Effect of membrane environment on inhibition of acyl-CoA:cholesterol acyltransferase by a range of synthetic inhibitors

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Abstract

The effect of the membrane environment of acyl-CoA:cholesterol acyl transferase (ACAT), an important intracellular enzyme of cholesterol metabolism, on the properties of a range of inhibitors of varying potencies was studied. ACAT activity from rat liver was solubilised with 3% deoxycholate (97% solubilised activity). After dilution into cholesterol/phosphatidylcholine liposomes (molar ratio 0.35), the assay of this reconstituted system showed linearity with protein and time. Saturation with oleoyl-CoA was achieved at 10 μM. Comparison of the potency of the ACAT inhibitors in the reconstituted assay and in a microsomal assay revealed a relationship between the lipid content of the assay and the inhibitory activity for potent inhibitors of ACAT (CI976, CL277,082, YM17E and DuP128). This relationship was unrelated to lipophilicity of the drugs. Octimibate, lovastatin and progesterone, none of which is a potent ACAT inhibitor but which have all been described as ACAT inhibitors in the literature, all had low potencies in both assay systems. These results suggest that the lipid concentration must be taken into account when comparing potencies of ACAT inhibitors. The present data also indicate that some compounds which inhibit cholesterol esterification may do so by an indirect mechanism.

Keywords: Inhibition; Acyl CoA:cholesterol acyltransferase; Membrane environment

1. Introduction

Acyl-CoA:cholesterol acyltransferase (ACAT) is a central enzyme of intracellular cholesterol metabolism. It is an integral membrane protein found in the endoplasmic reticulum which esterifies cholesterol with long chain fatty acids. The resulting cholesteryl ester is frequently found stored in the cell as lipid droplets. In this way the concentration of intracellular free cholesterol is regulated within a narrow range thereby maintaining the integrity of cell membranes [1–4].

In monocyte-derived macrophages ACAT esterifies cholesterol which has been taken up from oxidised LDL and released to the cell after lysosomal hydrolysis. The resulting accumulation of cholesteryl ester is characteristic of the macrophage derived foam cell found in atherosclerotic lesions [5,6]. Hence inhibitors of ACAT might be expected to prevent the formation of lipid droplets in macrophages and prevent the formation of foam cells. Numerous ACAT inhibitors have been synthesized and some have reached clinical studies but up to the present none has shown success in the clinic [7].

ACAT has resisted purification for very many years. Recently Chang et al. [8] have reported the cloning and expression of a cDNA considered to be that of human ACAT. However, the activity was detected as increased synthesis of cholesteryl ester in cells and purified protein is not yet available. Because of the absence of a purified preparation ACAT inhibitors have only been studied either in cell culture or in vitro using a microsomal preparation from the tissue of interest. In cell culture activity is measured indirectly by the incorporation of labelled fatty acid into cholesteryl ester [9], whereas in the microsomal system radiolabelled fatty acyl CoA can be used as substrate [10].

Since these assay systems are complex the precise environment of ACAT cannot be defined. For example, the concentration of substrate cholesterol is not known and
may not be present at saturating levels [11]. The nature of the membrane phospholipids can affect ACAT activity [11–14] and there some evidence that modulators of ACAT activity are present in the intracellular membranes [15]. Saturation of ACAT with substrate cholesterol can be achieved to a certain extent by adding exogenous cholesterol to a microsomal assay in liposomes [11] or as a dispersion in Triton WR1339 [16] but even here the concentration of cholesterol in the immediate environment of the enzyme is not defined.

An earlier approach to produce a more controlled system involved the solubilisation of ACAT from the microsomal membrane using detergent, followed by reconstitution of the solubilised proteins into liposomes of defined lipid composition. Such a procedure was used with Ehrlich Ascites cell microsomes [17,18] and rat liver microsomes [11]. In both cases Triton X-100 was used for solubilisation. In other studies cholate or deoxycholate was used for solubilisation [15,19]. Detergents such as these inhibit ACAT activity and must be removed to allow activity to be assayed. These early methods were very time consuming and a more rapid method [20] was devised that allowed for removal of the detergent by dilution of the solubilised microsomal protein into liposomes.

ACAT inhibitors have only been studied in in vitro systems using assays based on microsomal fractions or intact cells in culture. Since it is clear that the membrane environment of ACAT has a significant effect on its in vitro activity and since the membrane environment of ACAT in a reconstituted system can be controlled to some extent, we compared a range of ACAT inhibitors in several systems. An understanding of the mechanism of action of the inhibitors is critically dependent on the interpretation of the quantitative data obtained in these assay systems and the differences reported here show that quantitative comparisons need to be done bearing the assay system more fully in mind than has been the case in the literature. Some of the data in this paper have been reported in abstract form (Biochemical Society Transactions (1993) 21, 325S).

2. Materials and methods

2.1. Materials

L-α-Phosphatidylcholine (type V-E from egg yolk), cholesterol, Ficoll (Type 400), cholestyramine, sodium deoxycholate, dimethylsulfoxide, oleoyl CoA and oleic acid were from Sigma (Poole, Dorset). [1–14C]Oleoyl CoA (52.7 mCi/mmol), [1α, 2α(α±)-3H]cholesterol oleate (49.6 mCi/mmol), [9,10(α−3H]oleic acid (10 mCi/mmol) and cholesteryl [1–14C]oleate (52 mCi/mmol) were supplied by Amersham (Amersham, Bucks.). Fatty acid-free BSA (fraction V), phospholipase D (20 U/mg from Streptomyces chromofuscus), peroxidase (1000 U/mg from horseradish) and choline oxidase (10 U/mg protein from Athrobacter globiformis) were from Boehringer Mannheim (Lewes, East Sussex). Techelut silical gel columns were from HPLC Technology (Macclesfield, Cheshire). Gibco BRL (Uxbridge, Middlesex) supplied foetal calf serum, trypsin-EDTA and L-glutamine. ICN Flow (High Wycombe, Bucks.) supplied DMEM, DMEM(Hepes) and PBS, TCA and ATP monitoring reagent came from Lab-systems Group (UK) Ltd. (Basingstoke, Hants.). Ames Sera-Pak cholesterol assay kits were from Bayer diagnostics (Basingstoke, Hants.). Protein assay dye concentrate was supplied by Bio-Rad laboratories (Hemel Hempstead, Herts.). CaCo2 cells were from the European Collection of Animal Cell Cultures. ACAT inhibitors were synthesised in the Medicinal Chemistry Laboratories of SmithKline Beecham Pharmaceuticals. All compounds were fully characterised by elemental analysis, 1H-NMR, IR and mass spectra.

2.2. Animals

Male Wistar rats were fed a standard laboratory diet. Male Sprague-Dawley rats were also used and were maintained on the same diet alone or supplemented with 2% cholesterol.

2.3. Preparation of microsomes

The livers of male Wistar rats (killed by cervical dislocation) were homogenised in four volumes of buffer A (0.25 M sucrose, 10 mM Tris-HCl, 1 mM EDTA, 0.1 mM benzamidine, pH 7.4) using a Polytron homogeniser. Sprague-Dawley rats were anaesthetised with diethyl ether and the livers perfused with 0.104 M KCI/0.05 M NaF, pH 7.4. The liver was homogenised, using three strokes of a glass-teflon homogeniser, in the same solution used for perfusion. Microsomes were then prepared by the method of Boyd et al. [21]. Wistar rat microsomes were stored in buffer A at −70°C and Sprague-Dawley rat microsomes were stored freeze-dried at −20°C. Protein was measured using the method of Bradford [22]. In the reconstituted ACAT assay (see below) microsomes from Sprague-Dawley rats gave an activities within the range 100–245 pmol/min/mg protein, depending on the activity of the original preparation. Microsomes from Wistar rats gave a range of 80–700 pmol/min/mg protein.

2.4. Microsomal assay

The microsomal assay was that used by Suckling, Boyd and Smellie [11]. Assays contained 0.5 mg microsomal protein, 0.5 mg fatty acid-free BSA and 0.5 mg reduced glutathione in 0.05 M KPO4/0.05 M NaF buffer (pH 7.4). After a preincubation of 5 min at 37°C with compounds
added in DMSO (controls were present both without DMSO and with DMSO alone), 38.8 nmol of [14C]oleoyl CoA was added to start the assays (final concentration 78 μM, 0.020 μCi/ml). The total assay volume was 0.5 ml. Assays were then incubated for a further 3 min and the reactions were stopped by the addition of 1 ml methanol. A recovery marker of [3H]cholesteryl oleate (approx. 25000 dpm) was added and lipid was extracted with dichloromethane/methanol (2:1, v/v) [23]. Extracts were dried down under nitrogen and the reaction products separated on silica gel columns.

2.5. Solubilisation and reconstitution

This method was based on that used by Cadigan and Chang [20]. Liposomes were prepared by the method of Ventimiglia et al. [24]. They contained 1.5 mg/ml cholesterol and 10 mg/ml phosphatidylcholine. For solubilisation, a deoxycholate/phosphatidylcholine mixture was added to microsomes to give final concentrations of 3% (w/v) deoxycholate, 4 mg/ml phosphatidylcholine and 10 mg/ml microsomal protein. This was incubated on ice for 20 min and then an aliquot of this solubilised protein was reconstituted by dilution by a factor of 1 in 240 into cholesterol/phosphatidylcholine liposomes. The molar ratio of cholesterol/phosphatidylcholine was 0.35 using 894 as the average molecular weight for phosphatidylcholine. Reconstitution was carried out for 10 min on ice.

2.6. Reconstituted ACAT assay

ACAT assays contained 5 μg fatty acid-free BSA, 0.1 mM dithiothreitol and 3.3 μg reconstituted protein in 50 mM Tris-HCl, 1 mM EDTA (pH 7.7). The assays were started by the addition of 1 nmol [14C]oleoyl CoA (final concentration 10 μM, 0.5 μCi/ml) in a total assay volume of 0.1 ml. Incubations were for 10 min at 37°C. Where compounds were being tested, they were added in 5 μl DMSO and a 5 min preincubation was carried out. Reactions were terminated by the addition of propan-2-ol/heptane (4:1 (v/v), 1 ml) with a recovery marker of [3H]cholesteryl oleate (approx. 25000 dpm). Lipids were then extracted by the addition of heptane (0.4 ml) and water (0.6 ml), removing 0.4 ml of the upper layer [25].

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

![Graph D](image4.png)

Fig. 1. Density gradient ultracentrifugation of microsomal protein after reconstitution. Samples were applied to a discontinuous two-step Ficoll gradient (10% Ficoll and 6% Ficoll on a 50% sucrose bed) and centrifuged at 50000 rpm in a Beckman SW60 rotor for 6 h at 4°C. Six fractions of 0.8 ml were collected from each gradient and ACAT activity and phosphatidylcholine were measured as described in the Materials and methods. Bar graphs show ACAT activity (unfilled bars) and phosphatidylcholine content (filled bars) of the fractions. (A) Liposomes only. (B) Rat liver microsomal protein only. (C) A mixture of liposomes and microsomal protein. (D) Microsomal protein reconstituted into liposomes. Assays of ACAT activity were carried out after reconstitution of the protein obtained from the fractions. The specific activity was 1230 pmol/min/mg protein. B contained 1 mg protein. C and D contained 0.32 mg protein. A, C and D contained 0.67 mg cholesterol and 4.48 mg phosphatidylcholine. Recoveries of phosphatidylcholine for each sample were: (A) 97%; (C) 94% and (D) 89%. Results shown are those from one representative experiment.
which was then applied to silica gel Techelut columns, primed with 1 ml petroleum spirit. Cholesteryl esters were eluted with petroleum spirit/diethyl ether (95:5 (v/v), 2 ml) and radioactivity was determined by scintillation counting after evaporation of the organic solvent [26].

2.7. Density gradient ultracentrifugation

To prepare the gradient, 0.27 ml 50% sucrose was pipetted into a centrifuge tube and 0.5 ml 10% Ficoll layered on top of this and a further 3 ml 6% Ficoll was added at the top. For samples containing liposomes, 0.48 ml was added to 0.24 ml of 50% sucrose and this was layered onto the sucrose bed before the addition of 10% Ficoll. Samples containing protein alone were added to the top of the gradient in a volume of 0.72 ml [20]. Gradients were centrifuged at 50000 rpm for 6 h in a Beckman SW60 rotor at 4°C.

2.8. Culture of CaCo2 cells

CaCo2 cells (a human colonic cell line that expresses some intestinal cell characteristics at confluence, obtained from ECACC) were cultured in DMEM supplemented with 20% foetal calf serum and 2 mM glutamine in 75 cm² flasks. Cells were passaged at a dilution of 1 to 10 using 0.05% trypsin-0.02% EDTA for cell detachment and then cells were collected by centrifugation. Cells were grown for 5–6 days until confluent and then plated out (2 ml per well) on 12 well Costar plates and grown again for 7 days until confluent.

2.9. Measurement of oleic acid esterification in CaCo2 cells

The incorporation of oleic acid into cholesteryl ester in CaCo2 cells was based on the method described by Murphy et al. [27] Briefly, the medium was replaced with 600 µl DMEM(Hepes) containing 1% (w/v) fatty acid-free BSA and supplemented with 2 mM glutamine (which had been filtered through a 0.2 µm filter); plates were incubated for 20 min at 37°C. Compounds were added in 10 µl DMSO to wells in triplicate; control wells either contained DMSO or no additions. After an incubation of 1 h, medium was added containing [3H]oleic acid (900 nmol, 5 µCi/ml) in DMEM(Hepes) with 2 mM glutamine and 10.5% (w/v)
fatty acid-free BSA. Plates were further incubated for 2 h. Each well was then washed with 1 ml phosphate-buffered saline (PBS) and cells were removed into 1 ml 0.05% trypsin-0.02% EDTA. Lipids were extracted using dichloromethane/methanol (2:1 v/v) and extracts dried down under nitrogen and added to silica gel columns in 1 ml hexane. Cholesteryl oleate was eluted with 3 ml hexane/diethyl ether (95:5, v/v). For protein determination [28] cells were removed with 0.5 ml 0.2 M NaOH and diluted 1 in 3 with distilled water. Toxicity of compounds to the cells was evaluated by the measurement of ATP levels in the cells. ATP was measured by the luciferase technique, measuring light emission in a LKB Wallac 1250 Luminometer.

2.10. Determination of cholesterol and phosphatidylcholine concentrations in liposomes and microsomes

Total cholesterol was measured using an Ames Sera Pak cholesterol kit. Phosphatidylcholine was measured by the method of Takayama et al. [29].

3. Results

3.1. Solubilisation and reconstitution of ACAT

In preliminary experiments several detergents were tested for their ability to solubilise ACAT activity [30]. Triton X-100, Triton N-101 and deoxycholate were compared. Of these deoxycholate was the most effective at solubilisation of activity and activity was most reliably recovered from the soluble fraction. Since significant changes were made over published procedures, it was necessary to characterise the reconstituted preparation fully. The microsomal fraction from Wistar rat liver was incubated with varying concentrations of deoxycholate and the ACAT activity determined after reconstitution in phosphatidylcholine/cholesterol liposomes. Virtually all the ACAT activity (97.3%) was found in the supernatant when 3% deoxycholate was used. A smaller fraction of activity was found after treatment of microsomes with 1% or 2% deoxycholate (87.4% and 91.2%, respectively). Solubilised

![Fig. 3. Structures of ACAT inhibitors. (A) CI 976; (B) octimidate; (C) CL 277,082; (D) YM 17E; (E) DuP 128.](image-url)
Table 1
Potency of compounds for the inhibition of cholesterol esterification in microsomal, reconstituted and cellular systems

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC_{50} (μM)</th>
<th>IC_{50} drug/lipid (μmol/g)</th>
<th>IC_{50} (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>microsomal</td>
<td>reconstituted</td>
<td>microsomal</td>
</tr>
<tr>
<td>CI 976</td>
<td>0.15 ± 0.01</td>
<td>6.3 ± 1.2</td>
<td>0.41 ± 0.03</td>
</tr>
<tr>
<td>Octimibate</td>
<td>39.6 ± 2.4</td>
<td>40.8 ± 4.9</td>
<td>110.0 ± 5.6</td>
</tr>
<tr>
<td>CL 277,082</td>
<td>0.65 ± 0.04</td>
<td>24.4 ± 3.1</td>
<td>1.80 ± 0.11</td>
</tr>
<tr>
<td>YM 17E</td>
<td>0.011 ± 0.001</td>
<td>0.16 ± 0.02</td>
<td>0.03 ± 0.002</td>
</tr>
<tr>
<td>DuP 128</td>
<td>0.004 ± 0.0002</td>
<td>0.11 ± 0.02</td>
<td>0.01 ± 0.001</td>
</tr>
<tr>
<td>Lovastatin</td>
<td>115 ± 7.7</td>
<td>549 ± 53</td>
<td>274 ± 18</td>
</tr>
<tr>
<td>Progesterone</td>
<td>44.1 ± 4.5</td>
<td>173 ± 21</td>
<td>105 ± 11</td>
</tr>
</tbody>
</table>

Values are means ± S.E. and are for a typical experiment where assays were carried out in duplicate. Progesterone and lovastatin were studied only in the microsomal and reconstitution assays as a comparison to the more potent, specific ACAT inhibitors. Total lipid for the microsomal assay was 0.2 mg and for the reconstitution assay it was 0.92 mg. IC_{50} values were calculated by a Grafit software package, version 3.01, with a defined endpoint at 0 and simple weighting for microsomal and reconstituted ACAT assays and using a 4 parameter logistic with simple weighting for CaCo2 cell assays for cholesterol esterification.

protein alone showed no activity, consistent with previous findings [20]. The reconstitution was carried out for periods of time from 5–75 min and gave similar results over this range of times.

The reconstitution of enzyme activity into liposomes was demonstrated using a 2-step Ficoll gradient (Fig. 1A–D). Liposomes were found to remain at the top of the gradient (Fig. 1A) and microsomal protein at the bottom.
(Fig. 1B). An unreconstituted mixture of microsomal protein and liposomes separated into the 2 component fractions (Fig. 1C). However, following reconstitution the ACAT activity remained associated with the liposomes (Fig. 1D).

3.2. Optimisation of the assay of ACAT activity in the reconstitution system

The effect of varying the extent of dilution of the solubilised microsomal fraction into liposomes on ACAT activity is shown in Fig. 2A. The activity increased linearly as the detergent concentration was reduced by dilution, up to 120-fold. At a dilution of 1 in 240 the activity had become maximal. In this experiment the protein/lipid ratio was decreased as the dilution increased. In a parallel experiment, in which the protein/lipid ratio was maintained at a constant level, activity still increased, although at a much lower rate, up to a dilution of 1 in 720. A dilution of 1 in 240 was chosen as a compromise between dilution and measurable ACAT activity.

The synthesis of cholesteryl ester was linear with respect to time up to 15 min and with respect to protein up to 7.5 μg. The detergent/protein ratio was maintained at 1.5%/mg.

Saturation of activity with oleyl-CoA occurred at approx. 10 μM (Fig. 2B) with a calculated (calculated by Grafit Software package) $K_m$ of $5.3 \pm 1.3 \, \mu M$ (means ± S.E.). Saturation of the enzyme with cholesterol was demonstrated by reconstituting the solubilised fraction into liposomes of increasing cholesterol/phosphatidylcholine molar ratio (Fig. 2C), the cholesterol content of each preparation of liposomes being determined by colorimetric enzymatic assay. The activity was maximal at a cholesterol/phosphatidylcholine molar ratio of $0.43 \pm 0.03$ (S.D.) and remained at this level up to a ratio of $0.48 \pm 0.02$. ACAT activity at a molar ratio of $0.66 \pm 0.06$ was significantly lower than that at a molar ratio of $0.33 \pm 0.03$ (Student’s t-test for 2 independent samples ($P < 0.05$)).

The reproducibility of the reconstituted assay was demonstrated by making 10 microsomal preparations from different rats and assaying ACAT activity in the reconstrui-
Table 2
Ratios of IC_{50} values in the microsomal and reconstitution assays and the calculated values for lipophilicity (clog P) of the compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ratio of microsomal/reconstituted IC_{50} values</th>
<th>Lipophilicity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>concentration drug/lipid</td>
<td>clogP</td>
</tr>
<tr>
<td>CI 976</td>
<td>0.02 ± 0.005</td>
<td>6.8</td>
</tr>
<tr>
<td>Octimibate</td>
<td>1.0 ± 0.1</td>
<td>8.6</td>
</tr>
<tr>
<td>CL 277,082</td>
<td>0.03 ± 0.004</td>
<td>8.5</td>
</tr>
<tr>
<td>YM 17E</td>
<td>0.07 ± 0.01</td>
<td>9.7</td>
</tr>
<tr>
<td>DuP 128</td>
<td>0.04 ± 0.008</td>
<td></td>
</tr>
<tr>
<td>Lovastatin</td>
<td>0.2 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>Progesterone</td>
<td>0.3 ± 0.04</td>
<td></td>
</tr>
</tbody>
</table>

Values are shown as means ± S.E. clog P is the log of the calculated partition coefficient for compounds between water and an organic solvent such as cyclohexane. Log P values were calculated using the CLOGP module of the Pamona Med Chem Software, Version 3.5.

3.3. Comparison of the effect of ACAT inhibitors in different assay systems

The effects of five ACAT inhibitors were compared: CI-976, octimibate, CL-277,082, YM17E and DuP 128 [7]. The structures are shown in Fig. 3. Progesterone has been used as an inhibitor of ACAT activity in cell culture experiments for many years and lovastatin, an HMG-CoA reductase inhibitor has been reported to affect cholesterol esterification in cell culture so these two compounds were also studied, although their inhibitory activity in vitro is very weak. IC_{50} values were determined in the microsomal fraction for all of these compounds (Table 1). The order of potency was DuP 128 > YM17E > CI-976 > CL 277,082 > octimibate > progesterone > lovastatin. Typical concentration response curves are shown in Fig. 4. All the inhibitors were found to be less potent in the reconstituted assay except for octimibate which had approximately the same IC_{50} in the microsomal and reconstituted systems (39.6 and 41.8 μM, respectively). The overall order of potency remained the same. The reconstituted system contains significantly more lipid than the microsomal assay (0.92 mg/assay compared with 0.2 mg). Clearly this increased amount of lipid could affect the effective concentration of the inhibitor in the membrane. The results were therefore recalculated as a function of the drug to lipid ratio rather than the nominal concentration. Typical curves are shown in Fig. 5. When this was done the IC_{50} values of DuP 128 and CL 277,082 became similar but there was still a significant difference between the values for CI-976 and YM17E (Table 2). For octimibate the difference in potency between the 2 assay systems was 25-fold and for progesterone and lovastatin the differences were even larger.

3.4. Studies of inhibitors in CaCo2 cells

The 5 ACAT inhibitors were also examined for their effect on cholesterol esterification in CaCo2 cells (Table 1). The potency of all the inhibitors was lower in the cell culture system suggesting that the effective concentration of inhibitor achieved inside the cell is less than that in the microsomal system. The IC_{50} values in the CaCo2 cells were of a similar order of magnitude to those in the reconstituted assay. The effect of the compounds on the viability of the cells was assessed by the measurement of intracellular ATP concentrations. 1 mM octimibate reduced intracellular ATP concentrations to 77% of controls and at 3 mM the ATP concentrations had dropped to 20% of controls. The other compounds showed no effect on intracellular ATP content at the concentrations used.

4. Discussion

We have developed and optimised a highly reproducible and rapid technique based on published methods for reconstituting rat liver microsomal ACAT. Very small amounts of protein are required for the measurement of activity. Previous methods [11,17] used 0.1 to 1 mg of protein and more recently [20] 30 μg of protein from CHO cells and 60 μg from human skin fibroblasts was required. In the present system as little as 3.3 μg were needed.

The comparison of the potency of the ACAT inhibitors in the microsomal and reconstituted assay systems raises some interesting points. Clearly octimibate, progesterone and lovastatin are not very potent ACAT inhibitors and may well affect activity by indirect mechanisms. The more potent ACAT inhibitors are highly hydrophobic molecules. It is therefore reasonable that their potencies as inhibitors should depend markedly on the drug to lipid ratio. It is probable that this ratio is a more meaningful measure of concentration than the nominal concentrations that are commonly used in published reports. Factors other than lipophilicity clearly participate since the potency of the...
compounds does not relate directly to the calculated log $P$ (Table 2). For example CL-277,082 and YM17E have quite different IC$_{50}$ values but their calculated logP values are the same. The situation is clearly more complex in assays in cell culture where there are many more pools into which the inhibitor can partition and more membranes that must be penetrated for the compounds to gain access to the enzyme.

The literature on ACAT inhibitors contains many estimates of potencies of inhibitors measured in assay systems with widely varying lipid and protein content [7,31,32]. In some cases authors attempt to draw conclusions as to relative potencies and activities of these ACAT inhibitors [31,32]. The present work shows that comparisons of such data can be misleading. The dependence of the IC$_{50}$ on the lipid composition of the assay system shows that the hydrophobicity of the inhibitor plays a major role in the measured activity. Some compounds frequently referred to as ACAT inhibitors may not even interact directly with the enzyme (ocitamate and progesterone) [33]. Given the complex nature of all the assay systems for ACAT activity it is unlikely that meaningful kinetic parameters ($K_i$ for example) can be obtained for any ACAT inhibitor and it would be more correct to refer to these many of compounds as ‘inhibitors of cholesterol esterification’ rather than ‘ACAT inhibitors’. There has been considerable interest in the measurement of ACAT activity and interest has increased with the discovery of compounds that appear to inhibit this enzyme at very low concentrations in both subcellular fractions and in cultured cells. However, there is still little known about the enzyme itself. Attempts to purify and characterise this protein have proved unsuccessful although two reports on cloning and expression have been published [8,34], interestingly claiming two quite different proteins as ACAT. As a result the concentration of substrates, cofactors and potential inhibitors in the immediate environment of the enzyme remain unknown. Recently the gene described by Chang et al. [8] has been expressed in an insect cell line Sf9 [35], which has no endogenous ACAT activity. The IC$_{50}$ values for CI 976 and for ocitamate are similar to those reported here for the reconstituted assay. That for DuP 128 is somewhat higher, but of the same order of magnitude. In this work [35] a reconstituted system was used for the measurement of ACAT activity. No data are available on the effect of inhibitors of ACAT on the gene cloned by Becker et al. [34]. Inhibition of the rate of intracellular cholesterol esterification is recognised as a possible target for intervening in the formation of atherosclerotic plaques. Compounds that either reduce the rate of progression of lipid deposition in arteries, or cause regression of those deposits could form the next generation of drugs for the treatment of the major cause of death in the western world. We have shown in this report that the basis for selecting compounds for further development may have been flawed, in that the reduction in the rate of cholesterol esterification by so-called ACAT inhibitors depends very largely on the lipid content of the assay system used, and that some compounds may reduce cholesterol esterification by indirect mechanisms, and not by directly interacting with the enzyme. This reconstituted assay system may be the method of choice for determining the relative potencies of inhibitors of cholesterol esterification until it is possible to use the purified enzyme, which itself may well need to be reconstituted in a similar system.

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