Effects of NTE-122, an Acyl-CoA:Cholesterol Acyltransferase Inhibitor, on Cholesterol Esterification and Lipid Secretion From CaCo-2 Cells, and Cholesterol Absorption in Rats

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Received February 1, 1999  Accepted March 10, 1999

ABSTRACT—The effect of NTE-122 (trans-1,4-bis[[1-cyclohexyl-3-(4-dimethylamino phenyl)ureido]methyl]cyclohexane), an acyl-CoA:cholesterol acyltransferase (ACAT) inhibitor, on cholesterol absorption was investigated. NTE-122 inhibited whole-cell ACAT activity in CaCo-2 cells, a human intestinal cell line, with an IC_{50} value of 4.7 nM. In CaCo-2 cells cultured on a membrane filter, NTE-122 pronouncedly inhibited the basolateral secretion of newly synthesized cholesteryl esters, and significantly reduced the basolateral secretion of newly synthesized triglycerides without influencing the cellular triglyceride synthesis. Furthermore, NTE-122 (1 mg/kg, p.o.) inhibited [^{3}H]cholesterol absorption in rats. These results suggest that NTE-122 is capable of exhibiting anti-hyperlipidemic effects by reducing the absorption of dietary cholesterol.

Keywords: NTE-122, Acyl-CoA:cholesterol acyltransferase (ACAT) inhibitor, Cholesterol absorption.

Acyl-CoA:cholesterol acyltransferase (ACAT, EC 2.3.1.26) is a microsomal enzyme catalyzing the intracellular formation of cholesteryl esters from acyl-coenzyme A and free cholesterol in such tissues as intestine, liver and arterial wall (1). In the intestine, ACAT facilitates the absorption of exogenous cholesterol, which is incorporated into chylomicron (CM). In the liver, ACAT is thought to play an important role in the assembly of very low density lipoprotein, which is secreted into the blood. In the arterial wall, ACAT is presumed to play a crucial role in foam cell formation from macrophages and vascular smooth muscle cells (1), which is a characteristic feature of atherosclerotic lesions. Therefore, ACAT inhibitor may exhibit cholesterol-lowering and anti-atherosclerotic activities by blocking intestinal absorption of dietary cholesterol, inhibiting hepatic secretion of VLDL, and preventing formation of foam cells in the arterial wall (1,2).

NTE-122, trans-1,4-bis[[1-cyclohexyl-3-(4-dimethylamino phenyl)ureido]methyl]cyclohexane, is a potent and selective inhibitor of ACAT (3). We previously reported that NTE-122 suppressed lipid secretion from HepG2 cells, a human hepatocyte cell line, and caused cholesterol lowering in cholesterol diet-fed rats and rabbits (3, 4). Furthermore, NTE-122 prevented the foam cell formation and enhanced the foam cell regression in human THP-1 macrophages (5). In the present study, we investigated the effects of NTE-122 i) on intracellular cholesterol esterification and lipid secretion from CaCo-2 cells, a human intestinal cell line, and ii) on cholesterol absorption in rats.

First, we evaluated the inhibitory effect of NTE-122 (Nissin Food Products Co., Ltd., Kusatsu) on whole-cell ACAT activity in CaCo-2 cells using [^{3}H]oleate as a tracer, compared with other ACAT inhibitors, E5324 (n-butyl-N-[2-[3-(5-ethyl-4-phenyl-1H-imidazol-1-yl)propoxy]-6-methylphenyl]urea) (6) and CI-976 (2,2-dimethyl-N-(2,4,6-trimethoxyphenyl)-dodecanamide) (7), which were also synthesized at Central Research Institute, Nissin Food Products, Co., Ltd. After CaCo-2 cells had grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, NY, USA) containing 10% fetal bovine serum, 50 units/ml penicillin, 50 µg/ml streptomycin and 1% non-essential amino acids to a post-confluent state in a 12-well plate at 37°C in a humid atmosphere containing 95% air and 5% CO_{2}, the cells were cultured in 0.9 ml of 199 medium (Gibco BRL) containing 1 mM HEPES, 0.2 mM cholesterol, 5 mM sodium taurocholate...
and 0.05 mM L-α-phosphatidylcholine, followed by incubation for 2.5 hr. Then, 0.1 ml of various concentrations of each test compound dissolved in 199 medium containing 1 mM HEPES and 1% DMSO was added to the medium. After incubation for 30 min, 20 μl of [9,10(9)-3H]oleic acid-BSA complex (1H)oleate, 5 mM, specific radioactivity of 296 MBq/mmol (Amersham, Little Chalfont, UK); BSA: 120 mg/ml) was added, followed by incubation for 2 hr. Cellular [3H]cholesterol esters (nmol [3H]oleate incorporation/mg cell protein) were determined as described previously (4, 5).

NTE-122, E5324 and CI-976 inhibited whole-cell ACAT activity in CaCo-2 cells in a dose-dependent manner with the IC50 values of 4.7, 220 and 180 nM, respectively. These results were consistent with our previous report, which indicated that NTE-122 was one of the strongest ACAT inhibitors (3–5).

Next, we examined the effect of NTE-122 on the basolateral secretion of cholesteryl esters and triglycerides in CaCo-2 cells cultured on a membrane filter. After CaCo-2 cells were cultured to a post-confluent state on micropore polyester filter (Traswell, 0.4-μm pore size; Corning Costar, Cambridge, MA, USA) in a 12-well plate, media in the upper (apical side, 0.5 ml) and lower (basolateral side, 1.5 ml) were changed to 0.45 ml of 199 medium containing 1.5% BSA, 1 mM HEPES, 0.2 mM cholesterol, 5 mM sodium taurocholate and 0.05 mM L-α-phosphatidylcholine and 1.5 ml of 199 medium containing 1.5% BSA and 1 mM HEPES, respectively, and incubated for 2.5 hr. Then, 50 μl of various concentrations of each test compound dissolved in 199 medium containing 1.5% BSA, 1 mM HEPES and 1% DMSO was added to the medium in the apical side. After incubation for 30 min, 20 μl of the [3H]oleate-BSA complex described above was added to the apical side, followed by incubation for 18 hr. The [3H]cholesterol esters and [3H]triglycerides in the cells and the basolateral medium were determined as described previously (4, 5). The data represent the mean ± S.E.M. of triplicate assays. Statistical significance was evaluated by Dunnett’s multiple comparison test, at P < 0.05.

NTE-122 remarkably reduced both the cellular content and the basolateral secretion of [3H]cholesterol esters in CaCo-2 cells in a dose-dependent manner. When the compound was added at the final concentration of 1 μM, the cellular content of [3H]cholesterol esters was decreased by 99.1% and the secretion was decreased by 99.9% (Fig. 1). The secretion of [3H]cholesterol esters by CaCo-2 cells appeared to follow the intracellular [3H]cholesterol esterification. E5324 and CI-976 also reduced the cellular content and the secretion of [3H]cholesterol esters at the dose of more than 0.1 and 1 μM, respectively (Fig. 1). Moreover, NTE-122 significantly reduced [3H]triglyceride secretion without any influence on the triglyceride synthesis. However, the inhibitory effect of NTE-122 on the triglyceride secretion was weaker than that on cholesteryl ester secretion (by 26.7% at 1 μM) (Fig. 2). E5324 also slightly reduced the [3H]triglyceride secretion without any influence on the triglyceride synthesis in CaCo-2 cells at doses from 1 to 10 μM. CI-976 slightly increased the [3H]triglyceride content and secretion (Fig. 2).

(a) Cellular cholesteryl esters

(b) Secreted cholesteryl esters

![Fig. 1. Effects of NTE-122, E5324 and CI-976 on [3H]oleate incorporation into cellular (a) and basolateral secreted (b) cholesteryl esters in CaCo-2 cells cultured on a membrane filter. CaCo-2 cells were incubated in 199 medium containing 1.5% BSA, 1 mM HEPES, 0.2 mM cholesterol, 5 mM sodium taurocholate and 0.05 mM L-α-phosphatidylcholine for 2.5 hr at 37°C, followed by addition of drugs and [3H]oleate-BSA complex to the cell culture. After incubation for 18 hr, [3H]oleate incorporations into both cellular and basolateral secreted cholesteryl esters were determined. Each value represents the mean ± S.E.M. of triplicate assays. **Significantly different from control cells, P < 0.05, P < 0.01.](image-url)
Finally, we investigated the effect of NTE-122 on cholesterol absorption in rats. Male Sprague-Dawley rats (Charles River Japan, Inc., Tokyo) were housed under controlled temperature (23.5 ± 2°C), humidity (55 ± 10%) and light (8:00–20:00 hr), and free access to tap water and commercial chow, CRF-1 (Oriental Yeast Co., Ltd., Tokyo). This study was performed in accordance with the "Guiding Principles for the Care and Use of Laboratory Animals" approved by The Japanese Pharmacological Society. Rats (180–220 g) fed CRF-1 containing 1% cholesterol, 0.5% cholic acid and 5% olive oil for 3–4 days were administered p.o. with 1 mg/kg NTE-122 (5% arachidonic acid suspension) or vehicle, followed by p.o. administration of the oral tracer containing 6 mg of cholesterol, 185 kBq of [4-14C]cholesterol (2.0 GBq/mm, Amersham), 156 mg of triolein and 7.9 mg of cholic acid. Blood samples were collected from the tail vein and radioactivities of plasma (50 μl) were determined. The data represent the mean ± S.E.M. of 4 animals. Statistical significance was evaluated by Student’s t-test or Aspin-Welch’s t-test, at P < 0.05.

In control rats, the radioactivity of [14C]cholesterol in the plasma increased rapidly and reached 153,899 ± 25,227 dpm/ml 8 hr after application of tracer. In contrast, NTE-122 at a dose of 1 mg/kg, p.o. strongly prevented that transient increase in plasma radioactivity, and the plasma radioactivity in NTE-122 treated rats was significantly lower up to 48 hr (Fig. 3).

These results suggest that intestinal ACAT inhibition by NTE-122 decreases cholesterol absorption via the intestine. In fact, NTE-122 increased the cholesterol excretion in feces of cholesterol diet-fed rats (data not shown). Dietary cholesterol molecules taken up by intestinal cells are intracellularly esterified by ACAT and packed into the lipid cores of CM particles, which are secreted into the mesenteric lymph (8). NTE-122 did not affect the synthesis of triglycerides, the major core lipid of CM (8). Therefore, the decrease of triglyceride secretion in CaCo-2 cells by NTE-122 (Fig. 2) suggests that this compound not only decreases cholesterol content in CM but also

**Fig. 2.** Effects of NTE-122, E5324 and CI-976 on [1H]oleate incorporation into cellular (a) and basolateral secreted (b) triglycerides in CaCo-2 cells cultured on a membrane filter. After performing the procedures described in Fig. 1, [1H]oleate incorporations into both cellular and basolateral secreted triglycerides were determined. Each value represents the mean ± S.E.M. of triplicate assays. *Significantly different from control cells, P < 0.05.

**Fig. 3.** Inhibitory effect of NTE-122 on the dietary [14C]cholesterol absorption in rats. Rats fed the diet containing 1% cholesterol, 0.5% cholic acid and 5% olive oil for 3–4 days were administered p.o. with 1 mg/kg NTE-122 (○) or vehicle (●), followed by p.o. administration of 185 kBq of [14C]cholesterol. Plasma samples were collected 1, 2, 4, 8, 24 and 48 hr after the administration of the tracer. Each value represents the mean ± S.E.M. (N = 4). *Significantly different from the control, P < 0.05.
suppress the secretion of CM by the intestine. Triglycerides play a key role in the assembly and secretion of CM in the intestine (8). Several studies have suggested that esterification of cholesterol is also critical for CM assembly (9, 10). On the contrary, Field et al. (11) reported that an ACAT inhibitor, PD128042 (CI-976), inhibited the basolateral secretion of cholesteryl esters in CaCo-2 cells, but not the secretion of triglycerides and phospholipids, and we also obtained a similar result (Fig. 2). Therefore, it has been unknown whether direct inhibition of ACAT results in a decreased amount of CM secretion, changes of the lipid composition of the CM, or both. Our present study indicates that the inhibition of cholesteryl esterification in intestine by NTE-122 reduced both the cholesterol content and amount of secreted CM, leading to a decrease in cholesterol absorption.

Hypercholesterolemia is frequently associated with diabetes mellitus. One of the causes for hypercholesterolemia in the diabetic state is thought to be an increase in cholesterol absorption from the intestine. In diabetic animal models such as streptozotocin-induced diabetic rats (12) and Wistar fatty rats (13), ACAT activity in the intestine is increased. In addition, increased of intestinal CM secretion was observed in the postprandial state in non-insulin-dependent diabetic patients (14). These facts suggest that suppression of cholesterol absorption in the intestine by ACAT inhibition may be an effective treatment of hypercholesterolemia in diabetic patients. Therefore, NTE-122 could be useful for the treatment of hyperlipidemia in diabetic patients.

In conclusion, NTE-122 is one of the most strong and selective ACAT inhibitors, being able to exert its main action in the intestine. NTE-122 substantially reduced both the cholesterol content and the amount of secreted CM from CaCo-2 cells. Furthermore, NTE-122 suppressed the absorption of [14C]cholesterol in rats. Therefore, we suggest that NTE-122 has therapeutic potential for dietary hyperlipidemic subjects.

REFERENCES