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INHIBITORS OF ACYL-CoA: CHOLESTEROL *O*-ACYL TRANSFERASE (ACAT) AS HYPOCHOLESTEROLEMIC AGENTS. THE SYNTHESIS AND BIOLOGICAL ACTIVITY OF A SERIES OF MALONESTER AMIDES

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Abstract The synthesis and structure–activity relationships of a series of malonester amide ACAT inhibitors are described. One of these compounds, **4s**, was shown to be a potent inhibitor of both the intestinal and arterial enzymes, bioactive upon oral dosing (ex vivo bioassay) and efficacious in a clinically relevant rodent model of preestablished hypercholesterolemia.

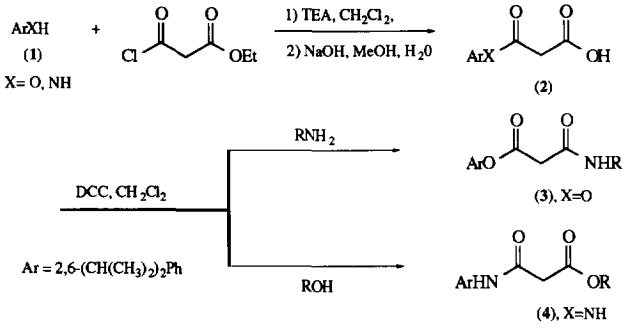
The enzyme acyl-CoA: cholesterol *O*-acyltransferase (ACAT) esterifies free cholesterol in the intestinal epithelia of man and animal species during the process of cholesterol absorption.[1] Thus, when ACAT is inhibited absorption from the intestine is impaired and plasma cholesterol levels decrease. There has been a vigorous search for inhibitors of this enzyme for the treatment of hypercholesterolemia.[2] Such compounds have been uniformly lipophilic in nature and although they inhibit cholesterol absorption in rodent species, most are poorly or erratically absorbed, especially in nonrodent species. Since clinical success with such inhibitors has been poor,[3] there has been an intense search for inhibitors that are well absorbed and thus exhibit plasma levels of active drug that may inhibit the enzyme in the cells of the artery wall.[4] Inhibition of ACAT in monocyte-macrophages of the arterial wall is now a prevailing trend in the field of atherosclerosis prevention, since direct inhibition of arterial ACAT may reduce the formation of macrophage-enriched fatty streaks and contribute to plaque stabilization by direct lipid depletion.[5] Our continuing research has primarily focused on ACAT inhibitors that possess the necessary structural features to achieve sufficient plasma levels of active drug to inhibit the arterial enzyme. In this paper, we describe the synthesis and structure–activity relationships of a series of malonester amides and explain how our screening strategy identifies the best candidates for long-term antiatherosclerotic studies.

Biological Methods: ACAT inhibition was measured in intestinal microsomes isolated from cholesterol-fed rabbits (IAI) and in cultured murine IC-21 macrophages (MAI). Results from these in vitro assays are reported as the micromolar concentration required to inhibit ACAT activity by 50% (IC_{50}).[6] Acute in vivo activity (APCC) was measured in rats dosed with the test compound. After dosing, the ability of the compound to prevent the rise in plasma total cholesterol (TC) after the consumption of a single high fat, high cholesterol meal (termed the PCC diet consisting of peanut oil (PO) (5.5%), cholesterol (C) (1.5%), cholic acid (CA) (0.5%)) was assessed. TC concentration was measured and reported as the percent change from controls.[6] In a two week chronic model (CPCC) hypercholesterolemia was first established (one week, PCC diet) followed by administration of the compounds for one week. Efficacy in this model is defined as (1) a reduction in cholesterol in apo B containing

lipoproteins (non HDL-C), and (2) an elevation in the diet-induced low levels of HDL-C.[6] A rabbit bioassay (ABIO) assessed oral bioactivity by incubating extracts of plasma, from animals dosed with the inhibitors, with rat liver microsomes. The ability of the extract to inhibit the ACAT reaction was assessed and the data were expressed as relative percent inhibition. Adrenal toxicity was assessed in chow-fed guinea pigs. Compounds were given by gavage for two weeks at a dose of 100 mg/kg. Oleic acid was used as the vehicle to facilitate absorption. Toxicity was defined by the incidence, severity, and complexity of adrenal histopathologic alterations in the adrenal cortex of treated animals.

Chemistry: The synthetic route to the target compounds **3** and **4** is illustrated in Scheme 1. Acylation of the anilines (**1**, X = NH) or the phenols (**1**, X = O) with ethyl malonyl chloride in CH_2Cl_2 and triethylamine, followed by saponification of the resulting ester, gave the acids (**2**). Both types of acid (**2**, X = O, NH) were condensed with amines or alcohols, using DCC in CH_2Cl_2 at 0 °C, to give the desired compounds **3** (X = O) and **4** (X = NH), respectively. Racemic 2-tetradecanol (used in the synthesis of **4s**) was resolved by porcine pancreatic lipase in heptane using the method of Klibanov.[7] The resulting R ($[\alpha]^{23}_D$ = -6°, 1.07% in EtOH) and S ($[\alpha]^{23}_D$ = +4°, 1.02% in EtOH) enantiomers were used in the synthesis of **4t** and **4u**, respectively.

Scheme 1

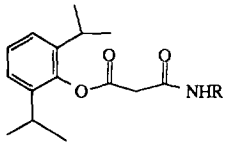


Results and Discussion (Part A): Structure–Activity Relationships: In previous studies with a series of fatty acid anilides, retaining significant ACAT inhibitory activity depended upon replacing the amide bond with isosteres containing hydrogen donor and acceptor functionalities.[8] Additionally, the 2,6-diisopropyl or 2,4,6-trimethoxy substitution pattern in the aniline moiety was necessary for potent ACAT inhibition.[9] Therefore, in vitro potency required both the secondary anilide moiety and the optimal phenyl substitution pattern discussed above.

Thus, reaction of 2,6-diisopropyl phenol with ethyl malonyl chloride, gave after saponification an acid (**2**, X = O), which was then coupled with amines to produce the secondary amides (**3**) necessary for activity. From Table 1, substitution of the anilide with fluorine, irrespective of position, gave compounds (**3b–d**) with weak in vitro and in vivo activities. Surprisingly, the trimethoxy compound (**3e**) was also a weak inhibitor in vitro. However, as expected, introduction of alkyl groups into the 2,6-positions of the anilide gave compound **3f**, which was

modestly potent in vitro but lacked in vivo activity. Increasing the steric bulk of the alkyl groups to the corresponding 2,6-diisopropyl analog (**3g**) gave a compound that was potent in vitro and displayed good efficacy in vivo. Interestingly, the much more hydrophilic compound (**3h**) retained in vitro potency but was less active in vivo. Table 2 shows the effects of retaining the 2,6-diisopropylanilide moiety, but varying the aryl substituent on the ester. Replacing the aryl ester isopropyl groups of compound **3g** by methoxy groups (**4a**) resulted in loss of potency and efficacy, however, the introduction of a third methoxy group at the 4-position **4b** did improve activity in vitro. The best improvements in activities were seen when bulky substituents were introduced at the 2,6-positions, (e.g., **4c**). The benzhydryl ester (**4d**) was very potent in vitro, but attempts to improve in vivo activity by replacing the phenyl groups with a much more hydrophilic 2-pyridyl group gave compounds **4e–f** which were less active both in vitro and in vivo. The cholesterol lowering observed with **4f** was due to severe anorexia.

Table 1



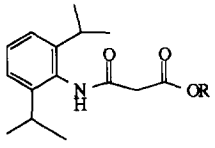
Example[a]	R	IAI (IC_{50}, μM)[b]	APCC(%Δ TC)[c]
3a	Ph	>5	NT[d]
3b	2,4-$(F)_2$Ph	6.4	NT[d]
3c	2,6-$(F)_2$Ph	6.2	+4*
3d	2,4,6-$(F)_3$Ph	>5	-9*
3e	2,4,6-$(OCH_3)_3$Ph	1.0	-29
3f	2,6-$(CH_3)_2$Ph	0.21	+5*
3g	2,6-$(CH(CH_3)_2)_2$Ph	0.042	-50
3h	2,6-$(N(CH_3)_2)_2$Ph	0.071	-29

[a] Analytical results are within ± 0.4% of the theoretical values. [b]ACAT inhibition in vitro, intestinal microsomes isolated from cholesterol-fed rabbits. Each determination performed in triplicate. [c] Denotes percent change in total cholesterol in cholic acid (0.5%)-cholesterol (1.5%)-peanut oil (5.5%)-fed rats. The standard dose was 30 mg/kg. Unless otherwise noted (by an asterisk as non significant), the changes were significantly different from control, $p<0.05$ using analysis of variance followed by Fisher's multiple range test. [d] Not tested.

Increasing the chain length of the alkyl esters, in general, led to increased in vitro potency and in vivo efficacy. The dodecyl ester (**4l**) was optimum for in vitro potency, however chain lengths varying from C_{12} (**4l**) to C_{16} (**4n**) all displayed similar in vivo activity at the screening dose of 30 mg/kg PO. When the chain length reached C_{18} (**4o**) the in vivo activity was greatly reduced, probably due to detrimental effects of extreme lipophilicity on in vivo absorption. In general, branching the alkyl chain resulted in much more potent compounds. As with the straight chain compounds, increasing the length improved the in vitro potency. Compounds **4r** and**4s** are two of the most potent ACAT inhibitors reported to date. The effect of chirality on biological activity for compound (**4s**) was examined and it was shown that there was little difference between the R (**4t**) and S (**4u**) forms, both in vitro and in vivo. Addition of another methyl group at the branch point of **4s** gave **4y**, which displayed equivalent potency and efficacy. In addition, by increasing the branching, shorter alkyl chains were used to yield much more potent

and efficacious inhibitors than was expected. Thus, compound **4x** was shown to be a potent and efficacious inhibitor, which is surprising when one considers the data for the straight chain alkyl esters of comparable length (**4i** and **4j**). Several of the more active compounds in Tables 1 and 2 were then evaluated in the much more clinically relevant model of pre-established hypercholesterolemia (CPCC). As can be seen from Table 3, only compounds **4r** and **4s** showed significant efficacy in this model. The striking difference in efficacy shown by compounds **4l** and **4m** when compared to efficacy displayed by **4r** and **4s** may be due to the beneficial steric effects of the α-substituents in **4r** and **4s** in the prevention of possible in vivo hydrolysis of the ester. The lack of efficacy observed with **4y** is surprising, but may be due to the increased lipophilicity and the possible detrimental

Table 2



Example[a]	R	IAI (IC_{50}, μM)[b]	APCC(%Δ TC)[c]
4a	2,6-$(OCH_3)_2$Ph	1.7	+5*
4b	2,4,6-$(OCH_3)_3$Ph	0.32	-9*
4c	2,6-$(Ph)_2$Ph	0.067	-41
4d	$CH(Ph)_2$	0.038	-41
4e	CH(Ph)2-Pyridyl	>5	NT[d]
4f	CH(2-Pyridyl)$_2$	>5	-55
4g	H	>5	NT[d]
4h	CH_2CH_3	1.8	NT[d]
4i	$(CH_2)_5CH_3$	0.81	-29
4j	$(CH_2)_7CH_3$	0.26	-43
4k	$(CH_2)_9CH_3$	0.053	-37
4l	$(CH_2)_{11}CH_3$	0.011	-52
4m	$(CH_2)_{13}CH_3$	0.015	-61
4n	$(CH_2)_{15}CH_3$	0.035	-56
4o	$(CH_2)_{17}CH_3$	0.053	-16
4p (RS)	$CH(CH_3)(CH_2)_4CH_3$	0.027	-46
4q (RS)	$CH(CH_3)(CH_2)_7CH_3$	0.012	-61
4r (RS)	$CH(CH_3)(CH_2)_9CH_3$	0.006	-70
4s (RS)	$CH(CH_3)(CH_2)_{11}CH_3$	0.009	-73
4t (R)	$CH(CH_3)(CH_2)_{11}CH_3$	0.004	-67
4u (S)	$CH(CH_3)(CH_2)_{11}CH_3$	0.004	-66
4v	$CH(CH_3)(CH_2)_{13}CH_3$	0.011	-60
4w	$C(CH_3)_3$	0.13	-24
4x	$C(CH_3)_2(CH_2)_3CH_3$	0.015	-52
4y	$C(CH_3)_2(CH_2)_{11}CH_3$	0.012	-72

See Table 1 for footnotes.

effects on absorption. Compound **4s** lowered non HDL-C by 83 % at a dose of 10 mg/kg and elevated HDL-cholesterol by 157 % at the same dose, even at doses as low as 1 mg/kg, **4s** lowered non HDL-C by 54 % and

elevated HDL-C by 64 %. In contrast, **4r** produced no significant decreases in non HDL-C or significant elevations in HDL at this dose.

In summary, the SAR studies show that the presence of a bulky 2,6-disubstituted anilide and a long chain alkyl ester moiety gave compounds that are potent ACAT inhibitors in vitro and are very effective at limiting the rise in plasma total cholesterol in rats fed a high fat, high cholesterol meal. The presence of branching in the alkyl ester gave compounds that were also very effective at decreasing plasma total cholesterol (and elevating HDL-cholesterol) in a model of preestablished hypercholesterolemia. After a consideration of the data shown in Tables 1, 2, and 3, compound **4s** was chosen for a more in-depth pharmacological evaluation.

Table 3

		CPCC
Example	**Dose (mg/kg)**	**%Δ non HDL-C(%Δ HDL)**
3g	30	-9 (NC[a])*
4l	30	-8 (-21)*
4m	30	-11(+6)*
4r	1	-15(NC[a])*
	3	-40(+29)
	10	-51(+36)
4s	1	-54(+64)
	3	-73(+114)
	10	-83(+157)
4v	0.1	-8 (-12)*
	1	+7(-24)*
	3	-2(+13)*
4y	0.1	-11 (-12)*
	1	-19(-13)*
	3	-19(+6)*

Unless otherwise noted (by an asterisk as non significant), the changes were significantly different from control, $p < 0.05$ using analysis of variance followed by Fisher's multiple range test. [a] No change.

(Part B): In-depth pharmacological evaluation of compound (4s): Compound **4s** is a potent inhibitor of microsomal ACAT and a potent hypocholesterolemic agent in two rodent models of hypercholesterolemia, both acutely and chronically. The compound was then evaluated in a cellular assay employing a cell line (murine IC-21 macrophages) relevant to the tissue of interest, in this case, the artery wall. In this assay, compound **4s** had an IC_{50} of 10 nM for inhibition of cholesterol esterification. Thus, we have established that if the drug (or active metabolite) reaches the target tissue it will inhibit potently the enzyme of interest. To measure this bioactivity, we incorporated a bioassay into our screening strategy. In this assay, compound **4s** was administered to rabbits at 50 mg/kg and four hours later plasma from the animals was extracted and the extracts evaluated for their ability to inhibit ACAT. At this dose, ACAT was inhibited 99.6 % at this timepoint. Similar data was obtained at a two-hour timepoint. The identity of the bioactive species (parent or active metabolite) was not established.Toxicological effects of a variety of ACAT inhibitors have most consistently been observed in the adrenal glands of some species.[10] Guinea pigs have been a suitable model for evaluating the adrenal effects of ACAT inhibitors. This species has a relatively small size that allows screening at relatively high doses using small quantities of drug. It was also found to be very sensitive (as were dogs and rabbits) to the adrenotoxic effects of ACAT inhibitors. Rats

and hamsters were shown to be relatively insensitive to adrenotoxic effects of this compound class.[11] Compound **4s** was classed as highly toxic to the adrenal gland of guinea pigs when dosed at 100 mg/kg PO. Cytotoxic zonal atrophy of the zona fasciculata (near complete loss of the zona fasciculata due to cortical cell necrosis and degeneration) was evident in all treated animals; atrophy of the zona reticularis was consistently observed. Inflammatory cell infiltrates were present in areas of cortical necrosis/degeneration. Coarse vacuoles were present in surviving cortical cells bordering degenerative zones in a small number of animals. Due to the adrenal toxicity compound development was discontinued and no further evaluations of this compound were performed.

Conclusions: We have identified a series of malonester amides that were shown to be potent ACAT inhibitors in vitro and hypocholesterolemic agents in vivo. We have also developed a screening strategy designed to identify inhibitors capable of inhibiting the enzyme at the target tissue, the artery wall. Before the initiation of long-term antiatherosclerotic studies, the toxicity of the test compound was assessed in guinea pigs. The pharmacological evaluation of **4s**, outlined in this paper, illustrates some of the difficulties faced in the identification of an ACAT inhibitor with activity at the artery wall. The major question facing most groups in this area is whether the adrenal toxicity observed is mechanism-related or compound-specific. If the toxicity is mechanism related, the design of a compound that reaches one desirable peripheral target (the artery wall) but does not reach another undesirable peripheral target (adrenal gland) may be a difficult one to achieve. Our efforts to identify such an agent will be the subject of further communications from these laboratories.

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