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where it inhibits carnitine palmitoyltransferase 1 and
decreases food intake and body weight**

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1 RUNNING TITLE: Hypothalamic inhibition of CPT1 by C75-CoA.
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4 ABBREVIATIONS: ARC, arcuate nucleus; CPT, carnitine palmitoyltransferase; CrAT,
5 carnitine acetyltransferase; FAS, fatty acid synthase;; wt, wild-type.
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11 KEYWORDS: Fatty acid metabolism, anti-obesity drugs, C75, CPT1 carnitine
12 palmitoyltransferase, FAS fatty acid synthase.
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ABSTRACT

1
2 Central nervous system administration of C75 produces hypophagia and weight loss in
3
4 rodents identifying C75 as a potential drug against obesity and type 2 diabetes.
5
6 However, the mechanism underlying this effect is unknown. Here we show that C75-
7
8 CoA is generated chemically, *in vitro* and *in vivo* from C75 and that it is a potent
9
10 inhibitor of carnitine palmitoyltransferase 1 (CPT1), the rate-limiting step of fatty acid
11
12 oxidation. Three-D docking and kinetic analysis support the inhibitory effect of C75-
13
14 CoA on CPT1. Central nervous system administration of C75 in rats led to C75-CoA
15
16 production, inhibition of CPT1 and lower body weight and food intake. Our results
17
18 suggest that inhibition of CPT1, and thus increased availability of fatty acids in the
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20 hypothalamus, contribute to the pharmacological mechanism of C75 to decrease food
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22 intake.
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1. Introduction

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3 The brain plays an important role in the evaluation and control of energy homeostasis.
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5 Blood concentrations of glucose and fatty acids are sensed by neurons of the
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7 hypothalamus, which adjusts feeding behaviour and monitors fatty-acid metabolism.
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10 Several laboratories have attempted to design anti-obesity drugs and modulate fatty acid
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12 metabolism to inhibit food intake. C75 is a synthetic inhibitor of fatty acid synthase
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14 (FAS) [1] and has been proposed as an anti-obesity agent since its administration
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16 decreases food intake and body weight in rodents [2-5]. C75 can alter the metabolism of
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18 neurons in the hypothalamus, where an increase in the level of malonyl-CoA due to
19
20 FAS inhibition serves as a secondary messenger of nutrient status, thereby mediating
21
22 the suppression of food intake [6, 7]. Hypothalamic levels of long-chain fatty acyl-CoA
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24 (LCFA-CoA) also signal nutrient availability and control food intake [8].
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27 Malonyl-CoA signals the availability of lipid and carbohydrate fuels [9] and acts as a
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29 physiological inhibitor of the enzyme carnitine palmitoyltransferase 1 (CPT1). CPT1
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31 catalyzes the first step in the transport of LCFA from the cytoplasm to the mitochondria,
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33 and is the rate-limiting step in β -oxidation. Mammalian tissues express three CPT1
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35 isoforms: CPT1A, CPT1B and CPT1C which differ in their sensitivity to malonyl-CoA
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37 and tissue distribution [10, 11]. CPT1A and CPT1C are expressed in the brain. CPT1A
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39 is located in the mitochondrial membrane and CPT1C is expressed in the endoplasmic
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41 reticulum of neurons where although it has CPT1 activity, it does not participate in
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43 mitochondrial fatty-acid oxidation [12]. Interestingly, our group has generated a mutant
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45 form of CPT1A insensitive to malonyl-CoA (CPT1A M593S), and a mutant form of
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47 carnitine acetyltransferase (CrAT) that swaps its preference from short to LCFA-CoA
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49 (CrAT D356A/M564G). These mutants have allowed us to examine the structural
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51 requirements of substrates and inhibitors [13-15].
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1 The inhibition of FAS by C75 produces an accumulation of malonyl-CoA which is
2 difficult to reconcile with the activation of CPT1 reported by others [2, 16-18]. To
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4 unravel this paradox the mechanism of action of C75 needs to be examined. We
5
6 recently demonstrated that C75 is converted *in vitro* to C75-CoA, a potent inhibitor of
7
8 CPT1 [19]. CPT1 activity was also inhibited in mitochondria from pancreas-, muscle-,
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10 and kidney-derived cell lines incubated with C75, which indicates that C75-CoA is
11
12 produced in these cells. This inhibition was followed by a decrease in fatty-acid
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14 oxidation. The role of CPT1 in heart, liver and pancreatic β -cells makes it a potential
15
16 target in the treatment of diabetes, obesity, and other human diseases.
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19 Here we show that C75-CoA, and not C75, directly inhibits CPT1 activity. We also
20
21 show that C75-CoA is formed in the hypothalamus in a dose-dependent way, where it
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23 inhibits CPT1 activity and decreases food intake and body weight. These results
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25 indicate that direct inhibition of CPT1 by C75-CoA in the hypothalamus could control
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27 body weight and feeding behaviour.
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2. Materials and methods

2.1. Materials

L-[methyl-³H]carnitine hydrochloride was purchased from Amersham Biosciences (GE Healthcare, Europe, Barcelona, Spain). Yeast culture media products were from Difco™ Laboratories (Detroit, MI). Bradford solution for protein assays was from Bio-Rad Laboratories (Barcelona, Spain). RPMI 1640 was from Gibco-Invitrogen Corporation (Barcelona, Spain). C75, C₁₇-CoA, defatted bovine serum albumin (BSA), palmitoyl-CoA, malonyl-CoA, and other chemicals were from Sigma-Aldrich (Madrid, Spain). Acyl-CoA synthetase from *Pseudomonas sp.* was from Sigma (Madrid, Spain). Etomoxir was provided by H.P.O. Wolf (GMBH, Allensbach, Germany).

2.2. Animals

Sprague-Dawley female rats (210-230 g) were bred in our laboratory. Animals were maintained under a 12 h dark/light cycle with free access to food (2014, Harlan) and water. All experimental protocols were approved by the Animal Ethics Committee at the University of Barcelona, in accordance with current legislation.

2.3. Cannulation surgery

Chronic (i.c.v.) cannulae were stereotaxically implanted into lateral ventricle under ketamine (Imalgene, 90 mg/Kg) and xylazine (Rompun, 11 mg/Kg) anesthesia. The coordinates were: 1.0 mm posterior to bregma, 1.4 mm lateral of the sagittal sinus and 6.2 mm ventral to the dura mater. Rats received subcutaneous injection of buprenorphine (0.05 mg/Kg) for analgesia.

2.4. Stereotaxic microinjection

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2 Rats were anesthetized as previously described and immobilized in a stereotaxic
3 apparatus. Injections were given bilaterally into the ARC nucleus of the hypothalamus
4 (2.7 mm posterior to bregma, 0.2 mm lateral of the sagittal sinus and 9.8 mm ventral to
5 the dura mater). 30 minutes after the injection rats were sacrificed and hypothalamus
6 was dissected.
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2.5. Treatments

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18 After 1 week of postsurgical recovery, cannula placements were verified by assessing a
19 feeding response to ghrelin [20]. I.c.v. injection (5 μ l) of etomoxir (190 μ g), C75 (40
20 μ g) or vehicle (RPMI 1640) were performed with a microliter syringe (Hamilton). For
21 feeding experiments, rats received single injections of vehicle or compound dissolved in
22 vehicle 30 min before the light was turned off. We measured intakes of chow, corrected
23 for spillage, at 1, 2, 4 and 22 h. Body weight was measured after 22 h. For the activity
24 experiments 3 rats were microinjected (ARC) with 2 μ L of RPMI 1640 medium as a
25 control or with 2 μ L of C75 dissolved in the same medium at 33 mM final
26 concentration. For the LC-MS/MS analysis, 2 rats were microinjected (ARC) with 2 μ L
27 of RPMI 1640 medium as control or with 2 μ L of C75 dissolved in the same medium at
28 15, 30 or 60 mM final concentration. 30 minutes after injection rats were killed and
29 hypothalamus was excised and stored at -80 °C for acyl-CoA extraction.
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2.6. C75-CoA and acyl-CoAs extraction and quantification

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52 Acyl-CoAs were extracted as described elsewhere [21], with some modifications. All
53 procedures were performed at 0-4 °C. Hypothalamus from control- and C75-
54 microinjected rats was thawed and 1 mL of 100 mM KH_2PO_4 pH 4.9 was added. 16
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1 nmol of heptadecanoyl-CoA (C_{17} -CoA), as an internal standard, was added to all
2 samples, which were then sonicated for 20s. One millilitre of n-propanol was added and
3
4 the emulsions were sonicated for a further 20s, left on ice for 20s, and sonicated again
5
6 for 20s. To this solution, 0.125 mL of saturated $(NH_4)_2SO_4$ was added, followed by 2
7
8 mL of 100% acetonitrile, and the mixture was then vortexed for 5 min at 4 °C. The
9
10 tubes were then centrifuged for 5 min at 2,100xg at 4 °C. The supernatant was removed
11
12 and passed through a sterile 0.2 μ m filter (Millipore). The filtrate was lyophilized and
13
14 re-dissolved in the same mobile phase (buffer B: 10% acetonitrile in 10 mM ammonium
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16 acetate buffer (pH 5.3)) used for the LC-MS/MS analysis. C_{17} -CoA and C_{75} -CoA were
17
18 used as standards to produce the calibration curves from which we quantified
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20 production of C_{75} -CoA *in vivo*. The straight lines obtained had a regression coefficient
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22 of 99%.
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31 **2.7. Liquid chromatography-mass spectrometry (LC-MS/MS)**

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33 LC analyses were performed using a Perkin Elmer series 200 equipped with a
34
35 quaternary pump and thermostatted autosampler. An XBridge (Waters) C_{18} column (50
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37 x 2.1 mm, 3.5 μ m) was used at room temperature and the volume injected was 15 μ L.
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39 The mobile phases were buffer A, 50% acetonitrile in 10 mM ammonium acetate buffer
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41 (pH 5.3); buffer B, 10% acetonitrile in 10 mM ammonium acetate buffer (pH 5.3) and
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43 C, 100% acetone. The column was equilibrated with 15% buffer A for 10 min, and then
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45 the eluting gradient was as follows: 15% buffer A to 90% buffer A in 5.5 min, then 90%
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47 buffer A to 50% buffer A, 5% C to 45% C in 15.5 min, and 50% buffer A, 45% C in 25
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49 min. The flow rate was 0.4 mL/min.
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55 MS and MS/MS experiments were performed on an API 3000 triple-quadrupole mass
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57 spectrometer equipped with a Turbo Ion spray source. All the analyses were performed
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in positive mode with the following settings: capillary voltage, +3000 V; nebulizer gas (N₂), 10 (arbitrary units); curtain gas (N₂), 12 (arbitrary units); collision gas (N₂), 4 (arbitrary units); declustering potential, +30 V; focusing potential, +200 V; entrance potential, +10 V; collision energy, +40 V; collision cell exit potential, +15 V. Drying gas (N₂) was heated to 300 °C and introduced at a flow-rate of 6000 cm³ min⁻¹. Full scan data acquisition was performed scanning from *m/z* 500 to 1200 in profile mode and using a cycle time of 2 s with a step size of 0.1 u and a pause between each scan of 2 ms. In order to achieve maximum sensitivity, samples were injected in multiple reaction monitoring mode (MRM) by monitoring the 1022.5/515.6 and the 1020.7/513.5 transitions, obtained through product ion scan experiments with the C₇₅-CoA and C₁₇-CoA standard, respectively. To calculate the C₇₅-CoA concentration in the samples, 15 μL of increasing concentrations of C₇₅-CoA and C₁₇-CoA from 10 to 5000 nM was injected. The areas of the resulting peaks were used to produce the calibration curve. The C₁₇-CoA was used as internal standard to calculate the percentage of recovery in the acyl-CoA extraction.

2.8. Enzymatic synthesis of C₇₅-CoA and etomoxiryl-CoA

C₇₅ was converted first, *in vitro*, to its CoA derivative by long-chain acyl-CoA synthetase in the presence of CoA, as described previously [19]. The unreacted CoA from the C₇₅-CoA activation solution (1 mL) was removed by incubation for 30 min at 4 °C with 1.25 mL of SulfoLink Coupling Gel (Pierce Biotechnology), as carnitine acyltransferase activity is inhibited by CoA [22]. C₇₅-CoA synthesized in this enzymatic reaction was used as a standard for LC-MS/MS analysis. The conversion of etomoxir to etomoxiryl-CoA was nearly complete, as deduced from the fluorometric assay of the remaining free CoA, as described elsewhere [23].

2.9. Non-enzymatic synthesis of C75-CoA

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2 In the second experiment, a solution of C75 and CoA (pH 8.4) was stirred in the
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4 absence of acyl-CoA synthetase. The structure and relative stereochemistry of the C75-
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6 CoA adduct was determined by NMR. CoA sodium salt hydrate (8.6 mg), and
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8 $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$ (7.6 mg) were added to a solution of (\pm)-C75 (2.5 mg) in D_2O (0.8 mL)
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10 in an NMR tube. The structure of the C75-CoA adduct was fully determined by ^1H and
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12 ^{13}C NMR, gCOSY and gHSQC experiments.
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2.10. Expression of rat CrAT and CPT1 in *Saccharomyces cerevisiae*

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20 Plasmids pYESCrAT^{wt} [14], pYESCrAT^{D356A/M564G} [15], pYESLCPT^{wt} [24],
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22 pYESLCPT^{M593S} [13] containing CrAT wt, CrAT D356A/M564G mutant, CPT1A wt and
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24 CPT1A M593S mutant, respectively, were expressed in yeast cells, and mitochondrial cell
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26 extracts were prepared as previously described [24]. An *S. cerevisiae* strain devoid of COT
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28 and CPT1 activity and lacking the endogenous *CAT2* gene (FY23 Δ cat2 (MATa trp1 ura3
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30 Δ cat2::LEU2)) was used as an expression system [25].
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2.11. Determination of carnitine acyltransferase activity

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40 Mitochondrial-enriched fractions were obtained by differential centrifugation [26], with
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42 minor modifications. All protein concentrations were determined using the Bio-Rad
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44 protein assay with bovine serum albumin as a standard.
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49 Two methods were used for the assay of carnitine acyltransferase: a radiometric
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51 method [24] and an endpoint fluorometric method [14, 23]. The radiometric assay was
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53 used in all cases, unless otherwise indicated. *Radiometric method:* The forward reaction
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55 of carnitine acyltransferase activity was assayed in mitochondrial-enriched fractions
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57 obtained from yeast (5 μg protein for CrAT D356A/M564G and 3-4 μg protein for
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CPT1A) and from rat hypothalamus (100 µg protein for CPT1). Enzyme activity was assayed for 4 min at 30 °C in a total volume of 200 µL. The substrates were 400 µM L-[methyl-³H]carnitine, and 50 µM myristoyl-CoA (for CrAT D356A/M564G) or palmitoyl-CoA (for CPT1A). Drugs were preincubated with the enzyme for 1-5 min depending on the assay. Drug concentrations ranging from 0.01 to 60 µM were used to calculate the IC₅₀. Enzyme assays were carried out to deduce the effects of the free acid (C75) versus the CoA ester (C75-CoA), that is, the potency of C75-CoA as inhibitor relative to C75 as an activator. In this case, CPT1A wt overexpressed in yeast (8 µg protein) was preincubated for 5 min with increasing concentrations of C75 (5-100 µM), C75-CoA (5-50µM) with or without 100 µM C75 at each C75-CoA concentration. Enzyme activity was compared to the control (DMSO). In all cases, the molar ratio of acyl-CoA to albumin was kept at 5:1 to avoid the presence of free acyl-CoA and its deleterious detergent effects and to prevent the formation of micelles. *Fluorometric method:* The forward reaction of carnitine acyltransferase activity was assayed for 8 min at 30 °C in a solution containing 0.1 mM acyl-CoA, 1.5 mM EDTA, 1.5 mM L-carnitine, and 40 mM HEPES buffer, pH 7.8, in a total volume of 600 µL. Acetyl-CoA was used as substrate for CrAT wt. Reactions were started by the addition of 5 µg of yeast-expressed protein. Increasing concentrations of C75-CoA, etomoxiryl-CoA or malonyl-CoA were independently incubated with yeast-overexpressed mitochondrial CrAT wt. Parallel (blank) assays were run in the absence of L-carnitine.

2.12. Construction of rat CrAT and CPT1A models

A model of rat CrAT wt enzyme was constructed by homology modelling using as templates the structures deposited in the Protein Data Bank (PDB) corresponding to human (1NM8) [27], and mouse CrAT (1NDB, 1NDF and 1NDI) [28], essentially as

1 described elsewhere [14]. CrAT D356A/M564G was modelled by the same procedures
2 using rat CrAT wt as template. CPT1A was also modelled using as templates the
3 structures of mouse carnitine octanoyltransferase (1XL7, 1XL8) [29] and carnitine
4 palmitoyltransferase 2 (PDB entry 2H4T) [30], essentially as described elsewhere [31,
5 32]. The quality of the models was checked using the WHAT-CHECK routines [33]
6 from the WHAT-IF program [34] and the PROCHECK validation program from the
7 SWISS-MODEL server facilities [35]. To optimize geometries and release local
8 constraints or inappropriate contacts, the modelled structures were energy minimized
9 with the implementation of the GROMOS 43B1 force field in the program DeepView
10 [36], using 500 steps of steepest descent minimization followed by 500 steps of
11 conjugate-gradient minimization.
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29 **2.13. *In Silico* molecular docking**

30 Structural models of the molecular docking of the inhibitors C75-CoA and malonyl-
31 CoA to the active site of the receptor proteins CPT1A, and CrAT mutant
32 D356A/M564G were performed using the suite of programs included in the Autodock
33 3.0 package [35], as described elsewhere [14].
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44 **2.14. Statistical analysis**

45 Data are represented as mean \pm S.E.M. Student *t*-test was used for statistical analysis.
46 Different experimental groups were compared with a one-way ANOVA followed by
47 Turkey's test for comparisons post hoc. A probability level of $P < 0.05$ was considered
48 significant.
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3. Results

3.1. Synthesis and chemical characterization of C75-CoA by NMR

We have shown that C75-CoA can be synthesized from C75 and CoA in the presence of acyl-CoA synthetase from *Pseudomonas sp.* [19]. Here we show that the MS spectrum of C75-CoA gave an $[M + H]^+$ ion at m/z 1022.5, corresponding to the protonated molecule of C75-CoA. This ion corresponded to the formation of the C75-CoA molecule by conjugation of the CoA group without the loss of a water molecule. The product showed the following: a fragmentation ion at m/z 515.6, which was assigned to the C75-pantethenoic group, and an ion at m/z 428.1, which corresponds to adenosine 3'-diphosphate (Supplemental Fig. S1).

We hypothesised that the $-SH$ group in CoA would be sufficiently nucleophilic to add to the electrophilic methylene group in C75 without any external agent (acyl-CoA synthetase) in an alkaline medium in which the thiol group is partially deprotonated. We mixed both reagents, C75 and HSCoA in a deuterated water solution at pH 8.4 (Fig. 1A) and the mixture was analyzed by 1H NMR, comparing the results with the unreacted C75 and CoA. The peaks assigned to the methylene group (black triangle) progressively decreased and a new signal at ~ 2.9 ppm appeared due to the formation of the new saturated methylene group (white triangle) (Fig.1B). These changes were fast (30 min). In a parallel experiment at neutral pH the same transformation was observed, albeit much more slowly (data not shown).

The structure and relative stereochemistry of the C75-CoA adduct shown in Figure 1C were determined by one- and two-dimensional 1H and ^{13}C NMR techniques in order to assign every signal (gCOSY and gHSQC experiments) [37]. In particular, the *trans-trans* relative configuration on the lactone ring shown in the Figure 1C is well

1 established on the basis of the observed coupling constants (J), especially $J_{d,e}$. These
2 coupling constants depend on the dihedral angle (H-C-C-H) of the protons involved and
3
4 give information about their relative position. When we compared the coupling
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6 constants of the lactone ring moiety of our adduct with those reported [38, 39] for the
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8 related compounds I and II (Fig. 1C) high correlation with II was observed, suggesting
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10 the same relative configuration.
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14 As described above, we used two methods to obtain C75-CoA. In one, a solution of C75
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16 and CoA was incubated in the presence of acyl-CoA synthetase. In the other, the adduct
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18 was synthesised in the absence of the enzyme. We aimed to determine whether the
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20 structural and inhibitory properties of the C75-CoA adducts depended on the presence
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22 of acyl-CoA synthetase in their synthesis. Two analytical tests were applied. First, a
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24 comparative mass/mass spectroscopy analysis showed that the C75-CoA synthesised in
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26 the presence of the enzyme (Supplemental Fig. S1) had the same ion fragmentation
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28 profile as that synthesised in the absence of the enzyme (data not shown). Second, we
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30 assayed their capacity to inhibit CPT1, obtaining identical results for both adducts (Fig.
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32 1D). We conclude that the two adducts synthesized in the presence or absence of the
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34 enzyme have identical structure and properties.
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44 **3.2. Effect of C75 and C75-CoA on CPT1 activity**

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46 To examine the controversial effect of C75 on CPT1 activity we incubated yeast
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48 mitochondria with increased concentrations of C75 (Fig. 2A). The results reported
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50 indicate that free C75 alone is neither an activator nor an inhibitor of CPT1. We also
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52 performed kinetic inhibitory experiments in which, in addition to increasing
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54 concentrations of C75-CoA, a fixed concentration of C75 (100 μ M) was added to the
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56 mixture. CPT1 inhibition was not counteracted by the fixed amount of free C75 at any
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1 concentration of C75-CoA. In other words, even at the highest C75 concentrations, the
2 free product did not overcome the inhibition by C75-CoA (Fig. 2B).
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7 **3.3. Inhibitory effects of C75-CoA on yeast-expressed mutated CPT1A and CrAT**

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9 To examine the structural requirements of the interaction between C75-CoA and CPT1
10 we carried out C75-CoA inhibitory experiments where we used specific acyltransferase
11 mutants. We used CrAT D356A/M564G, which has a deep hydrophobic pocket for the
12 binding of LCFA-CoAs instead of short acyl-CoAs and behaves like CPT1A in terms of
13 acyl-CoA specificity [15]. We also used CPT1A M593S, which is active but insensitive
14 to malonyl-CoA inhibition [13]. Experiments with etomoxiryl-CoA, a pharmacological
15 inhibitor of CPT1, and malonyl-CoA, its physiological inhibitor, were carried out as
16 controls.
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28 C75-CoA and etomoxiryl-CoA inhibited CrAT D356A/M564G but had little effect on
29 CrAT wt (Fig. 3A and 3B). At 60 μ M of C75-CoA, the remaining CrAT activity was
30 83% for CrAT wt, and only 21% of the original level for CrAT D356A/M564G. For
31 etomoxiryl-CoA, at a concentration of 60 μ M, the remaining activity was 86% for CrAT
32 wt, and 46% for the CrAT mutant. These results indicate that CrAT wt is insensitive to
33 these drugs because the long aliphatic chain of C75-CoA and etomoxiryl-CoA does not
34 fit in the shallow cavity of the wt enzyme, where only the acyl group of short-chain
35 acyl-CoAs such as acetyl- and butyryl-CoA can enter. However, when this cavity was
36 open and accessible to longer acyl-CoAs, as in the CrAT D356A/M564G mutant, the
37 enzyme became sensitive to these inhibitors. In contrast, malonyl-CoA had little effect
38 on mutant or wt CrAT (Fig. 3A and 3B). These results show that CrAT D356A/M564G,
39 like the wt enzyme, does not contain the structural determinants needed for the initial
40 interaction with malonyl-CoA and enzyme inhibition.
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Next we compared the inhibitory effect of C75-CoA on CPT1A wt and CPT1A M593S. At 50 μM of C75-CoA, the remaining activity of the M593S mutant was about 45% of the original level (Fig. 3D), whereas the activity of the CPT1A wt was almost abolished (Fig. 3C). A similar effect was observed with etomoxiryl-CoA. Malonyl-CoA, as expected, inhibited CPT1A wt while the activity of the malonyl-CoA-insensitive enzyme (CPT1A M593S) was unaffected. The IC_{50} for C75-CoA and CPT1A M593S was 108-fold higher than that for CPT1A wt (25.9 μM vs. 0.24 μM). In the case of etomoxiryl-CoA the IC_{50} increased 31-fold compared with the wt enzyme (168 μM vs. 4.1 μM). This indicates that CPT1A inhibitors in addition to fitting in the hydrophobic pocket also need to interact with the malonyl-CoA site of CPT1A to produce potent inhibition.

3.4. Molecular model of docking of C75-CoA into CPT1A wt, and CrAT D356A/M564G

Since CPT1 has not yet been crystallized, we do not know the location of C75-CoA in the CPT1 crystal. Therefore, we used computational docking methods to identify a putative location of C75-CoA molecule in the 3-D models of the catalytic core of CPT1A wt and of CrAT D356A/M564G. The model locates the C75-CoA molecule (Fig. 4A) in CPT1A wt in a position that coincides with the site of palmitoyl-CoA substrate [32], thus introducing the aliphatic chain into the hydrophobic cavity in the protein active centre. The carboxyl group in the head of the C75-CoA molecule is positioned at the equivalent locus for the carnitine substrate in the CPT1A wt models, partially filling it. This indicates that this inhibitor could hamper the correct positioning of the carnitine substrate. Moreover, the head of C75-CoA is also located near the catalytic His residue (His⁴⁷³), which would impair the catalytic activity of CPT1A. The

1 position of the carboxyl group on C75-CoA (Fig. 4A) is similar to that proposed for the
2 dicarbonyl moiety of malonyl-CoA on CPT1A [32], which thus interferes with the
3 correct positioning of carnitine substrate (Fig. 4C). This shared mode of action could be
4 explained in part by the presence of a carboxyl group in carnitine, C75-CoA and
5 malonyl-CoA molecules (Fig. 4D), putatively located in the active centre of the enzyme.
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7 An analogous model was constructed for the location of C75 in CrAT D356A/M564G
8 mutant structure. The position of the C75 aliphatic chain moiety is similar to that
9 proposed for stearyl-CoA in the same enzyme derivative [15], in which the
10 hydrocarbon chain is located in the cavity that opens up when Asp³⁵⁶ and Met⁵⁶⁴ are
11 replaced by Ala and Gly respectively (Fig. 4B). The carboxyl group at the head of C75
12 is located in the carnitine locus, close to the catalytic His³⁴³ residue as mentioned above
13 for CPT1A.
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32 **3.5. C75 is converted to C75-CoA in rat hypothalamus**

33 Since C75 has been proposed as a regulator of food intake through its action on the
34 hypothalamus, we examined whether C75-CoA is produced in the hypothalamus
35 following direct injection of C75. We injected 7.6, 15.2 and 30.4 μg of C75 in the
36 arcuate (ARC) nucleus by stereotaxic surgery. Thirty minutes after injection the rats
37 were killed and acyl-CoAs, including C75-CoA, were extracted from the hypothalamus.
38 Analysis of the samples by LC-MS/MS showed a peak corresponding to C75-CoA (Fig.
39 5). The production of C75-CoA increased with the amount of C75 injected. Considering
40 that in our conditions the average weight of the rat hypothalamus was 50 mg, the
41 concentrations of C75-CoA produced in the hypothalamus after an injection of 7.6, 15.2
42 and 30.4 μg of C75 were 3.7, 15.9 and 25.1 nmol/g tissue respectively.
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3.6. Central nervous system administration of C75 inhibits CPT1 activity and decreases food intake and body weight

Having demonstrated the conversion of C75 into C75-CoA in the rat hypothalamus, we then studied the effect of a stereotaxic microinjection of C75 into the rat ARC nucleus on CPT1 activity. Hypothalamic mitochondrial fractions from rats injected with 16.8 μ g of C75 were assayed for CPT1 activity and the value was compared to hypothalamic mitochondrial fractions from rats injected with RPMI medium. Activity was decreased by 46% in C75-treated rats (Fig. 6A).

Next we administered C75 in the lateral cerebral ventricle of the rat and measured food intake and body weight. Consistent with previous results [2-5], C75 produced a decrease in food intake and body weight (Fig. 6B and 6C). The anorectic action of etomoxir was seen at a later point (22h).

4. Discussion

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5 Energy balance is monitored by the hypothalamus, where inhibitors of FAS have been
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7 described to suppress food intake [40, 41]. C75 acts on fatty acid metabolism by
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9 inhibiting FAS activity. Inhibition of FAS produces accumulation of malonyl-CoA,
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11 which, as a physiological inhibitor of CPT1, prevents the oxidation of newly
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13 synthesized fatty acids. Moreover, C75 may activate CPT1 [2, 18]. The effect of C75
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15 on FAS is difficult to reconcile with the C75-activation of CPT1 as they have opposite
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17 effects: inhibition by malonyl-CoA and direct activation of CPT1. The simultaneous
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19 activation and inhibition of CPT1 by C75 appears paradoxical, and it has not been
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21 addressed satisfactorily. Kuhajda *et al.* suggested that C75 could modulate the inhibition
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23 of AMP-activated protein kinase (AMPK) which would lead to an increase in acetyl-
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25 CoA carboxylase (ACC) activity and a subsequent increase in malonyl-CoA levels [42].
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27 This does not solve the paradox, since there would be an increase in malonyl-CoA
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29 (putatively inhibiting CPT1 activity) and a simultaneous activation of CPT1 produced
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31 by C75 itself (Fig. 7).

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33 Here we attempt to explain the anorectic effects of C75 in terms of its inhibitory action
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35 on CPT1. Inhibition of CPT1 could prevent the oxidation of fatty acids of the ARC
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37 nucleus in the hypothalamus leading to a local accumulation of LCFA-CoAs. The
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39 increase in LCFA-CoA is a central signal of 'nutrient abundance' which in turn
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41 activates a chain of neuronal events, via up-regulation of anorexigenic genes and down
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43 regulation of orexigenic genes, that would promote a switch in fuel sources from
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45 carbohydrates to lipids and limit food intake [8]. The detailed mechanism of this up- or
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47 down-regulation has not been addressed yet. Likewise, central administration of fatty
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49 acid suppresses food intake. Here we demonstrated that C75 is transformed into C75-
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CoA in the hypothalamus where it inhibits CPT1 activity. We propose that this direct effect *in vivo* of C75-CoA on CPT1 would explain by itself the inhibition of CPT1 activity. However, FAS could be inhibited by C75 in the hypothalamus. Therefore, malonyl-CoA could be in excess, inhibiting CPT1, together with C75-CoA. The anorectic effects of C75 are similar to those produced by other CPT1 inhibitors such as ST1326 and tetradecylglycidic acid, and to those of a riboprobe that specifically cleaves CPT1A mRNA [8]. The CPT1 inhibitor etomoxir also decreased feeding and reduced body weight in rats supporting those previous results [8].

It was important to discern whether C75 was an inhibitor or an activator of CPT1. Our results indicate that C75 is neither an activator nor an inhibitor of CPT1 when incubated with yeast-expressed CPT1A. C75 did not overcome the inhibition caused by C75-CoA. This agrees with the observed net effect of inhibition of CPT1 activity. Results shown here and elsewhere [19] demonstrate that C75-CoA inhibits CPT1A *in vitro* more strongly than etomoxiryl-CoA (IC₅₀ values are 0.24 μM and 4.1 μM respectively).

The finding that C75 forms a CoA ester before it can inhibit CPT1 is reminiscent of the case of etomoxir. McGarry and co-workers [43, 44] showed that the CPT1 inhibitory molecules were neither tetradecylglycidic acid nor etomoxir themselves, but rather their CoA esters produced in the presence of acyl-CoA synthetase. The CoA group may direct and fix the drug molecule in the cavities of CPT1, as it does with the natural physiological inhibitor malonyl-CoA and with the substrate palmitoyl-CoA. The synthesis of C75-CoA is produced stereo specifically through the electrophilic methylene group of C75. Moreover, our finding that C75-CoA is produced in absence of acyl-CoA synthetase indicated that it could be synthesised in the hypothalamus, irrespective of whether it is expressed in hypothalamic neurons.

1 Our results indicate that C75-CoA is formed in the hypothalamus following stereotaxic
2 injection of C75. Inhibition of hypothalamic CPT1 by C75-CoA *in vivo*, as seen in our
3 experiments, is independent of the putative inhibition by malonyl-CoA, which may be
4 formed after the action of C75 either on FAS or on AMPK. Hypothalamic CPT1
5 activity was determined in twice-washed mitochondria. Therefore, malonyl-CoA was
6 unlikely to remain within CPT1, as this metabolite leaves CPT1 freely when
7 mitochondria are washed. In contrast, C75-CoA, as it is a tight-binding inhibitor,
8 remains bound to the enzyme after washing, which means that its inhibition is persistent
9 [19]. Hence, C75-CoA not only inhibits CPT1 *in vitro*, but it also inhibits CPT1 activity
10 in the hypothalamus after C75 has been converted to its CoA derivative.

11 That C75-CoA is an inhibitor of CPT1A is also supported by the docking analysis of the
12 CPT1A model. Comparison of computer-calculated docking models shows that CoA is
13 bound at the same site, whether it belongs to C75-CoA or malonyl-CoA, or palmitoyl-
14 CoA. The carboxylic acid bound to the lactone of C75 protrudes into the carnitine site.
15 The inhibitory mechanism of C75-CoA resembles that observed for malonyl-CoA (Fig.
16 4C) [32]. Several authors reported the competition between malonyl-CoA and carnitine
17 [45, 46]. The carboxylate group of malonyl-CoA and C75-CoA may partially mimic the
18 interaction between the enzyme and the carboxylate group of carnitine, thus preventing
19 the positioning of this substrate and inhibiting the catalytic activity of the enzyme.
20 Several authors [47, 48] suggest that the presence of two carbonyl groups in close
21 juxtaposition in the malonyl-CoA molecule might be responsible for the interaction and the
22 inhibitory effect on CPT1A. Because C75-CoA also has these two carbonyl groups, it may
23 behave like malonyl-CoA. Indeed the docking models show that C75-CoA could bind to
24 the malonyl-CoA site of the enzyme [32].

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Moreover, the hydrocarbon chain of C75 is located at the same site as the hydrocarbon long-chain of palmitic acid. The kinetic experiments of inhibition of C75-CoA against palmitoyl-CoA [19] are confirmed by the docking studies, and both support the notion that C75-CoA is a strong inhibitor of CPT1A.

To assess the involvement of the hydrocarbon chain of C75-CoA positioning in the acyl group binding pocket, we carried out C75-CoA inhibitory experiments with the new mutated protein, CrAT D356A/M564G. This protein has a deep hydrophobic pocket for the binding of long-chain instead of short-chain acyl-CoAs and shows CPT1-like behaviour in terms of acyl-CoA specificity, although unlike CPT1A, it is not inhibited by malonyl-CoA [15]. The inhibition by C75-CoA of the mutant CrAT D356A/M564G suggests that C75-CoA fits in the large hydrophobic pocket of this enzyme, as in CPT1A wt, and that the presence of this pocket is necessary for C75-CoA inhibition. However, CrAT D356A/M564G is not as sensitive as CPT1A wt to C75-CoA, since the IC_{50} for C75-CoA acting on CPT1A wt is 50-fold lower than that observed for the CrAT double mutant. These results indicate that factors other than the presence of a hydrophobic pocket contribute to the inhibitory potency of C75-CoA toward CPT1. CPT1A M593S, which is insensitive to malonyl-CoA inhibition, shows limited sensitivity towards C75-CoA, but its IC_{50} for C75-CoA is similar to that of CrAT double mutant (25.9 vs. 12.8 μ M, respectively). Therefore, the lack of a “malonyl-CoA-like” interaction between CrAT double mutant or CPT1A M593S and the carbonyl groups in the polar head of C75-CoA might explain their limited sensitivity to the inhibitor.

We conclude that C75 is converted into C75-CoA and that it strongly inhibits CPT1 *in vitro* and *in vivo*. Docking and kinetic analysis revealed the molecular basis by which C75-CoA interacts with the enzyme and its substrates. We also show that C75-CoA is

1 formed *in vivo* in the hypothalamus, where it inhibits CPT1. Here the inhibition of
2 CPT1 could alter fatty acid oxidation, thus putatively promoting down-regulation of
3 orexigenic genes and up-regulation of anorexigenic genes, which induces restriction in
4 food intake. These results point to the potential use of drugs to inhibit CPT1 activity,
5 and control food intake in the treatment of obesity and diabetes.
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REFERENCES

- 1
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4
5 [1] Kuhajda FP, Pizer ES, Li JN, Mani NS, Frehywot GL, Townsend CA. Synthesis
6 and antitumor activity of an inhibitor of fatty acid synthase. *Proc Natl Acad Sci*
7 *U S A* 2000;97:3450-4.
8
9 [2] Thupari JN, Landree LE, Ronnett GV, Kuhajda FP. C75 increases peripheral
10 energy utilization and fatty acid oxidation in diet-induced obesity. *Proc Natl*
11 *Acad Sci U S A* 2002;99:9498-502.
12 [3] Thupari JN, Kim EK, Moran TH, Ronnett GV, Kuhajda FP. Chronic C75
13 treatment of diet-induced obese mice increases fat oxidation and reduces food
14 intake to reduce adipose mass. *Am J Physiol Endocrinol Metab* 2004;287:E97-
15 E104.
16 [4] Clegg DJ, Wortman MD, Benoit SC, McOsker CC, Seeley RJ. Comparison of
17 central and peripheral administration of C75 on food intake, body weight, and
18 conditioned taste aversion. *Diabetes* 2002;51:3196-201.
19 [5] Kumar MV, Shimokawa T, Nagy TR, Lane MD. Differential effects of a
20 centrally acting fatty acid synthase inhibitor in lean and obese mice. *Proc Natl*
21 *Acad Sci U S A* 2002;99:1921-5.
22 [6] Loftus TM, Jaworsky DE, Frehywot GL, Townsend CA, Ronnett GV, Lane MD,
23 et al. Reduced food intake and body weight in mice treated with fatty acid
24 synthase inhibitors. *Science* 2000;288:2379-81.
25 [7] Hu Z, Cha SH, Chohnan S, Lane MD. Hypothalamic malonyl-CoA as a
26 mediator of feeding behavior. *Proc Natl Acad Sci U S A* 2003;100:12624-9.
27 [8] Obici S, Feng Z, Arduini A, Conti R, Rossetti L. Inhibition of hypothalamic
28 carnitine palmitoyltransferase-1 decreases food intake and glucose production.
29 *Nat Med* 2003;9:756-61.
30 [9] McGarry JD, Brown NF. The mitochondrial carnitine palmitoyltransferase
31 system. From concept to molecular analysis. *Eur J Biochem* 1997;244:1-14.
32 [10] Esser V, Brown NF, Cowan AT, Foster DW, McGarry JD. Expression of a
33 cDNA isolated from rat brown adipose tissue and heart identifies the product as
34 the muscle isoform of carnitine palmitoyltransferase I (M-CPT I). M-CPT I is
35 the predominant CPT I isoform expressed in both white (epididymal) and brown
36 adipocytes. *J Biol Chem* 1996;271:6972-7.
37 [11] Price N, van der Leij F, Jackson V, Corstorphine C, Thomson R, Sorensen A, et
38 al. A novel brain-expressed protein related to carnitine palmitoyltransferase I.
39 *Genomics* 2002;80:433-42.
40 [12] Sierra AY, Gratacos E, Carrasco P, Clotet J, Urena J, Serra D, et al. CPT1c is
41 localized in endoplasmic reticulum of neurons and has carnitine
42 palmitoyltransferase activity. *J Biol Chem* 2008;283:6878-85.
43 [13] Morillas M, Gomez-Puertas P, Bentebibel A, Selles E, Casals N, Valencia A, et
44 al. Identification of conserved amino acid residues in rat liver carnitine
45 palmitoyltransferase I critical for malonyl-CoA inhibition. Mutation of
46 methionine 593 abolishes malonyl-CoA inhibition. *J Biol Chem* 2003;278:9058-
47 63.
48 [14] Cordente AG, Lopez-Vinas E, Vazquez MI, Swiegers JH, Pretorius IS, Gomez-
49 Puertas P, et al. Redesign of carnitine acetyltransferase specificity by protein
50 engineering. *J Biol Chem* 2004;279:33899-908.
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

- 1 [15] Cordente AG, Lopez-Vinas E, Vazquez MI, Gomez-Puertas P, Asins G, Serra D,
2 et al. Mutagenesis of specific amino acids converts carnitine acetyltransferase
3 into carnitine palmitoyltransferase. *Biochemistry* 2006;45:6133-41.
- 4 [16] Nicot C, Napal L, Relat J, Gonzalez S, Llebaria A, Woldegiorgis G, et al. C75
5 activates malonyl-CoA sensitive and insensitive components of the CPT system.
6 *Biochem Biophys Res Commun* 2004;325:660-4.
- 7 [17] Aja S, Bi S, Knipp SB, McFadden JM, Ronnett GV, Kuhajda FP, et al.
8 Intracerebroventricular C75 decreases meal frequency and reduces AgRP gene
9 expression in rats. *Am J Physiol Regul Integr Comp Physiol* 2006;291:R148-54.
- 10 [18] Aja S, Landree LE, Kleman AM, Medghalchi SM, Vadlamudi A, McFadden
11 JM, et al. Pharmacological stimulation of brain carnitine palmitoyl-transferase-1
12 decreases food intake and body weight. *Am J Physiol Regul Integr Comp*
13 *Physiol* 2008;294:R352-61.
- 14 [19] Bentebibel A, Sebastian D, Herrero L, Lopez-Vinas E, Serra D, Asins G, et al.
15 Novel effect of C75 on carnitine palmitoyltransferase I activity and palmitate
16 oxidation. *Biochemistry* 2006;45:4339-50.
- 17 [20] Lopez M, Lelliott CJ, Tovar S, Kimber W, Gallego R, Virtue S, et al.
18 Tamoxifen-induced anorexia is associated with fatty acid synthase inhibition in
19 the ventromedial nucleus of the hypothalamus and accumulation of malonyl-
20 CoA. *Diabetes* 2006;55:1327-36.
- 21 [21] Golovko MY, Murphy EJ. An improved method for tissue long-chain acyl-CoA
22 extraction and analysis. *J Lipid Res* 2004;45:1777-82.
- 23 [22] Zierz S, Engel AG. Different sites of inhibition of carnitine palmitoyltransferase
24 by malonyl-CoA, and by acetyl-CoA and CoA, in human skeletal muscle.
25 *Biochem J* 1987;245:205-9.
- 26 [23] Hassett RP, Crockett EL. Endpoint fluorometric assays for determining activities
27 of carnitine palmitoyltransferase and citrate synthase. *Anal Biochem*
28 2000;287:176-9.
- 29 [24] Morillas M, Gomez-Puertas P, Roca R, Serra D, Asins G, Valencia A, et al.
30 Structural model of the catalytic core of carnitine palmitoyltransferase I and
31 carnitine octanoyltransferase (COT): mutation of CPT I histidine 473 and
32 alanine 381 and COT alanine 238 impairs the catalytic activity. *J Biol Chem*
33 2001;276:45001-8.
- 34 [25] Swiegers JH, Dippenaar N, Pretorius IS, Bauer FF. Carnitine-dependent
35 metabolic activities in *Saccharomyces cerevisiae*: three carnitine
36 acetyltransferases are essential in a carnitine-dependent strain. *Yeast*
37 2001;18:585-95.
- 38 [26] Rickwood D, Graham JM. Subcellular fractionation : a practical approach.
39 Oxford: IRL Press at Oxford University Press, 1997.
- 40 [27] Wu D, Govindasamy L, Lian W, Gu Y, Kukar T, Agbandje-McKenna M, et al.
41 Structure of human carnitine acetyltransferase. Molecular basis for fatty acyl
42 transfer. *J Biol Chem* 2003;278:13159-65.
- 43 [28] Jogl G, Tong L. Crystal structure of carnitine acetyltransferase and implications
44 for the catalytic mechanism and fatty acid transport. *Cell* 2003;112:113-22.
- 45 [29] Jogl G, Hsiao YS, Tong L. Crystal structure of mouse carnitine
46 octanoyltransferase and molecular determinants of substrate selectivity. *J Biol*
47 *Chem* 2005;280:738-44.
- 48 [30] Hsiao YS, Jogl G, Esser V, Tong L. Crystal structure of rat carnitine
49 palmitoyltransferase II (CPT-II). *Biochem Biophys Res Commun* 2006;346:974-
50 80.
- 51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

- 1 [31] Morillas M, Lopez VE, Valencia A, Serra D, Gomez-Puertas P, Hegardt FG, et
2 al. Structural model of carnitine palmitoyltransferase I based on the carnitine
3 acetyltransferase crystal. *Biochem J* 2004;379:777-84.
- 4 [32] Lopez-Vinas E, Bentebibel A, Gurunathan C, Morillas M, de Arriaga D, Serra
5 D, et al. Definition by functional and structural analysis of two malonyl-CoA
6 sites in carnitine palmitoyltransferase 1A. *J Biol Chem* 2007;282:18212-24.
- 7 [33] Hooft RW, Vriend G, Sander C, Abola EE. Errors in protein structures. *Nature*
8 1996;381:272.
- 9 [34] Vriend G. WHAT IF: a molecular modeling and drug design program. *J Mol*
10 *Graph* 1990;8:52-6, 29.
- 11 [35] Goodsell DS, Morris GM, Olson AJ. Automated docking of flexible ligands:
12 applications of AutoDock. *J Mol Recognit* 1996;9:1-5.
- 13 [36] Guex N, Peitsch MC. SWISS-MODEL and the Swiss-PdbViewer: an
14 environment for comparative protein modeling. *Electrophoresis* 1997;18:2714-
15 23.
- 16 [37] d'Ordine R, Paneth P, Anderson VE. ¹³C NMR and ¹H-¹H NOEs of Coenzyme-
17 A: Conformation of the pantoic acid moiety. *Bioorg Chem* 1995;23:169-81.
- 18 [38] Amador M, Ariza X., García J. A versatile stereoselective approach to paraconic
19 acids *Heterocycles* 2006:705-20.
- 20 [39] Ariza X, García J, López M, Montserrat L. A concise synthesis of (-)-
21 methylenoactocin and (-)-Phaseolinic acid from (6S,9S)-tetradec-7-yne-6,9-diol.
22 *Synlett* 2001:120-2.
- 23 [40] Shimokawa T, Kumar MV, Lane MD. Effect of a fatty acid synthase inhibitor
24 on food intake and expression of hypothalamic neuropeptides. *Proc Natl Acad*
25 *Sci U S A* 2002;99:66-71.
- 26 [41] Lane MD, Wolfgang M, Cha SH, Dai Y. Regulation of food intake and energy
27 expenditure by hypothalamic malonyl-CoA. *Int J Obes (Lond)* 2008;32 Suppl
28 4:S49-54.
- 29 [42] Kuhajda FP, Landree LE, Ronnett GV. The connections between C75 and
30 obesity drug-target pathways. *Trends Pharmacol Sci* 2005;26:541-4.
- 31 [43] Weis BC, Cowan AT, Brown N, Foster DW, McGarry JD. Use of a selective
32 inhibitor of liver carnitine palmitoyltransferase I (CPT I) allows quantification
33 of its contribution to total CPT I activity in rat heart. Evidence that the dominant
34 cardiac CPT I isoform is identical to the skeletal muscle enzyme. *J Biol Chem*
35 1994;269:26443-8.
- 36 [44] Declercq PE, Falck JR, Kuwajima M, Tyminski H, Foster DW, McGarry JD.
37 Characterization of the mitochondrial carnitine palmitoyltransferase enzyme
38 system. I. Use of inhibitors. *J Biol Chem* 1987;262:9812-21.
- 39 [45] McGarry JD, Mills SE, Long CS, Foster DW. Observations on the affinity for
40 carnitine, and malonyl-CoA sensitivity, of carnitine palmitoyltransferase I in
41 animal and human tissues. Demonstration of the presence of malonyl-CoA in
42 non-hepatic tissues of the rat. *Biochem J* 1983;214:21-8.
- 43 [46] Bird MI, Saggerson ED. Interacting effects of L-carnitine and malonyl-CoA on
44 rat liver carnitine palmitoyltransferase. *Biochem J* 1985;230:161-7.
- 45 [47] Cook GA, Mynatt RL, Kashfi K. Yonetani-Theorell analysis of hepatic carnitine
46 palmitoyltransferase-I inhibition indicates two distinct inhibitory binding sites. *J*
47 *Biol Chem* 1994;269:8803-7.
- 48 [48] Kashfi K, Mynatt RL, Cook GA. Hepatic carnitine palmitoyltransferase-I has
49 two independent inhibitory binding sites for regulation of fatty acid oxidation.
50 *Biochim Biophys Acta* 1994;1212:245-52.
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- [49] Lopez M, Lage R, Saha AK, Perez-Tilve D, Vazquez MJ, Varela L, et al. Hypothalamic fatty acid metabolism mediates the orexigenic action of ghrelin. *Cell Metab* 2008;7:389-99.

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Figure Legends

Fig. 1. NMR structural studies

(A) Incubation of a ~1:1 mixture of C75 and CoA in D₂O (with 10% of DMSO-d₆ in order to increase the solubility of C75). Peaks assigned to the methylene group (black triangles), peaks assigned to the saturated methylene groups (white triangles). (B) ¹H NMR (300 MHz) of a ~1:1 mixture of C75 and CoA in D₂O at pH ~8 at time 0 min (upper panel) and ¹H NMR (400 MHz) of a ~1:1 mixture of C75 and CoA in D₂O at pH ~8 after 30 min (lower panel). (C) Comparison of coupling constants (*J*) of C75-CoA adduct lactone ring with those reported early for related compounds I and II. (D) Comparison between the CPT1 inhibitory properties of C75-CoA synthesized in absence (●) and in presence (○) of acyl-CoA synthetase.

Fig. 2. CPT1A wt overexpressed in yeast is inhibited by C75-CoA in presence of C75

(A) Mitochondrial fractions from yeast overexpression CPT1A wt were preincubated for 5 min with increasing concentrations of C75 and activity was measured. (B) Comparative CPT1A wt activity of mitochondrial fractions from yeast, incubated with increasing concentrations of C75-CoA and in presence or absence of a fixed concentration of C75 (100 μM). Data are represented as a percentage of the control value in the presence of DMSO and the results are the average of three independent experiments.

Fig. 3. Effects of malonyl-CoA, etomoxiryl-CoA and C75-CoA on the activity of yeast-expressed wt CPT1A and CrAT, and the point mutants CPT1A M593S and CrAT D356A/M564G

1 Mitochondrial extracts from yeast expressing (A) CrAT wt, (C) CPT1A wt, and point
 2 mutants (B) CrAT D356A/M564G and (D) CPT1A M593S, were preincubated with
 3
 4 increasing concentrations of malonyl-CoA (●), etomoxiryl-CoA (○) and C75-CoA (▼).
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 6 Carnitine acyltransferase activity was measured, and data are expressed relative to
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 8 control values in the absence of drugs (100%) as the mean of at least two independent
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 10 experiments.
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17 **Fig. 4. *In silico* docking of inhibitors to 3-D models for the active centres of wt**
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 19 **CPT1A and CrAT D356A/M564G mutant**

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 21 (A) Predicted docked solution for C75-CoA molecule in the active centre of wt CPT1A.
 22 Location of catalytic residue His⁴⁷³ is indicated. Carboxylate group of C75-CoA
 23 partially occupy the volume corresponding to the carnitine substrate molecule
 24 (magenta). (B) Putative position for C75-CoA docking solution for C75-CoA in the
 25 active centre of CrAT D356A/M564G. The aliphatic chain of the inhibitor is located in
 26 the tunnel opened by mutated residues A356 and G564. The carboxylate group of C75-
 27 CoA is placed in the carnitine locus (magenta). Location of catalytic His³⁴³ is also
 28 indicated. (C) Docking of malonyl-CoA on CPT1A wt showing a similar positioning to
 29 C75-CoA, locating the dicarbonyl moiety in the space usually occupied by carnitine.
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 31 (D) Molecular structures of malonyl-CoA, carnitine and C75-CoA, showing a similar
 32 arrangement of their corresponding carboxyl groups. Molecular representations were
 33 performed using PyMOL (DeLano Scientific, San Carlos CA).
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52 **Fig. 5. Analysis of the C75-CoA levels after hypothalamic C75 injections**

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 54 (A) LC-MS/MS representative chromatograms of C75-CoA and C₁₇-CoA. Five
 55 concentrations of C75-CoA: 50, 100, 250, 500 and 1000 nM and seven concentrations
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 57 of C₁₇-CoA: 50, 100, 250, 500, 1000, 2500 and 5000 nM were injected and the areas
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1 below the resulting peaks were calculated. The calibration curves are shown in insets.
2 Representative LC chromatograms for C75-CoA obtained from rat hypothalamus after
3 the injection in the hypothalamic ARC nucleus of 2 μ l of C75 at various concentrations,
4 (B) 15 mM, (C) 30 mM and (D) 60 mM. 30 minutes after injection rats were
5 decapitated, and the hypothalamus was excised, immediately frozen and stored at -80°C.
6 Acyl-CoAs were extracted as described in Experimental Procedures, and C₁₇-CoA was
7 used as internal standard. Peak areas are shown in arbitrary units.
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19 **Fig. 6. Central nervous system administration of C75 inhibits CPT1 activity and**
20 **decreases food intake and body weight**

21 (A) Determination of CPT1 activity after the injection of C75 in ARC nucleus. Control
22 injections (n = 3; 2 μ l of RPMI 1640 medium as a control, black bar). C75 injections (n
23 = 3; 2 μ l of C75 33 mM in RPMI 1640 medium, white bar). (B) Food intake measured
24 in rats at 1, 2, 4 and 22h after i.c.v. injection of C75 (white bars), etomoxir (grey bars)
25 and control (RPMI medium, black bars). n = 5. (C) Body weight measured in rats at 22h
26 after i.c.v. injection of C75 (white bar), etomoxir (grey bar) and control (RPMI medium,
27 black bar). n = 5. * P < 0.05.
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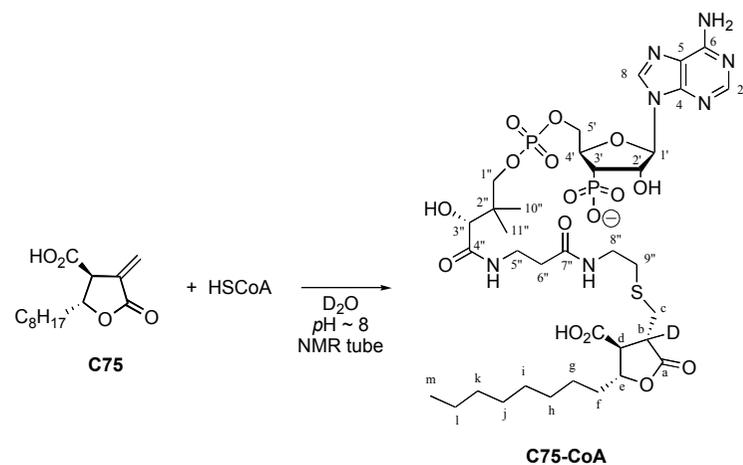
44 **Fig. 7. Model of action of C75 on hypothalamic fatty acid metabolism**

45 Energy balance is monitored by the hypothalamus. The accumulation of malonyl-CoA
46 due to FAS-inhibition by C75 leads to a reduction in CPT1 activity. This would increase
47 the cytosolic pool of LCFA-CoA and thus a decrease of food intake. In addition, C75
48 has been suggested to inhibit AMPK resulting in an activation of ACC and consequent
49 increase of malonyl-CoA levels [42]. Here we have shown that C75 is converted into
50 C75-CoA in the hypothalamus contributing to the direct inhibition of CPT1. Our
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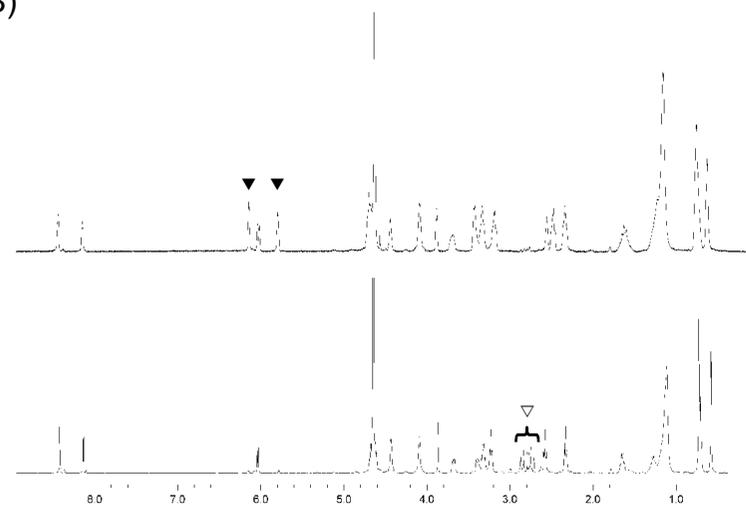
1 experiments also demonstrate that other CPT1 inhibitors such as etomoxir produce
2 similar effects decreasing food intake. On the other hand, AMPK-activators such as
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4 ghrelin [49] lead to ACC inactivation reducing malonyl-CoA levels and CPT1
5
6 inhibition. Thus, increased β -oxidation produces a reduction on LCFA-CoAs promoting
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FIGURE 1

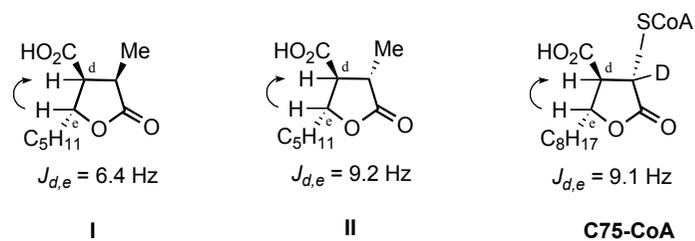
A)



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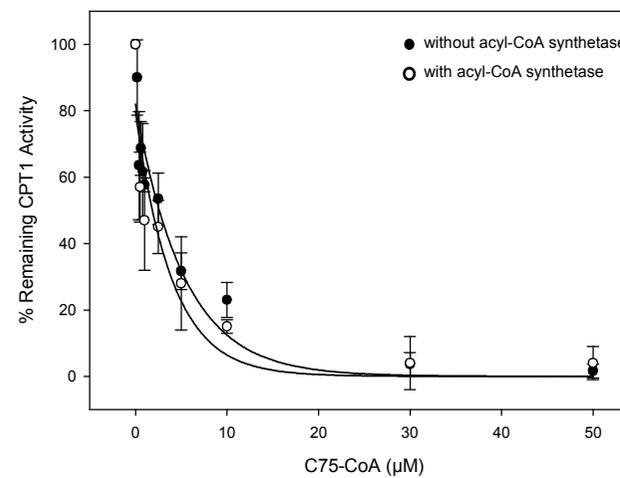


FIGURE 2

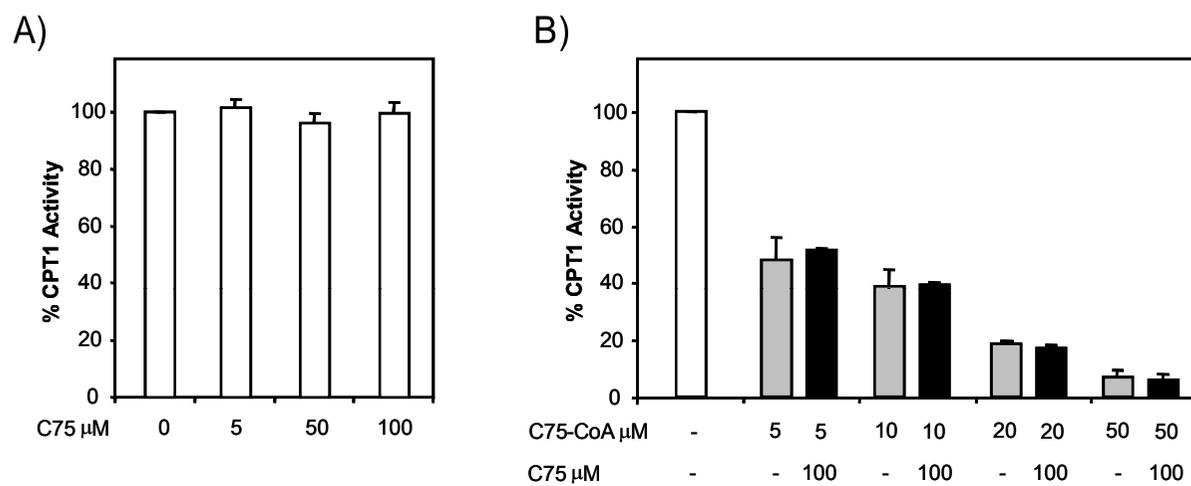


FIGURE 3

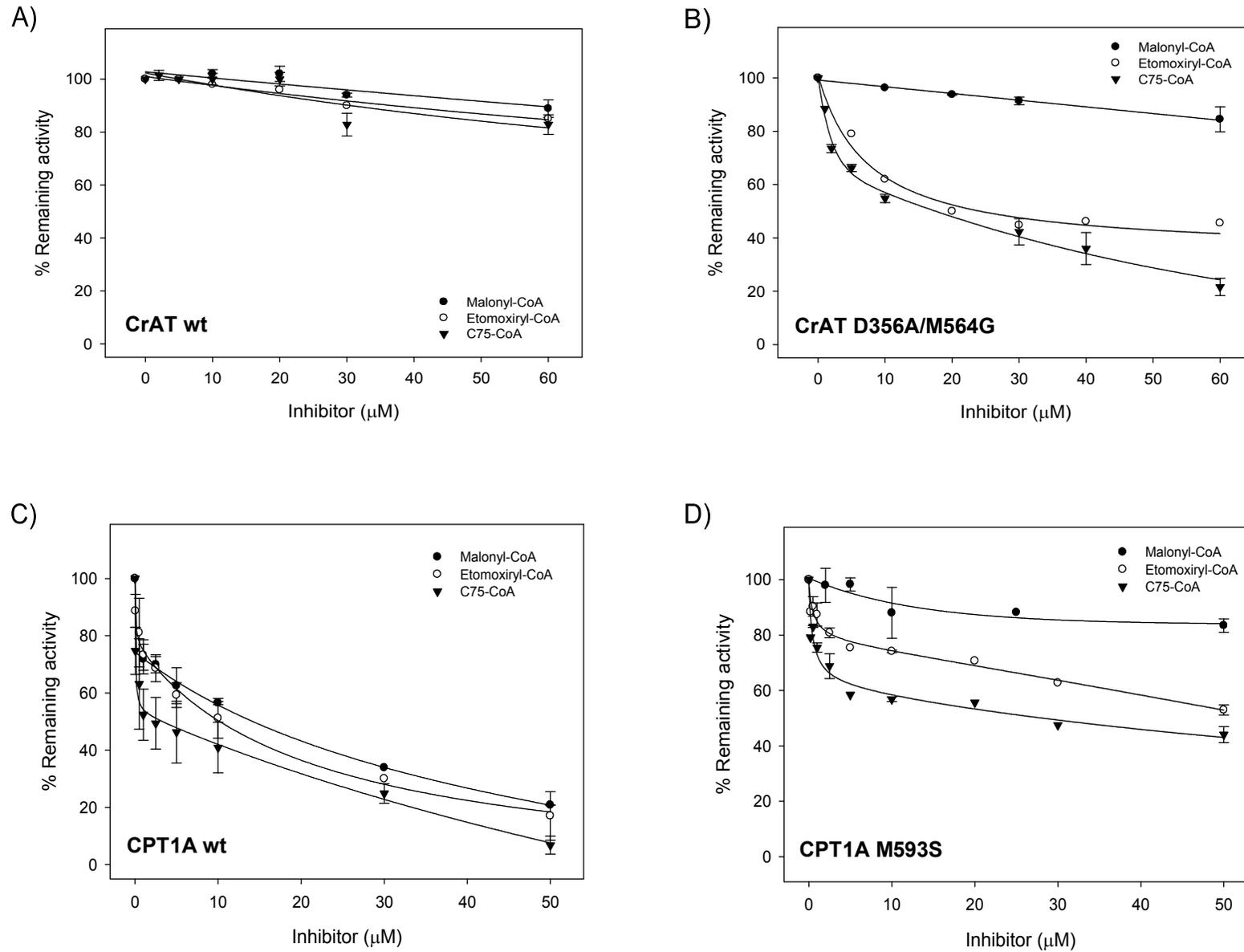


FIGURE 4

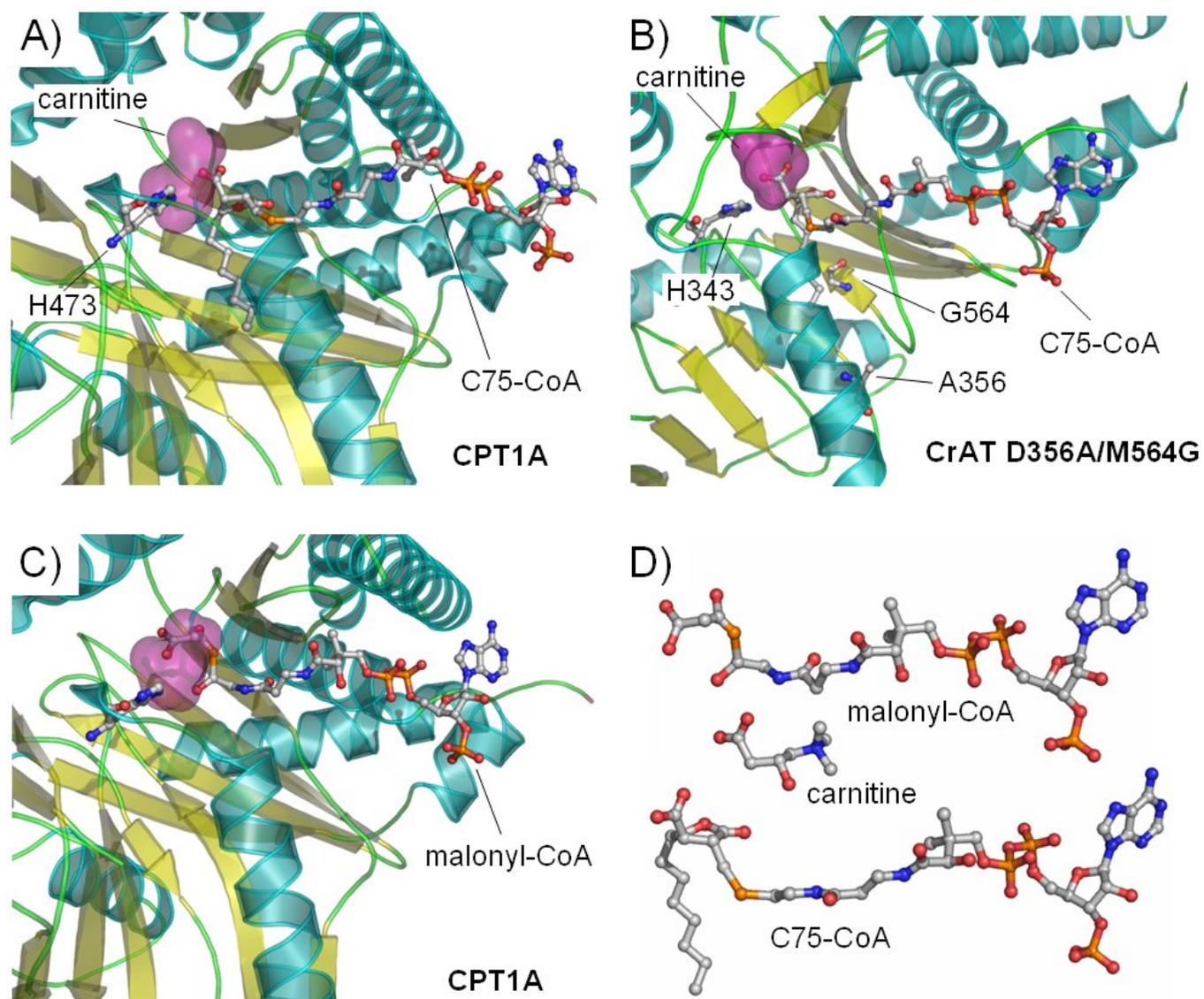


FIGURE 5

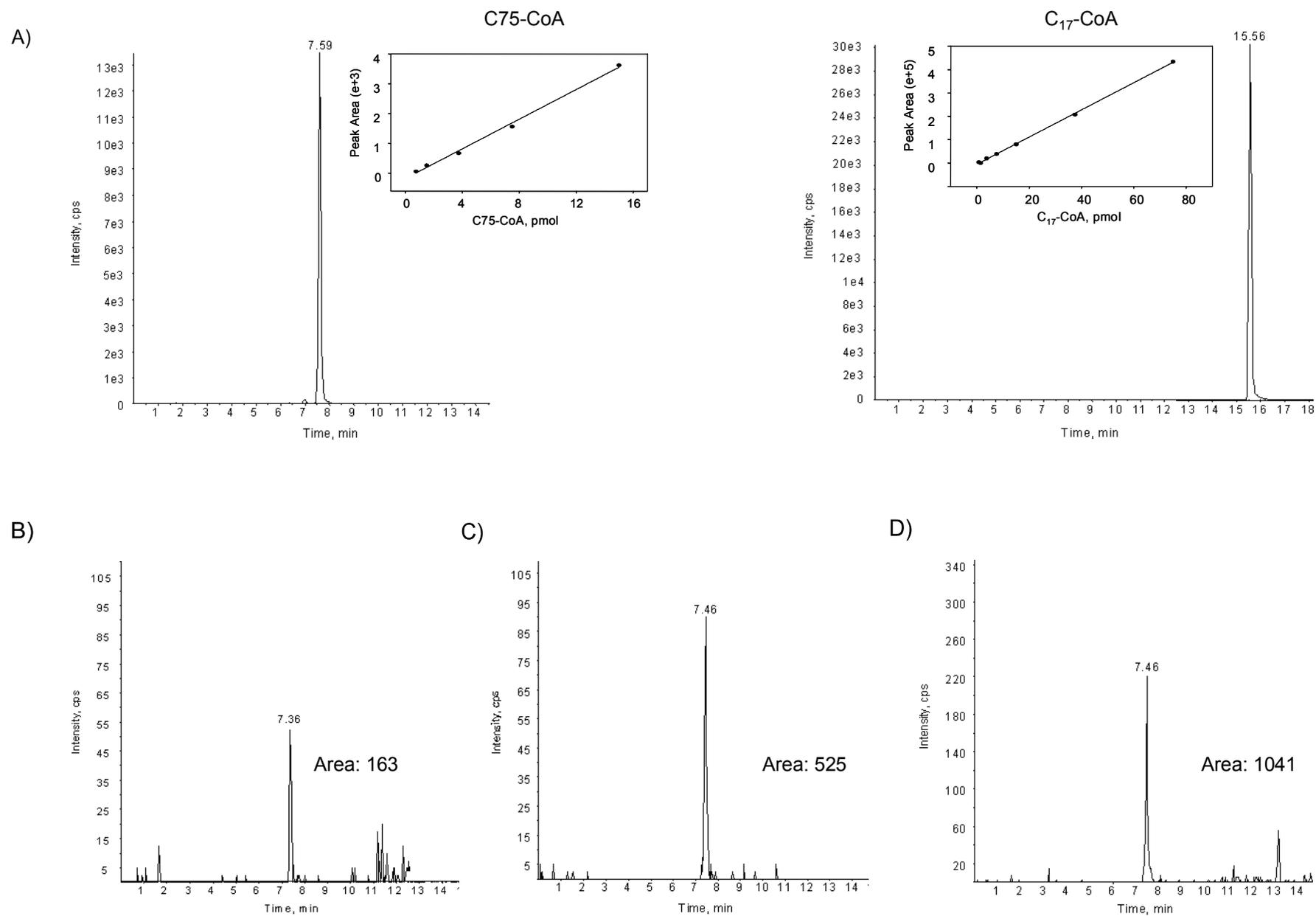


FIGURE 6

