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POLYUNSATURATED FATTY ACID ANILIDES AS INHIBITORS OF ACYL-COA:CHOLESTEROL ACYLTRANSFERASE (ACAT)

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Abstract: A series of polyunsaturated fatty acid anilides were synthesized and evaluated as ACAT inhibitors. Compound **24** had potent inhibitory activity against microsomal ACAT derived from U937, HepG2 and Caco-2 cell lines. Therefore, it might be expected to act as an antiarteriosclerotic and hypocholesterolemic agent. Interestingly, the ACAT inhibitory potency of **24** varied significantly depending on the source of the enzyme.

Introduction

ACAT is an intracellular enzyme responsible for cholesterol esterification in the intestine, liver and arterial wall. Intestinal epithelial ACAT catalyzes re-esterification of a dietary cholesterol taken into the cells, providing the chylomicron cholesteryl ester component. It is believed that ACAT plays a key role in the assembly and secretion of very low density lipoprotein(VLDL) in the liver, and also in the accumulation of cholesterol esters in macrophages and arterial vascular smooth muscle cells in atherosclerotic lesions. Therefore, ACAT inhibitors may be expected to have potent hypocholesterolemic and antiarteriosclerotic actions.[1]

In recent years, fatty acid anilides have been reported as potent ACAT inhibitors.[2] And the n-3 polyunsaturated fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have also been reported to have many physiological activities such as plasma lipid-lowering activity, antithrombotic activity and antiinflammatory activity.[3] Moreover, EPA ethyl ester (EPADEL®, Mochida) has already been used as a hypolipidemic agent in Japan. Therefore, we prepared a series of polyunsaturated fatty acid anilides, expecting the synergic hypolipidemic and antiarteriosclerotic effect caused by ACAT inhibitory activities and above-mentioned unique physiological activities derived from the characteristics of EPA or DHA.

This report describes ACAT inhibitory activities of a series of polyunsaturated fatty acid anilides.

Chemistry

The polyunsaturated fatty acid anilides were all prepared by reacting the appropriate anilines with linoleic acid, EPA and DHA respectively in Chemical Synthesis Research, Azwell Inc.

N-(2,6-diisopropylphenyl)-4,7,10,13,16,19-docosahexaenamide (**24**), for example, was synthesized as follows: DHA(1.31 g, 4 mmol) and oxalyl chloride(0.761 g, 6 mmol) were dissolved in chloroform and reacted for about 2 hours while cooling in a nitrogen stream. The mixture was concentrated and dissolved in anhydrous tetrahydrofuran(THF, 5 mL). This was added to a solution of 2,6-diisopropylaniline (709 mg, 4 mmol) and triethylamine(405 mg, 4 mmol) dissolved in THF(3 mL), and the mixture reacted overnight cooling in a nitrogen stream and then filtered. The filtrate was concentrated in vacuo and dissolved in ethyl acetate(120 mL). It was washed with 2N HCl and saturated brine, and then ethyl acetate layer was concentrated. The concentrate was applied to a silica gel(60 g) column and eluted with a hexane-ethyl acetate solvent to give **24** (1.37 g).

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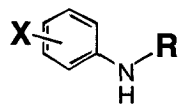
Table 1 Structure and human macrophage ACAT inhibition



Compound	X	R	formula	Percentage of ACAT inhibition 0.5 μM	5 μM
1	2,4,6-trimethoxy	linoleoyl	$C_{27}H_{43}NO_4$	34	84
2		eicosapentaenoyl	$C_{29}H_{41}NO_4$	57	92
3		docosahexaenoyl	$C_{31}H_{43}NO_4$	47	92
4	3,4,5-trimethoxy	linoleoyl	$C_{27}H_{43}NO_4$	19	51
5		eicosapentaenoyl	$C_{29}H_{41}NO_4$	42	62
6		docosahexaenoyl	$C_{31}H_{43}NO_4$	24	52
7	2,4,6-trifluoro	linoleoyl	$C_{24}H_{34}F_3NO$	57	85
8		eicosapentaenoyl	$C_{26}H_{32}F_3NO$	66	96
9		docosahexaenoyl	$C_{28}H_{34}F_3NO$	40	98
10	2,6-difluoro	linoleoyl	$C_{24}H_{35}F_2NO$	9	67
11		eicosapentaenoyl	$C_{26}H_{33}F_2NO$	14	76
12		docosahexaenoyl	$C_{28}H_{35}F_2NO$	12	76
13	2,4,6-trimethyl	linoleoyl	$C_{27}H_{43}NO$	36	76
14		eicosapentaenoyl	$C_{29}H_{41}NO$	38	81
15		docosahexaenoyl	$C_{31}H_{43}NO$	62	81
16	2,6-dimethyl	linoleoyl	$C_{26}H_{41}NO$	75	83
17		eicosapentaenoyl	$C_{28}H_{39}NO$	72	91
18		docosahexaenoyl	$C_{30}H_{41}NO$	59	89
19	2,6-diethyl	linoleoyl	$C_{28}H_{45}NO$	88	95
20		eicosapentaenoyl	$C_{30}H_{43}NO$	91	100
21		docosahexaenoyl	$C_{32}H_{45}NO$	92	97
22	2,6-diisopropyl	linoleoyl	$C_{30}H_{49}NO$	92	97
23		eicosapentaenoyl	$C_{32}H_{47}NO$	96	99
24		docosahexaenoyl	$C_{34}H_{49}NO$	95	98
linoleic amide			$C_{18}H_{33}NO$	-7	3
eicosapentaenamide			$C_{20}H_{31}NO$	-9	14
docosahexaenamide			$C_{22}H_{33}NO$	9	5
Melinamide				11	46

Each concentration was run in single.

Melinamide[4], HL-004[5] and CI-976[4] were also synthesized in-house as positive controls.

Biological method

Microsome preparation and ACAT assay were performed by a modification of the method of Roth et al.[2] Human macrophage ACAT inhibition of the compounds was determined using [1-^{14}C]oleoyl-CoA and microsomes derived from U937 cell which was differentiated to macrophage by phorbol myristate acetate. The inhibitory activity of each compound was tested at the concentrations of 0.5 and 5 μM. **24** was selected and further in vitro investigations of this compound were performed. Microsomes prepared from Caco-2, HepG2 and differentiated U937 cells were used for assays of human intestinal, hepatic and macrophage ACAT inhibitory activities, respectively. Microsomes from intestine, liver and adrenal gland of cholesterol-fed rabbit and beagle dog were also used. Each assay was performed at six dilutions and each concentration was run in triplicate or single. IC_{50} values were calculated by linear regression after probit conversion.

Table 2 ACAT inhibitory activities of 24, HL-004 and CI-976

(IC_{50}, nM)

microsome origin		24	HL-004	CI-976
human	Caco-2	43	113 *	838 *
	HepG2	36	89 *	926 *
	U937	35	130 *	619 *
rabbit	intestine	45	6 *	152 *
	liver	56	15 *	191 *
	adrenal gland	228	554 *	1,952 *
canine	intestine	7,236	281	5,866
	liver	3,567	141	4,590
	adrenal gland	36	137	4,654

Assay was performed at six dilutions and each concentration was run in triplicate or in single (*).

Results and Discussion

Because ACAT plays a central role in foam cell formation from macrophages and smooth muscle cells in atherosclerotic lesions, ACAT inhibition may lead to the prevention and/or treatment of arteriosclerosis. Therefore, we evaluated the compounds for their ability to inhibit human macrophage ACAT activity in the first step. The inhibition percentages of the compounds at 0.5 and 5 μM are shown in Table 1. Anilides containing 2,6-diethyl and 2,6-diisopropyl group (**19-24**) had potent ACAT inhibitory activities. On the contrary, linoleic amide, eicosapentaenamide and docosahexaenamide themselves lacked inhibitory activities. Many anilides containing 2,6-substitution in the aryl ring were reported as ACAT inhibitors.[6,7,8] Similar results were observed in the ACAT inhibitory activities in this study. In preliminary study, **23** and **24** did show the hypocholesterolemic effect after oral administration in cholesterol-fed rat and hamster models (data not shown).

Secondly, the characteristics of **24** were investigated in detail. IC_{50} values of the compound for microsomal ACAT from human cell lines, and those for intestinal, hepatic and adrenal ACAT from rabbit and canine are presented in Table 2. In comparison to HL-004 and CI-976, **24** possessed potent inhibitory effects against human ACAT derived from U937, HepG2 and Caco-2 cells with IC_{50} values of 35, 36 and 43 nM, respectively. **24** also maintained strong inhibitory action against ACAT from rabbit liver and intestine with IC_{50} values of 56 and 45 nM, respectively, suggesting that this animal was suitable as an experimental model to evaluate the compound. It showed less in vitro activity with IC_{50} value of 228 nM against rabbit adrenal ACAT and similar effects on the enzyme activities were observed in the case of HL-004 and CI-976. **24** also showed potent inhibitory activity against canine adrenal ACAT with IC_{50} values of 36 nM, but interestingly, the compound was almost one hundred fold less active against canine hepatic and intestinal ACAT with IC_{50} values of 3,567 and 7,326 nM, respectively. On the other hand, HL-004 and CI-976 did not show such organ-specificity.

The suggestion that multiple cholesterol esterification enzymes may exist in mice, rabbits and rats has been reported. Mouse macrophage ACAT mRNA was expressed highly in the adrenal gland, ovary and macrophage, but relatively low expression was observed in the liver and intestine which were generally known to have significant ACAT activities.[9] Similar findings in rabbits[10] and rats[11] were also reported. In addition, disruption of the gene for mouse macrophage ACAT resulted in markedly reduced cholesterol ester levels in adrenal glands and peritoneal macrophages, while the livers contained substantial amounts of cholesterol esters and exhibited no

reduction in cholesterol esterification activity.[12] Furthermore, ACAT-2, which possibly accounts for the mouse[13,14], primate[15] and human[16] hepatic and intestinal ACAT activity, have recently been cloned. According to the studies, there are at least two subtypes of ACAT in these animals. In the present study, it became clear that canines might also have such kinds of ACAT subtypes.

In this communication, a series of polyunsaturated fatty acid anilides were synthesized and examined as ACAT inhibitors. Because compound **24** possessed strong inhibitory activities against human macrophage, hepatic and intestinal ACAT, it is expected as antiarteriosclerotic and hypocholesterolemic agent for use in humans. It also had similar inhibitory action against ACAT from rabbit liver and intestine, so in vivo hypocholesterolemic effect of **24** in cholesterol-fed rabbit model is under investigation. The synergic hypolipidemic and antiarteriosclerotic effects caused by ACAT inhibitory activities and the physiological activities derived from the characteristics of DHA should be investigated. In addition, since the ACAT inhibitory potency of **24** varied significantly depending on the source of the enzyme, the compound will be useful for the investigations of ACAT subtypes.

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