein B-containing lipoprotein, such as 25-hydroxycholesterol sensitive (ACAT inhibitor sensitive) and non-sensitive (ACAT inhibitor non-sensitive) processes. It is plausible that NTE-122 decreases the number of the secreted apolipoprotein B-containing lipoproteins for 25-hydroxycholesterol-sensitive secretion of apolipoprotein B-containing lipoprotein, while it modifies the lipid composition for 25-hydroxycholesterol non-sensitive secretion.

There remains a possibility that NTE-122 has therapeutic potential for cholesterol lowering because, in fact, this compound significantly inhibited the secretion of cholesterol esters, triglycerides and apolipoprotein B in the presence of 25-hydroxycholesterol. Although ACAT inhibitors have been reported to cause a pronounced lowering of the level of blood cholesterol (13, 19, 21, 22), it remains controversial whether these exert their main action in liver or in small intestine. Recently, Krause et al. (32) showed that orally-administered CI-976 caused a dose-dependent decrease in the hepatic ACAT activity and cholesteryl ester mass. In addition, when radiolabeled CI-976 was orally administered to the rat, the liver was very highly labeled (19). Similar results were obtained using NTE-122 (data not shown), suggesting that NTE-122 can act directly not only in the small intestine but also in the liver.

The liver plays a central role in changes in the levels of lipoproteins, bile acids and cholesterol in the bile, which may be dependent on the balance between the utilization of free cholesterol and cholesteryl esters (18). Free cholesterol is a polar lipid, so that the excess of free cholesterol induced cytotoxicity (33). In hepatocytes, bile acid esters are synthesized from free cholesterol and excreted into the bile (16). Therefore, the stimulation of bile acid secretion accelerates the regression of hypercholesterolemia, for instance, the effect of probucol (17). With respect to the clinical application of ACAT inhibitors, it is of interest to determine whether the inhibition of hepatic ACAT affects the free cholesterol level and even the excretion of free cholesterol in the form of bile acids into the bile. Sampson et al. (18) showed that the ACAT inhibitor 58-035 increased bile acid excretion in rat hepatocytes. Recently, Murakami et al. (20) showed that the ACAT inhibitor, HL-004, increased bile acid secretion in rat and HepG2 cells due to the increased supply of free cholesterol caused by ACAT inhibition. In the present study, NTE-122 also increased bile acid secretion in HepG2 cells more effectively than the reported compounds.

In conclusion, NTE-122 is one of the most strong and selective ACAT inhibitors, being able to exert its main action even in the liver, as well as in the small intestine. NTE-122 substantially reduced both the cholesterol content and the number of particles in VLDL secreted by HepG2 cells. Furthermore, NTE-122 stimulated the production of bile acids through an increase in the free cholesterol pool by ACAT inhibition. Therefore, we are convinced that NTE-122 produces its cholesterol-lowering effect through the inhibition of VLDL secretion and enhancement of bile acid excretion in the liver.

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