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Selectivity of Pyripyropene Derivatives in Inhibition toward Acyl-CoA:cholesterol Acyltransferase 2 Isozyme

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Abstract Selectivity of 96 semisynthetic derivatives prepared from fungal pyripyropene A, originally isolated as a potent inhibitor of acyl-CoA:cholesterol acyltransferase (ACAT), toward ACAT1 and ACAT2 isozymes was investigated in the cell-based assay using ACAT1- and ACAT2-expressing CHO cells. Eighteen derivatives including PR-71 (7-*O*-isocaproyl derivative) showed much more potent ACAT2 inhibition (IC_{50}: 6.0 to 62 nM) than pyripyropene A (IC_{50}: 70 nM). Among them, however, natural pyripyropene A showed the highest selectivity toward ACAT2 with a selectivity index (SI) of >1000, followed by PR-71 (SI, 667).

Keywords semisynthetic pyripyropene derivative, acyl-CoA:cholesterol acyltransferase (ACAT), ACAT isozyme, atherosclerosis

Introduction

Acyl-CoA:cholesterol acyltransferase (ACAT) plays important roles in cholesterol metabolism in mammals. Therefore, a large number of synthetic ACAT inhibitors such as ureas, imidazoles and amides have been reported [1]. However, pharmaceutical companies have so far failed to develop them as new types of cholesterol-lowering or anti-atherosclerotic agents [2]. Recently, the development of avasimibe [3] and pactimibe [4] has also failed.

Our research group started screening for ACAT inhibitors of natural origin with a chemical structure different from synthetic ones from the 1990s, and discovered a number of compounds [5]. Among them, pyripyropenes, fungal metabolites, were one of the most potent ACAT inhibitors in an enzyme assay using rat liver microsomes [6~8]. Accordingly, about 200 derivatives were semisynthetically prepared from pyripyropene A. Certain derivatives were shown to be more potent than pyripyropene A, and proved to be active in reducing cholesterol absorption *in vivo* from the intestines of hamsters [9~14].

Recent molecular biological studies revealed the presence of two isozymes, ACAT1 and ACAT2 [15~18]. ACAT1 is ubiquitously expressed, and high-level expression is observed in sebaceous glands, steroidogenic tissues, and macrophages, while ACAT2 is expressed predominantly in the liver and intestine [19]. In spite of this recent knowledge about ACAT isozymes, the selectivity of the inhibitors, even synthetic ones, toward the isozymes has not been fully studied. We established a cell-based assay using ACAT1- or ACAT2-expressing CHO cells and

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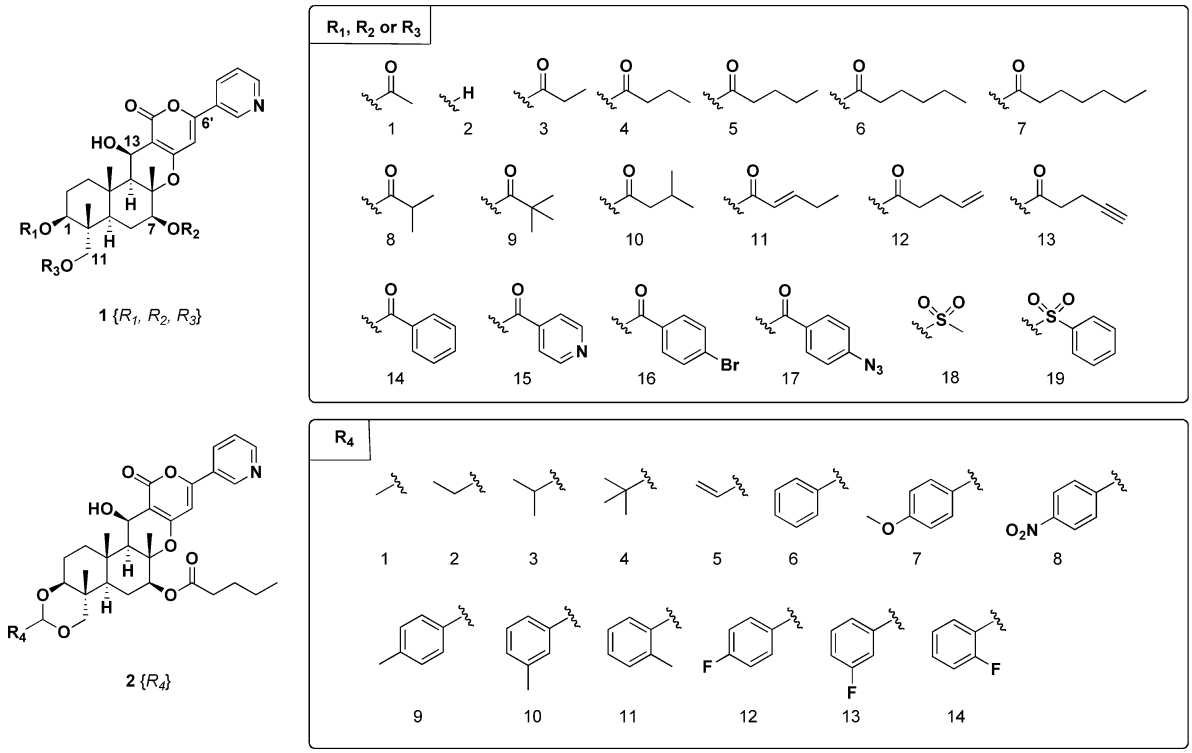

Fig. 1 Structures of pyripyropene derivatives.

Type **1** {R_1, R_2, R_3} has a diversity at R_1, R_2 and R_3. Type **2** {R_4} has a diversity at R_4.

studied the selectivity of microbial ACAT inhibitors we have discovered toward the isozymes [20, 21]. We confirmed that pyripyropenes showed a very unique characteristics, namely ACAT2-selective inhibition.

In this study, 96 pyripyropene derivatives now available were evaluated in this cell-based assay to investigate their selectivie inhibition toward the ACAT isozymes.

Materials and Methods

Materials

Pyripyropene A was purified from the culture broth of the producing microorganism, *Aspergillus fumigatus* FO-1289, according to the established methods [6～8]. [1-^{14}C]oleic acid was purchased from PerkinElmer Life and Analytical Sciences (USA). Fetal bovine serum (FBS) was from HyClone (USA). HAM's F12 was obtained from Sigma-Aldrich (USA). Geneticin (G-418 sulfate) and MEM vitamin solution were from Wako Pure Chemical Industries (Japan). Penicillin (10,000 units/ml)/streptomycin (10,000 mg/m) solution was from Invitrogen (USA). Plastic microplates (48-well) were purchased from Asahi Techno Glass (Japan). Ninety-six semisynthetic derivatives were prepared from pyripyropene A as reported previously [9～14]. Among them, the structures of pyripyropene A and 35 derivatives are summarized in Fig. 1 and Table 1.

Culture of ACAT1- and ACAT2-CHO Cells

Two cell lines, CHO cells expressing African Green monkey ACAT1 (ACAT1-CHO) and ACAT2 (ACAT2-CHO) [20], were maintained at 37°C in 5.0% CO_2 in Ham's F-12 medium supplemented with MEM vitamins, geneticin (300 μg/ml) and 10% heat inactivated FBS (hereafter referred to as medium A).

Assay for [^{14}C]CE Synthesis from [^{14}C]oleic Acid in ACAT1- or ACAT2-CHO Cells

The assay for the synthesis of [^{14}C]cholesteryl ester (CE) from [^{14}C]oleic acid in ACAT1- or ACAT2-CHO cells was carried out by our established method [21]. ACAT1- or ACAT2-CHO cells (1.25×10^5 cells in 250 μl of medium A) were cultured in a 48-well plastic microplate and allowed to recover overnight at 37°C in 5.0% CO_2. The assay was done with cells that were at least 80% confluent. Following overnight recovery, a test sample (2.5 μl in MeOH) and [1-^{14}C]oleic acid (1.85 kBq, 5.0 μl in 10% EtOH/phosphate

Table 1 Selectivity of semisynthetic derivatives toward ACAT isozymes

Derivative	Structure*	IC_{50} for CE synthesis (μM)			SI***
		Rat liver microsomes**	ACAT1-CHO	ACAT2-CHO	
Pyripyropene A	**1**{*1*, *1*, *1*}	0.058	>80	0.070	>1000
PR-71	**1**{*1*, *10*, *1*}	0.025	3.5	0.0060	667
PR-72	**1**{*1*, *11*, *1*}	0.019	4.2	0.010	420
PR-220	**2**{*9*}	0.11	3.8	0.010	380
PR-218	**2**{*11*}	0.025	3.2	0.010	320
PR-60	**1**{*4*, *6*, *1*}	0.013	2.8	0.012	233
PR-109	**2**{*6*}	0.052	4.0	0.020	200
PR-156	**2**{*5*}	0.22	3.2	0.020	160
PR-118	**1**{*1*, *12*, *1*}	0.050	5.0	0.050	100
PR-221	**2**{*14*}	0.056	4.0	0.040	100
PR-113	**1**{*1*, *13*, *1*}	0.028	6.5	0.070	93
PR-146	**2**{*2*}	0.069	1.8	0.020	90
PR-171	**2**{*3*}	0.0055	0.7	0.010	70
PR-141	**2**{*7*}	0.40	2.8	0.040	70
PR-45	**1**{*1*, *5*, *1*}	0.015	4.5	0.080	56
PR-186	**1**{*1*, *17*, *1*}	0.065	1.1	0.020	55
PR-70	**1**{*1*, *7*, *1*}	0.125	1.4	0.035	40
PR-25	**1**{*1*, *4*, *1*}	0.052	>16	0.450	>36
PR-165	**1**{*1*, *5*, *18*}	0.024	0.7	0.020	35
PR-223	**2**{*12*}	0.091	6.5	0.19	34
PR-159	**2**{*4*}	0.43	4.1	0.12	34
PR-222	**2**{*13*}	0.023	1.8	0.060	33
PR-26	**1**{*1*, *8*, *1*}	0.10	12	0.40	30
PR-219	**2**{*10*}	0.12	1.9	0.080	24
PR-163	**1**{*1*, *15*, *1*}	0.40	10	0.42	24
PR-86	**1**{*1*, *1*, *18*}	0.043	>22	1.0	>22
PR-69	**1**{*1*, *14*, *1*}	0.065	1.0	0.050	20
PR-59	**1**{*1*, *10*, *1*}	0.15	5.7	0.40	14
PR-155	**1**{*5*, *5*, *18*}	0.67	0.010	0.070	0.14
PR-164	**1**{*14*, *5*, *18*}	0.30	0.025	0.062	0.40
PR-75	**1**{*5*, *1*, *1*}	0.39	2.0	0.30	6.7
PR-34	**1**{*8*, *8*, *8*}	0.45	0.60	0.40	1.5
PR-10	**1**{*3*, *3*, *3*}	0.79	3.8	0.45	8.4
PR-31	**1**{*4*, *5*, *4*}	0.58	0.57	0.60	0.95
PR-54	**1**{*5*, *5*, *5*}	0.88	0.50	0.80	0.63
PR-55	**1**{*4*, *1*, *4*}	0.12	3.5	0.90	3.9

* See Fig. 1.

** Data of IC_{50} value in rat liver microsomes are cited from References [6~14].

*** Selectivity index (SI): IC_{50} for ACAT1/IC_{50} for ACAT2.

The data are expressed as the mean of N=5.

buffered saline (PBS) solution) were added to each culture. After a 6-hour incubation at 37°C in 5.0% CO_2, the medium was removed, and the cells in each well were washed twice with PBS. The cells were lysed by adding 0.25 ml of 10 mM Tris-HCl (pH 7.5) containing 0.1% (w/v) sodium dodecyl sulfate (SDS), and the cellular lipids were extracted by the method of Bligh and Dyer [22]. After the organic phase was concentrated, the total lipids were separated on a thin layer chromatography (TLC) plate (silica gel F254, 0.5 mm thick, Merck, Germany) and

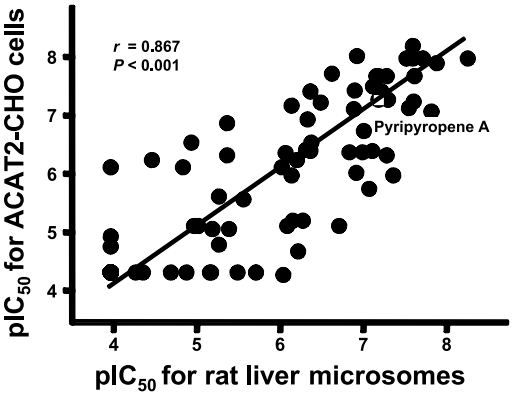

Fig. 2 Relationship between pIC_{50} of pyripyropene derivatives against ACAT activity in rat liver microsomes and in ACAT2-CHO cells.

pIC_{50} is defined as $-\log_{10}$ (IC_{50} for rat liver microsomes or ACAT2-CHO cells). pIC_{50} for rat liver microsomes and pIC_{50} for ACAT2-CHO cells of each derivative (■) were plotted on the X and Y axes, respectively. The coordinate of pyripyropene A (□) was also plotted for comparison.

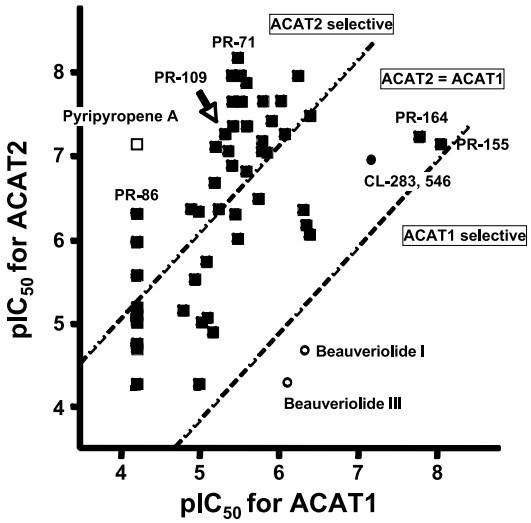

Fig. 3 Relationship between pIC_{50} of pyripyropene derivatives against ACAT1 and ACAT2 activity.

pIC_{50} is defined as $-\log_{10}$ (IC_{50} for ACAT1- or ACAT2-CHO cells). pIC_{50} for ACAT1 and pIC_{50} for ACAT2 of each derivative (■) were plotted on the X and Y axes, respectively. The coordinates of pyripyropene A (□), CL-283,546 (●) and beauveriolides (○) were also plotted for comparison.

analyzed with a bioimaging analyzer (BAS 2000, Fuji Film, Japan). In this cell-based assay, [^{14}C]CE was produced by the reaction of ACAT1 or ACAT2. ACAT inhibitory activity (%) is defined as (1−[^{14}C]CE-drug/[^{14}C]CE-control)×100. The IC_{50} value is defined as the drug concentration causing 50% inhibition of an enzyme (or biological) activity. Selectivity index (SI) is defined as IC_{50} for ACAT1/IC_{50} for ACAT2.

Results

All the semisynthetic pyripyropene derivatives (96 compounds) were tested in the cell-based assay using ACAT1- and ACAT2-CHO cells. Among them, 57 derivatives showed inhibitory activity against ACAT1 and/or ACAT2.

First, the IC_{50} values of the derivatives against ACAT2 in the assay using ACAT2-CHO cells and against ACAT in the rat liver microsomes previously reported [9∼14] are plotted in Fig. 2. A good correlation was obtained (Pearson r=0.867, P<0.001) between the two IC_{50} values. The result is reasonable because rat livers specifically express ACAT2 isozymes [19].

Next, the IC_{50} values of these 57 derivatives against ACAT1 and ACAT2 activities are plotted in Fig. 3. Among them, 27 derivatives showed ACAT2 selective inhibition with selectivity indexes (SI) of >10, 30 derivatives inhibited both isozymes (SI, <10), and no derivatives showed ACAT1 selective inhibition. These data indicate that the structural skeleton of pyripyropene fundamentally prefers the ACAT2 isozyme. Furthermore, 35 derivatives strongly inhibited with nano molar levels of IC_{50} values. The structures and the IC_{50} values are summarized in Fig. 1 and Table 1. Eighteen of the 35 derivatives showed more potent ACAT2 inhibition than pyripyropene A in the cell-based assay, and 10 derivatives (PR-71, 72, 218, 60, 109, 118, 221, 171, 165 and 222) were more potent in both cell-based and microsomal assays. PR-171 was the most potent in the microsomal assay, and PR-71 was the most potent in the cell-based assay.

To assess the selectivity toward ACAT2 isozyme, the 35 derivatives were ordered according to their SIs (Table 1). Among 27 derivatives having SIs of >10 as described, 9 derivatives (PR-71, 72, 220, 218, 60, 109, 156, 118, 221) were highly selective toward ACAT2 with SIs of >100. Surprisingly, pyripyropene A was the most selective in ACAT2 inhibition (SI, >1000), followed by PR-71 (SI, 667). CL-283, 546, a synthetic urea inhibitor, inhibited both ACAT1 and ACAT2, and beauveriolides I and III showed ACAT1 selective inhibition in the cell-based assay (Fig. 3).

Discussion

The relationships between the structures of pyripyropene

derivatives and their ACAT2 inhibitory activity in the cell-based assay were confirmed: 1) *O*-Acyl groups at the 1-, 11- and 7-positions are essential for inhibitory activity, 2) a free hydroxyl group at the 13-position is essential for the activity, and 3) the position of the nitrogen atom in the pyridine residue at the 6′-position is essential for the activity. These conclusions are comparative to those obtained in the rat liver mirosomal assay [9～14].

We have tested a number of ACAT inhibitors toward the ACAT1 and ACAT2 isozymes. Most of them were characterized into two groups; the first one includes CL-283, 546 [21], avasimibe [3] and pactimibe [4] showing inhibition of both isozymes and the second one includes beauveriolides [21] and Wu-V-23 [20] showing selective inhibition of ACAT1. As confirmed in this study, pyripyropene A and its derivatives (the third group) have very unique characteristics of selectively inhibiting the ACAT2 isozyme. Therefore, pyripyropenes are expected to be developed as a new type of anti-atherosclerotic agent.

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