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Differential pharmacologic properties of the two C75 enantiomers: (+)-C75 is a strong anorectic drug. (–)-C75 has antitumour activity

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SHORTENED TITLE

Stereoselectivity of C75 enantiomers

KEYWORDS

CPT1 carnitine palmitoyltransferase 1, FAS fatty acid synthase, obesity, cancer, stereoselectivity.

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ABSTRACT

C75 is a synthetic compound described to have antitumoural properties. It produces hypophagia and weight loss in rodents, limiting its use in cancer therapy but identifying it as a potential anti-obesity drug. C75 is a fatty acid synthase (FAS) inhibitor and, through its CoA derivative, it acts as a carnitine palmitoyltransferase (CPT) 1 inhibitor. Racemic mixtures of C75 have been used in all the previous studies; however, the potential different biological activities of C75 enantiomers have not been examined yet. To address this question we synthesized the two C75 enantiomers separately. Our results showed that (−)-C75 inhibits FAS activity *in vitro* and has cytotoxic effect on tumour cell lines, without affecting food consumption. (+)-C75 inhibits CPT1 and its administration produces anorexia, suggesting that central inhibition of CPT1 is essential for the anorectic effect of C75. The differential activity of C75 enantiomers may lead to the development of potential new specific drugs for cancer and obesity.

1. INTRODUCTION

C75 is a synthetic compound with several possible pharmacological applications. On the one hand, it is an inhibitor of fatty acid synthase (FAS).[1] Most tumour cells present a typical phenotype of abnormally elevated FAS activity, the inhibition of which triggers apoptosis.[2,3] This aspect makes FAS inhibitors potential chemotherapeutic compounds. In fact, C75 has antitumour activity in both tumour-cell lines and animal models.[1,3,4] On the other hand, C75 produces anorexia and weight loss in rodents, which limits its use in cancer therapy, but makes it a potential drug for the treatment of obesity and related diseases.[5-7]

The central nervous system (CNS), specifically the hypothalamus, plays a major role in the control of food intake and the maintenance of energy balance. It has been widely reported that C75-derived anorexia is due to its action on hypothalamic neurons; however the exact molecular mechanism underlying the central effect of C75 on food intake has not been fully elucidated. Recent evidences suggest that malonyl-CoA and long chain fatty acyl-CoAs (LCFA-CoAs) in hypothalamic neurons could be molecular signals for the regulation of appetite and energy homeostasis.[8-11] Importantly, it has been demonstrated that C75 raises hypothalamic malonyl-CoA levels.[8] This metabolite is the physiological inhibitor of carnitine palmitoyltransferase (CPT) 1, which catalyzes the first step in the transport of LCFA-CoAs into the mitochondria for β-oxidation.[12] Additionally, we previously demonstrated that central injection of C75 is followed by the formation of C75-CoA, which directly inhibits CPT1 activity in the hypothalamus.[13,14] Nevertheless further research is needed to clarify whether C75-induced hypophagia is directly related to hypothalamic inhibition of FAS, CPT1 or both enzymes.

A major problem associated with C75 research is that, since this compound was first synthesized,[1] only the racemic mixtures has been used in experiments. Indeed, the enzymatic

resolution of the two C75 enantiomers was not reported until very recently.[15] Significantly, it is well known that the stereochemistry of a drug can determine its biological action.[16] It is thus essential that each enantiomer should be studied separately. Taking advantage of our previous experience on asymmetric synthesis of paraconic acids[17-19] herein we describe an efficient stereoselective synthesis of (+)-C75 and (−)-C75 to study their possible differential biological activity. Our results show that the two enantiomers of C75 have selective effects on food intake, body weight and cytotoxicity, demonstrating that the absolute configuration of each molecule plays a crucial role in its respective pharmacological action. Furthermore, each C75 enantiomer is selective for its respective target, FAS and CPT1, which helps us to understand the central anorectic effect of this compound. This study provides results that could facilitate the search for more specific drugs for the treatment of obesity, cancer and other related diseases.

2. MATERIAL AND METHODS

2.1. Materials

L-[Methyl-^{3}H]carnitine hydrochloride was purchased from Amersham Biosciences. [Malonyl-2-^{14}C]- Malonyl-Coenzyme A was from PerkinElmer Health Sciences. Yeast culture media products were from DifcoTM Laboratories. Bradford solution for protein assays was from Bio-Rad Laboratories. RPMI 1640 was from Gibco-Invitrogen Corporation. Defatted bovine serum albumin (BSA), palmitoyl-CoA, malonyl-CoA, (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and other chemicals were from Sigma–Aldrich.

2.2. Synthesis of (+)-C75 and (–)-C75

(+)-C75 and (–)-C75 were stereoselectively prepared using Evans' auxiliaries derived from L-phenylalanine and D-phenylalanine respectively (Scheme 1).

2.2.1. Stereoselective synthesis of (+)-C75

(S)-Methyl 4-(4-benzyl-2-oxooxazolidin-3-yl)-4-oxobutanoate [(+)-**1**]*:*

BuLi in hexanes (16.3 ml, 40.7 mmol) was added dropwise over 15 min at –78 ºC under N_2 to a solution of the commercially available (*S*)-4-benzyloxazolidin-2-one (6.00 g, 33.90 mmol) in dry THF (250 ml). The resulting mixture was stirred for 35 min. Then, methyl 4-chloro-4-oxobutanoate (4.68 ml, 37.29 mmol) was added and the mixture was stirred for 30 min at –78 ºC and 30 min at rt. The reaction was quenched by adding saturated aq. NH_4Cl (30 ml), the volatiles were evaporated and the resulting residue was taken up in CH_2Cl_2 (50 ml). The aqueous layer was extracted with 50 ml of CH_2Cl_2 and then the combined organic extracts were washed with NaOH 1N (20 ml) and brine (20 ml). The solution was dried ($MgSO_4$) and concentrated under reduced pressure. The

resulting orange solid was recrystallized from AcOEt:hexane (1:1) to give 7.18 g (24.67 mmol, 73%) the almost pure product as a white solid.

White solid [(+)-**1**]; **mp**: 81-82 ºC; $\mathbf{R_f}$ (hexane/AcOEt 7:3) = 0.33; $\mathbf{[\alpha]_D}$ = + 55.3 (*c* 1.0, $CHCl_3$); **^{1}H NMR** ($CDCl_3$, 400 MHz): δ 2.69-2.75 (2 H, m, C*H*$_2$-CO-N), 2.77 (1 H, dd, *J*=13.4, 9.5 Hz, C*H*Ph), 3.24-3.31 (3 H, m, C*H*Ph and C*H*$_2$-COO), 3.72 (3 H, s, OC*H*$_3$), 4.21 (2 H, m, C*H*$_2$-O), 4.68 (1 H, m, C<u>H</u>-N), 7.20-7.36 (5 H, m, Ar); **^{13}C NMR** ($CDCl_3$, 101 MHz): δ 28.0, 30.8, 37.7, 51.9, 55.1, 66.3, 127.3, 128.9, 129.4, 135.1, 153.4, 171.9, 172.8; **IR** (film): 2953, 2928, 1780, 1737, 1697, 1390, 1213, 993, 761; **HRMS** (ESI+) calcd for $C_{15}H_{17}NO_5Na$ $[M+Na]^+$ = 314.0999, found: 314.0992.

(S)-4-Benzyl-3-((2R,3S)-2-octyl-5-oxotetrahydrofuran-3-carbonyl)oxazolidin-2-one [(+)-**2**]*:*

A solution of Bu_2BOTf 1 M in CH_2Cl_2 (0.57 ml, 0.57 mmol) was added dropwise via cannula to a mixture of methyl (*S*)-4-(4-benzyl-2-oxo-1,3-oxazolidin-3-yl)-4-oxobutanoate (150 mg, 0.515 mmol) and activated 4 Å molecular sieves (~0.8 g) in dry CH_2Cl_2 under N_2 at –20 ºC. In few minutes the solution became dark pink. The mixture was stirred for 30 min at the same temperature and then DIPEA (0.11 ml, 0.625 mmol) was added carefully. The resulting yellow solution was stirred 45 min at –20 ºC and then freshly distilled *n*-nonanal (0.13 ml, 0.76 mmol) was added dropwise. The mixture was further stirred at –20 ºC for 3 h and then quenched with saturated NH_4Cl (1.5 ml). The aqueous layer was extracted with CH_2Cl_2 (2 × 5 ml). The combined organic extracts were dried ($MgSO_4$) and the solvent was removed under vacuum. MeOH (5 ml) and a catalytic amount of *p*-toluensulfonic acid were added to the residue (278 mg) and the resulting solution was refluxed for 1 h. Then, the solvent was removed and the residue was dissolved in CH_2Cl_2 (10 ml). The organic layer was washed with $NaHCO_3$ (5 ml) and H_2O

(5 ml), dried ($MgSO_4$) and the volatiles were evaporated under vacuum. Purification of the residue by flash chromatography (hexane/AcOEt 6:4) furnished the desired product (0.130 g, 0.33 mmol, 65 %).

White solid [(+)-**2**]; **mp**: 55-57 ºC; $\mathbf{R_f}$ (hexane/AcOEt 6:4) = 0.61; $\mathbf{[\alpha]_D}$ = + 85.3 (*c* 1.0, $CHCl_3$); **^{1}H NMR** ($CDCl_3$, 400 MHz): δ 0.88 (3 H, t, *J* = 6.9 Hz, CH_3), 1.26-1.55 (12 H, m, CH_2), 1.71 (2 H, m, OCHC*H*$_2$), 2.71 (1 H, dd, *J* = 6.8, 17.6 Hz, C*H*H-CO), 2.83 (1 H, dd, *J* = 9.2, 13.2 Hz, *CH*H-Ph), 3.00 (1 H, dd, *J* = 9.6, 17.6 Hz, CH*H*-CO), 3.26 (1 H, dd, *J* = 3.2, 13.2 Hz, *C*H*H*-Ph), 4.16 (1 H, m, C*H*R-CO), 4.28 (2 H, m, C*H*$_2$-OCO), 4.71 (1 H, m, C*H*R-N), 4.80 (1 H, m, CHR-O-CO), 7.17-7.37 (5 H, m, Ar); **^{13}C NMR** ($CDCl_3$, 101 MHz): δ 14.0, 22.6, 25.3, 29.1, 29.2, 29.3, 31.7, 32.4, 35.1, 37.7, 45.0, 55.2, 66.7, 81.5, 127.6, 129.0, 129.3, 134.6, 153.0, 171.0, 174.3; **IR** (film): 2926, 2855, 1782, 1698, 1559, 1456, 1389, 1210, 1113, 1076, 1054, 762, 703; **HRMS** (ESI+) calcd for $C_{23}H_{32}NO_5$ $[M+H]^+$ = 402.2275, found: 402.2284.

(2R,3S)-2-Octyl-5-oxotetrahydrofuran-3-carboxylic acid [(+)-**3**]*:*

H_2O_2 (100 μL, 0.98 mmol) and $LiOH{\cdot}H_2O$ (6 mg, 0.25 mmol) were added to a solution of (*S*)-4-benzyl-3-((2*R*,3*S*)-2-octyl-5-oxotetrahydrofuran-3-carbonyl)-1,3-oxazolidin-2-one (50 mg, 0.125 mmol) in THF/H_2O 1:1 (5 ml) at 0 ºC. The resulting mixture was stirred at rt for 30 min. Then reaction was quenched with Na_2SO_3 1.5 M (0.4 ml). The mixture was treated with 1N NaOH until the solution was basic and the aqueous layer was washed with CH_2Cl_2 (5 × 10 ml) and then was acidified to *p*H=1–2 with conc. HCl. The aqueous layer was extracted with CH_2Cl_2 (5 × 10 ml). The combined organic extracts were washed with brine, dried ($MgSO_4$), and concentrated under vacuum to give 22 mg (0.091 mmol, 73 %) of product.

White solid [(+)-**3**]; **mp**: 98-100 ºC; $\mathbf{R_f}$ (hexane/AcOEt/AcOH 8:2:0.1) = 0.24; $\mathbf{[\alpha]_D}$ = + 34.0 (*c* 1.0, MeOH); **^{1}H NMR** ($CDCl_3$, 400 MHz): δ 0.88 (3 H, t, *J* = 6.4 Hz, CH_3), 1.28-1.56 (12 H, m, CH_2), 1.70-1.86 (2 H, m, CH_2), 2.82 (1 H, dd, *J*= 9.6, 17.6 Hz, C*H*H-CO), 2.95 (1 H, dd, *J*= 8.4, 17.9 Hz, CH*H*-CO), 3.10 (1 H, m, C*H*-COOH), 4.62 (1 H, m, CHR-O); **^{13}C NMR** ($CDCl_3$, 101MHz): δ 15.1, 23.6, 26.2, 30.1, 30.2, 30.3, 32.8, 32.9, 36.3, 46.4, 83.0, 175.7, 177.1; **IR** (film): 3000-3300, 2926, 2853, 1749, 1718, 1393, 1243, 1215, 1195, 759, 669; **HRMS** (ESI+) calcd for $C_{13}H_{22}NaO_4$ $[M+Na]^+$ = 265.1410, found: 265.1410.

*Synthesis of (2R,3S)-4-methylene-2-octyl-5-oxotetrahydrofuran-3-carboxylic acid [(+)-**C75**]:*

A sample of (2*R*,3*S*)-2-octyl-5-oxotetrahydrofuran-3-carboxylic acid (85 mg, 0.35 mmol) was heated in a 2M solution of MMC (magnesium methyl carbonate**)** in DMF (6 ml) at 130-135 ºC under N_2 for 45 h. Then, 6N HCl (10 ml) and CH_2Cl_2 (15 ml) were added carefully. The aqueous layer was extracted with CH_2Cl_2 (2 × 10 ml). The combined organic extracts were dried ($MgSO_4$) and the volatiles were removed to afford 100 mg of residue. The crude was stirred with 1.2 ml of a freshly prepared stock solution (1 ml AcOH, 0.75 ml formalin, 30 mg NaAcO and 0.26 ml *N*-methylaniline) for 1.45 h. To the resulting mixture, a (10:1) solution NaCl : conc. HCl (5 ml) and CH_2Cl_2 (12 ml) were added. The aqueous layer was extracted with CH_2Cl_2 (5 × 10 ml). The combined organic extracts were washed with LiCl 5% (2 × 4 ml), HCl 0.02 N (2 × 4 ml) and H_2O (3 × 5 ml). The organic layer was stirred with 5 ml of saturated $NaHCO_3$ for 5 min and then the aqueous layer was then treated with concentrated HCl until pH~ 1-2 and was extracted with CH_2Cl_2 (4 × 10 ml). The combined organic extracts were washed with brine and dried ($MgSO_4$) and the solvent was removed to give 54 mg (0.21 mmol, 60%) of (+)-C75.

White solid [(+)-**C75**]; **mp**: 88-89 ºC; $\mathbf{R_f}$ (CH_2Cl_2/MeOH 9:1) = 0.27; $\mathbf{[\alpha]_D}$ = + 11.4 (c 1.0, $CHCl_3$); **^{1}H NMR**: δ 0.88 (3 H, t, *J* = 6.9 Hz, CH_3), 1.20-1.53 (12 H, m, CH_2), 1.67-1.79 (2 H, m, CH_2) 3.63 (1 H, dt, *J*=5.6, 2.8 Hz, C*H*-COOH), 4.81 (1 H, td, *J*=7.2, 5.6 Hz, CHR-O), 6.02 (1 H, d, *J*= 2.7 Hz, =C*H*H), 6.46 (1 H, d, *J*=3.0 Hz, =CH*H*); **^{13}C NMR**: δ 14.0, 22.6, 24.7, 29.1, 29.3, 31.7, 35.7, 49.4, 78.8, 125.9, 132.4, 168.2, 174.5; **IR** (film): 3000-3400, 2924, 2852, 1743, 1717, 1660, 1621, 1460.

2.2.2. Stereoselective synthesis of (–)-C75

(R)-methyl 4-(4-benzyl-2-oxooxazolidin-3-yl)-4-oxobutanoate [(–)-**1**]*:*

Compound (–)-**1** was obtained from (*R*)-4-benzyloxazolidin-2-one as a white solid in 70% yield as described above for (+)-**1**. Compound (–)-**1: mp**: 81-82 ºC; $\mathbf{[\alpha]_D}$ = – 56.2 (*c* 1.0, $CHCl_3$); **HRMS** (ESI+) calcd for $C_{15}H_{17}NO_5Na$ $[M+Na]^+$ = 314.0999, found: 314.0989.

(R)-4-Benzyl-3-((2S,3R)-2-octyl-5-oxotetrahydrofuran-3-carbonyl) oxazolidin-2-one [(–)-**2**]*:*

Compound (–)-**2** was obtained from (–)-**1** as described above for its enantiomer in 59% yield. Compound (–)-**2:** white solid; **mp**: 55-57 ºC; $\mathbf{[\alpha]_D}$ = – 85.3 (*c* 1.0, $CHCl_3$); **HRMS** (ESI+) calcd for $C_{23}H_{32}NO_5$ $[M+H]^+$ = 402.2275, found: 402.2280.

(2S,3R)-2-Octyl-5-oxotetrahydrofuran-3-carboxylic acid [(–)-**3**]*:*

Compound (–)-**3** was obtained from (–)-**2** as described above for its enantiomer in 77% yield. Compound (–)-**3:** white solid; **mp**: 98-100 ºC; **[α]D** = – 39.6 (*c* 1.0, MeOH); **HRMS** (ESI+) calcd for $C_{13}H_{22}NaO_4$ $[M+Na]^+$ = 265.1410, found: 265.1414.

*Synthesis of (2S,3R)-4-methylene-2-octyl-5-oxotetrahydrofuran-3-carboxylic acid [(–)-**C75**]:*

(–)-**C75** was obtained from (–)-**3** as described above for its enantiomer in 55% yield. (–)-**75:** white solid; **mp**: 88-89 ºC; $[\alpha]_D$ = – 11.4 (c 1.0, $CHCl_3$).

2.3. Synthesis of (±)-C75-CoA, (+)-C75-CoA and (–)-C75-CoA

2.3.1. Non-enzymatic synthesis of C75-CoA adducts

Coenzyme A (HSCoA) sodium salt hydrate (8.6 mg), and $Na_3PO_4 \cdot 12H_2O$ (7.6 mg) were added to a solution of (±)-C75 (2.5 mg) in D_2O (0.8 ml) in an NMR tube (Figure 1). The structure of the C75-CoA adduct was fully determined by ^1H and ^{13}C NMR, gCOSY and gHSQC experiments.[13] Similarly, (+)-C75-CoA and (–)-C75-CoA were prepared respectively with (+)-C75 or (–)-C75 and HSCoA. The structure of the CoA adducts was subsequently determined as described above.

The most significant spectroscopic data of (+)-C75-CoA are (Figure 1): **^{1}H NMR** (500 MHz): δ 0.58 (s, 3H, H10"), 0.71 (t, *J*=6.9, 3H, Hm), 0.72 (s, 3H, H11"), 1.07-1.21 (m, 10H, Hh-l), 1.22-1.34 (m, 2H, Hg),1.62-1.67 (m, 2H, Hf), 2.33 (t, *J*=6.7, 2H, H6"), 2.58 (t, *J*=6.6, 2H, H9"), 2.73 (d, *J*=13.8,1H, Hc), 2.77 (d, *J*=9.1, 1H, Hd), 2.85 (d, *J*=13.8, 1H, Hc'), 3.22 (t, *J*=6.6, 2H, H8'), 3.32(q, *J*=6.7, 2H, H5"), 3.38 (dd, *J*=9.8, 5.0, 1H, H1"), 3.67 (dd, *J*=9.8, 5.0, 1H, H1"), 3.87 (s, 1H, H3"), 4.05-4.12 (m, 2H, H5'), 4.41-4.45 (m, 2H, H4', He), 4.60-4.70 (m, HDO,H2', H3'), 6.03 (d, *J*=7.1, 1H, H1'), 8.13 (s, 1H, H2), 8.42 (s, 1H, H8). **^{13}C NMR** (100MHz): δ 13.6 (Cm), 18.1 (C11"), 21.0 (C10"), 22.2 (Cl), 24.4 (Cg), 28.4, 28.5, 28.6 (Ch,i,j),30.4 (Cc), 31.3 (Ck), 31.7 (C9"), 34.0 (Cf), 35.5 (C5"), 38.4, 38.6 (C8", C6"), 54.7 (Cd),65.9 (C5'), 72.0 (C1"), 73.8, 74.2, 74.5 (C2', C3', C3"), 83.7 (C4'), 84.3 (Ce), 86.7

(C1'),118.8 (C5), 140.1 (C8), 149.6 (C4), 153.1 (C2), 155.9 (C6), 174.0 (C7"), 174.9 (C4"),178.0 (Ca), 179.8 (CO_2H).

(–)-C75-CoA and (+)-C75-CoA adducts showed identical ^{1}H and ^{13}C NMR spectra.

2.3.2. An attempt at enzymatic synthesis of C75-CoA adducts

All the components required for the preparation of the reaction buffer [0.1% (w/v) Triton X-100, 10mM ATP, 1 mM DTT, 10 mM $MgCl_2$, 100 mM MOPS-NaOH (pH 7.5)] were previously dissolved in D_2O and the ^{1}H NMR spectrum of the mixture was recorded. Then, HSCoA (5 mM) and (+)-C75, or the (–)-isomer, (4 mM) were added to 1 ml of the buffer. After each addition, ^{1}H NMR spectrum was recorded in order to assign representative signals to each component in the complex sample. Finally, to assess whether acyl-CoA synthetase (ACS) was a necessary step for the adduct formation, ACS from *Pseudomonas sp.* (0.25 unit) was added and the mixture was incubated at 35 °C for 2 h. recording the ^{1}H NMR spectrum for each enantiomer.

2.4. Animals and treatments

Sprague-Dawley male rats (260–290 g) were purchased from Harlan, and experiments were performed following 1 week's acclimatization. Six-wk-old C57/BL6J male mice were purchased from Janvier, and experiments were performed after 4 wk. 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.

Chronic intracerebroventricular (i.c.v.) cannulae were stereotaxically implanted into lateral ventricle of rats under ketamine (Imalgene, 90 mg/kg) and xylazine (Rompun, 11 mg/kg) anaesthesia. The coordinates were 1.0 mm posterior to bregma, 1.4 mm lateral of the sagittal sinus and 4 mm ventral to the dura mater.[20] Analgesics (buprenorphin, 0.3 mg/400 ml)

and antibiotics (enrofluoxacin, 10%) were added to the water for 7 days after surgery. I.c.v. injections (10 μl, 33 mM final concentration in DMSO:RPMI 1640 medium (1:3) of (+)-C75, (−)-C75 or vehicle (DMSO:RMPI 1640, 1:3)) were performed with a microliter syringe (Hamilton) after 1 wk of postsurgical recovery. For feeding experiments, rats received single injections 30 min before the light was turned off. We measured intakes of chow, corrected for spillage, and body weight after 22 h. For the CPT1 activity experiments rats were killed 1 h after injection. The hypothalamus was then excised and mitochondrial-enriched extract was obtained and assayed immediately. For determination of FAS activity rats were killed 1 h after injection, and the hypothalamus was excised and stored at -80 °C.

Intraperitoneal (i.p.) injections of of (+)-C75, (−)-C75 (100 μL in RPMI 1640 medium, 15 mg/kg) or vehicle were carried out daily in mice 3 h before the light was turned off, for 3 days. Body weight and food intake were measured after every injection.

2.5. Expression of CPT1 in *Saccharomyces cerevisiae*

Rat CPT1A was expressed in yeast cells and mitochondrial cell extracts were obtained as previously described.[21]

2.6. Determination of carnitine acyltransferase activity

Mitochondrial-enriched fractions were obtained by differential centrifugation,[22] with minor modifications. All protein concentrations were determined using the Bio-Rad protein assay with bovine serum albumin as a standard.

A radiometric method was used for the assay of carnitine acyltransferase as described previously.[21] The activity was assayed in mitochondrial-enriched fractions obtained from yeast (3–4 μg protein) and from rat hypothalamus (100 μg protein). Enzyme activity was assayed for 5 min at 30 °C in a total volume of 200 μL. The substrates were 400 μM L-

carnitine, and 50 μM palmitoyl-CoA. For the studies *in vitro* enzyme was preincubated with increasing concentration of drugs (0.1-100 μM) for 1 min. The values obtained were used to estimate the IC_{50} (the concentration that inhibits 50% of the enzymatic activity). 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.

2.7. Determination of fatty acid synthase activity

For experiments *in vitro*, FAS was purified from rat liver following the protocol described by Linn.[23] For the fatty acid synthase activity assay a spectrophotometric method was used.[24] Drugs concentrations ranging 100 to 5000 μM were used to estimate the IC_{50} value.

For experiments *in vivo*, frozen hypothalamus extracts were homogenized with 400 μL of buffer (0.25 M sucrose, 1 mM EDTA, 1mM DTT and protease inhibitors) then centrifuged (14,000x*g*) at 2°C for 30 min. Supernatant was assayed for fatty acid synthase activity using a radiometric method.[25]

2.7.1. Spectrophotometric method

Cytosolic hepatic extracts obtained from rat (315 μg) were preincubated at 30 °C for 30 min with increasing concentration of drugs; (+)-C75 or (−)-C75, dissolved in DMSO (in the main: 100 – 5000 μM) using DMSO for a blank, and (±)-C75-CoA dissolved in distilled water. 250 μM NADPH and 200 μM acetyl-CoA in potassium phosphate buffer (pH 7.2) were added to preincubated enzyme and equilibrated at 37 °C for 3 min. The reaction was initiated by the addition of 200 μM malonyl-CoA. Total reaction volume was 1 ml. The oxidation of NADPH was monitored at 340 nm at 37 °C for 10 min.

2.7.2. Radiometric method

100 µL of hypothalamic cytosolic extract was preincubated at 37 °C for 10 min and then a mixture of 225 µM NADPH, 24 µM Acetyl-CoA, 640 µM Malonyl-CoA and 0.05 µC ^{14}C-Malonyl-CoA in buffer (0.1 M K_2HPO_4 pH 7.2, 0.2 mM EDTA pH 8, 4 mM DTT and 0.2% BSA) was added. Total reaction volume was 500 µL. After 20 min at 37 °C reaction was arrested with 100 µL of NaOH 0.5 N. Afterwards, 200 µL of EtOH 96% was added and the mixture was heated to 100 ºC for 15 min to induce saponification. The solution was then acidified with 100 µL of HCl 1 N and fatty acids were extracted with 2 ml of pentane (3 washes). 5 ml of combined organic layer was washed with 2 ml of AcOH 0.1%, and then pentane extract was evaporated. The residue was redissolved in 0.5 ml of pentane and subjected to scintillation counting.

2.8. Cell cultures and viability assays

MCF7, SKBr-3 and OVCAR3 cell lines were used in all the studies. Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in complete medium composed of HAM'S F12 supplemented with 10% heat-inactivated foetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. The cultures were passaged once or twice a week by gentle trypsinization, and cells were grown to confluence in 10 cm culture dishes.

To evaluate the cytotoxic effect of the drugs, MTT-cytotoxicity assay was performed. 4-8 × 10^3 cells/well were plated in 96-well plates in 100 µl of culture medium. Once the cells were attached to the plate the medium was removed and cells were incubated for 72h in fresh medium with different concentrations (2.5, 5, 10, 15, 20 and 30 µg/ml) of (+)-C75 or (−)-C75. DMSO was used for a blank at a final concentration ≤ 0.2%. Then the cells were incubated for 3h with 100 µL of fresh medium and 20 µL of MTT (5 µg/ml). Following treatment, the supernatants were carefully removed and the MTT-formazan crystals, formed by

metabolically viable cells, were solubilised by adding 100 μL/well of DMSO and absorbance was measured at 570 nm.

2.9. Statistical analysis

Data are expressed as the mean ± SEM. Different experimental groups were compared using the unpaired Student's *t* test and one-way ANOVA followed by Bonferroni's test for comparisons *post hoc*. A probability level of $p < 0.05$ was considered to be statistically significant.

3. RESULTS AND DISCUSSION

3.1. Synthesis and characterization of (+)-C75, (–)-C75 and their Coenzyme A adducts

Enantiomers of C75 were synthesised in parallel (Scheme 1). We established that (+)-C75 is (2*R*,3*S*)-4-methylene-2-octyl-5-oxotetrahydrofuran-3-carboxylic acid and (–)-C75 is the (2*S*,3*R*) isomer. The synthesis of the C75-CoA adducts was performed by mixing both reagents in D_2O at pH~8. The structure and relative stereochemistry of the CoA adducts was determined by ^{1}H NMR spectroscopy (Figure 1). In a previous study[14] we hypothesized that the enzyme acyl-CoA synthase (ACS) could catalyze the formation of C75-CoA *in vivo*. Among the different ways in which HSCoA could be bound to C75, we envisaged that HSCoA could be added to the exocyclic double bond of C75. To confirm this hypothesis, we performed a series of structural ^{1}H NMR studies (data not shown). Thus, ^{1}H NMR spectra of the complex mixtures including ACS showed a significant reduction of the signals corresponding to the exocyclic methylene of C75 as well as the presence of conclusive signals assignable to the same product obtained in the former experiment in the absence of ACS enzyme (Figure 1). Therefore, we conclude that C75-CoA is the thioether adduct produced after the spontaneous nucleophilic attack of the SH group at the exocyclic methylene of C75.

3.2. C75 enantiomers have selective activities on FAS and CPT1 *in vitro*

Since previous studies had indicated that the CoA adduct is the form of C75 that inhibits CPT1,[13,14] the effect of the compounds (+)-C75-CoA and (–)-C75-CoA on yeast-overexpressed CPT1A activity was analyzed (Figure 2a). Results showed that (+)-C75-CoA inhibits CPT1 *in vitro* (IC_{50} = 0.68 ± 0.21 μM). In contrast, (–)-C75-CoA barely affected the activity of yeast-overexpressed CPT1A (IC_{50} > 50 μM). Consistent with our previous observations,[13] the free form of C75 had no effect on CPT1 activity. Next, the action of both C75 enantiomers on FAS activity was analyzed. Activity assays were performed with

cytosolic hepatic extracts containing FAS. Results demonstrated that (–)-C75 acts as a FAS inhibitor with an IC_{50} of 460 ± 44 μM. In contrast, (+)-C75 showed a much smaller effect on FAS activity (IC_{50} > 5000 μM) (Figure 2b). The adduct (±)-C75-CoA does not affect FAS enzymatic activity. Altogether, these data demonstrate for the first time that C75 enantiomers present stereoselectivity for the targets FAS and CPT1. These differences raised the idea that pharmacological actions of C75 enantiomers should be different. To test this hypothesis we evaluate the effect of these molecules on tumour-cell viability, food intake and body weight, all parameters affected by the racemic mixture of C75.

3.3. (–)-C75 has a cytotoxic effect on tumour-cell lines

In order to study the cytotoxicity of (–)-C75 and (+)-C75 we performed an MTT-cytotoxic assay with MCF7, SKBr-3 and OVCAR3 tumour-cell lines, all commonly used for cancer studies. Results showed that (–)-C75 has a cytotoxic effect on all the lines tested with an IC_{50} of 38 ± 2 μM, 46 ± 3 μM and 18 ± 3 μM, on MCF7, SKBr-3 and OVCAR3 cells respectively. However, (+)-C75 presented a higher IC_{50} (> 60 μM) in all cases (Figure 3). In the light of our results, the findings reported by different authors regarding the effect of racemic C75 on tumour cell growth and survival, could be attributed mainly to the (–)-C75 enantiomer. Many studies published during the last decade demonstrate a link between FAS inhibition and cytotoxicity in tumour cells.[26] Since the (+)-C75 enantiomer is a weak inhibitor of FAS, we conclude that the main critical effects on inhibition of growth malignancies, due to the C75-derived FAS inhibition, are probably produced by the enantiomer (–)-C75 when the racemic mixture is used. However, further research is needed to identify the molecular mechanism underlying (–)-C75-mediated cancer cell toxicity.

3.4. Central administration of (+)-C75 decreases food intake, body weight and hypothalamic CPT1 activity

Next, we analyzed the central effect of each C75 enantiomer on food consumption and body weight. Male adult Sprague-Dawley rats received intracerebroventricular (i.c.v.) injections of (+)-C75, (−)-C75 or vehicle (control animals). Interestingly, (+)-C75 caused a significant inhibition in chow intake (52 ± 12% respect to control animals, p □ 0.05) and reduction in body weight (2.7 ± 0.62% respect to control animals, p □ 0.05) while (−)-C75 did not produce significant changes (Figure 4a and b). We also examined the action of C75 enantiomers on CPT1 and FAS activities in the hypothalamus. The results indicated that only i.c.v. administration of (+)-C75 inhibited hypothalamic CPT1 activity (25.4 ± 3.6% respect to control animals, p □ 0.05) (Figure 4c). In a previous study we already demonstrated that formation of C75-CoA, essential for CPT1 inhibition, occurs in the hypothalamus after central administration of the free form C75.[13] Neither of the two enantiomers caused FAS inhibition in the hypothalamus after their i.c.v. injection (Figure 4d), although central inhibition of this enzyme has been proposed as a mechanism of C75-induced hypophagia.[5,8] We cannot rule out the possibility that C75 could act as a FAS inhibitor in different experimental conditions, but importantly at the dose tested here, C75 suppressed food intake without reducing central FAS activity. It is worth noting that hypothalamic FAS activity had not been previously measured after C75 i.c.v. administration. Instead, hypothalamic malonyl-CoA levels were considered to indicate FAS inhibition.[8,27] However, this is only an indirect measurement, since malonyl-CoA concentration does not depend exclusively on FAS enzymatic activity. Furthermore, other authors previously proved a disconnection between C75-induced anorexia and hypothalamic FAS inhibition,[28,29] suggesting that other enzymes could be implicated in the reduction of appetite caused by this compound.

It has been stated that pharmacological and genetic inhibition of CPT1 in the hypothalamus reduces food intake.[11,30] In the present study we demonstrate that only (+)-C75, through its CoA derivative, inhibits CPT1 activity both *in vitro* and *in vivo* and reduces body weight and food intake after i.c.v. administration. These results suggest that CPT1 inhibition might be the cause of the appetite loss produced by C75. It is known that malonyl-CoA accumulates in the hypothalamus after C75 central administration,[8] which may contribute to the inhibition of CPT1;[8,27] however, it is important to mention that hypothalamic CPT1 inhibition observed in our experiments is independent of the putative inhibition by increased malonyl-CoA. As we discussed previously,[13] hypothalamic CPT1 activity was measured in twice-washed mitochondria. Hence, malonyl-CoA was unlikely to remain within CPT1 after this procedure. In contrast, C75-CoA is a tight-binding inhibitor that remains bound to CPT1 after mitochondria have been washed.[14] This demonstrates that C75-CoA, after its formation in the hypothalamus, directly inhibits CPT1 activity *in vivo*. Pharmacologic inhibition of CPT1 in the hypothalamus leads to an accumulation of LCFA-CoA in hypothalamic neurons,[11] which has been proposed as a satiety signal that reduces food intake through down-regulation of orexigenic neuropeptides. We hypothesized that this mechanism may also underlie the central (+)-C75-induced hypophagia.

3.5. Peripheral administration of (+)-C75 decreases food intake and body weight

We also examined the effect of peripheral administration of each C75 enantiomer. Thus, we performed daily intraperitoneal (i.p.) injections of (+)-C75 and (–)-C75 on 10-wk-old C57/Bl6 mice. Our results showed a decrease in body weight and food intake after (+)-C75 injection (Figure 4e and f); however, consistent with previous results,[31] animals showed resistance to C75-induced anorexia after the second day of treatment (Figure 4f). It was previously reported that i.p. administration of C75 has the same aforementioned effect on

hypothalamic orexigenic neuropeptides,[31] since a small portion of C75 reaches the hypothalamus after i.p. injection;[5] however, a direct systemic effect of C75 cannot be excluded and might indirectly contribute to the observed anorexia.[32] Peripheral administration of (−)-C75 produced a mild reduction in body mass respect to control animals (4.4 ± 1% respect to control animals on day 1, p □ 0.05) (Figure 4e), although no effect was observed after central administration of the same compound (Figure 4c). Remarkably, this weight loss was significantly lower than that observed with (+)-C75 (Figure 4e), and it could not be attributed to a decrease in food consumption (Figure 4f). Given that we and others observed that C75-treated-animals produced watery stools[5,32] the reduction in body mass could be due to a toxic effect in the intestine, although this possibility requires further study.

4. CONCLUSIONS

Results presented here indicate that the two C75 enantiomers have different biological actions and show selectivity on their respective targets, FAS and CPT1 (Figure 5). (+)-C75 is anorectic and inhibits CPT1 activity. (–)-C75 is a FAS inhibitor and anti-tumour agent without affecting food intake, which confers an advantage for the use of this enantiomer as a chemotherapeutic agent. These results shed light into the central mechanism of C75-derived hypophagia, highlighting hypothalamic CPT1 as a potential therapeutic target for weight-loss treatments. The pharmacological effects of C75 can thus be separated, which may lead to more specific drugs for cancer and obesity.

FOOTNOTES

K.M. is a recipient of a fellowship from *Institut de Biomedicina de la Universitat de Barcelona* (IBUB), Barcelona, Spain. Research contract to P.M. is supported by CIBERObn, *Instituto de Salud Carlos III*, Spain. The pharmacological use of C75 enantiomers has been protected by the research group under patent application. The research team is interested in synthesizing and selling the products through the University of Barcelona Facilities.

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FIGURE LEGENDS

Scheme 1. The enantioselective synthesis of the two enantiomers of C75 was carried out by standard organic synthesis methodologies.[33]

Figure 1. (**a**) Synthesis of (+)-C75-CoA and (−)-C75-CoA. (**c**) ^{1}H NMR spectra of an equimolar mixture of C75 and HSCoA at t = 0 and after 4 h, when the reaction was completed. The signals of exocyclic double bond of starting C75 and the signals of saturated methylene group of the product are marked with triangles.

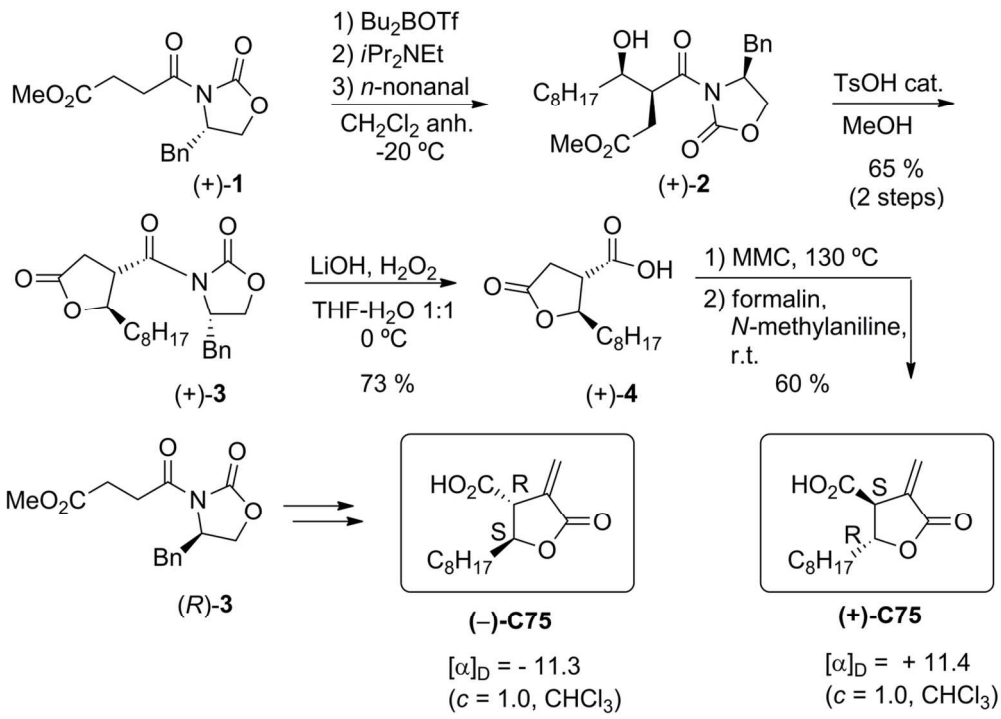
Figure 2. **Effects of (+)-C75-CoA and (−)-C75-CoA on CPT1A activity and (+)-C75 and (−)-C75 on FAS activity.** (**a**) Mitochondrial extracts from yeast expressing rat CPT1A were preincubated for 5 min with increasing concentrations of (±)-C75 (□), (+)-C75-CoA (●) and (−)-C75-CoA (▲), then CPT1 activity was measured. (**b**) Cytosolic hepatic extracts obtained from rat were preincubated 30 min with increasing concentrations of (±)-C75-CoA (■), (+)-C75 (○), (−)-C75 (Δ), then FAS activity was measured.

Figure 3. Effect of (+)-C75 and (−)-C75 on SKBr-3 MCF-7, and OVCAR3 cell viability. SKBr-3, MCF7 and OVCAR3 cells were incubated with increasing concentration of the compounds over 3 days. Then drug cytotoxicity was determined using a standard colorimetric MTT assay and the respectively IC_{50} were calculated.

Figure 4. Effect of central and peripheral administration of (+)-C75 and (−)-C75 on food intake, body weight and hypothalamic CPT1 and FAS activities. (**a**) Food intake and (**b**) body weight change measured in rats 22 h after i.c.v. injection of (+)-C75 (n = 8) and (−)-C75 (n = 7). Data expressed as percentage respective to control. (**c**) Determination of hypothalamic CPT1 activity after i.c.v. injection. Control injections (n = 13), (+)-C75 (n = 7)

and (–)-C75 (n = 5). (**d**) Determination of hypothalamic FAS activity after i.c.v. injection. Control injections (n = 5), (+)-C75 (n = 7) and (–)-C75 (n = 6). (**e**) Change of body weight and (**f**) food intake measured in 10-wk-old mice after daily i.p. injection (15 mg/kg) of (+)-C75 (n = 16) and (–)-C75 (n = 16). * $p < 0.05$, respect to control; ** $p < 0.05$, respect to control and (–)-C75; # $p < 0.05$, respect to control and (+)-C75.

Figure 5. Proposed action of C75 enantiomers on enzymes involved in lipid metabolism. Each C75 enantiomer is stereoselective for its respective target, FAS and CPT1. (–)-C75 enantiomer inhibits FAS irreversibly, probably through the formation of a covalent bond with a cystein of the enzyme. (+)-C75 enantiomer inhibits CPT1 through the derivative (+)-C75-CoA, produced by the reaction of (+)-C75 with HSCoA. Other possible targets of the C75 enantiomers should be examined in the future in order to use these compounds for therapeutic purposes.



Scheme 1. The enantioselective synthesis of the two enantiomers of C75 was carried out by standard organic synthesis methodologies.33
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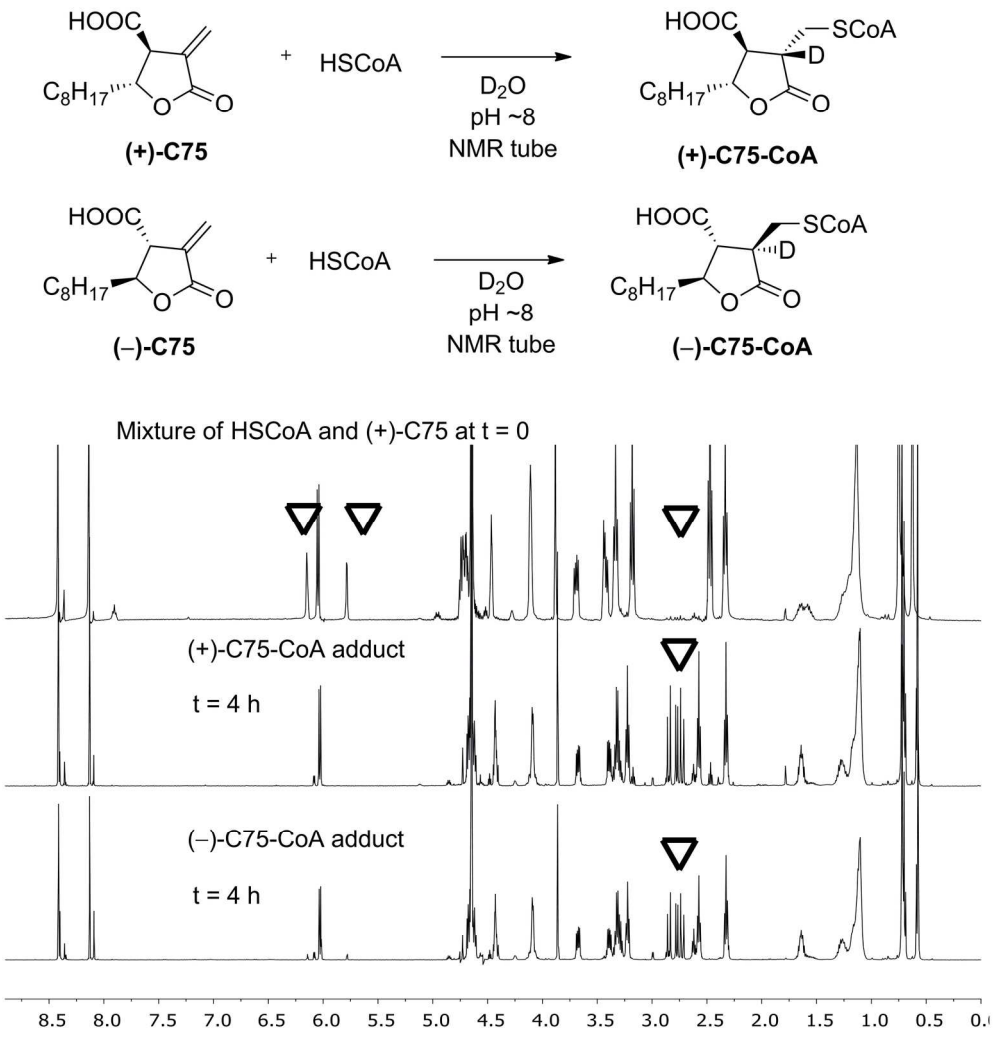

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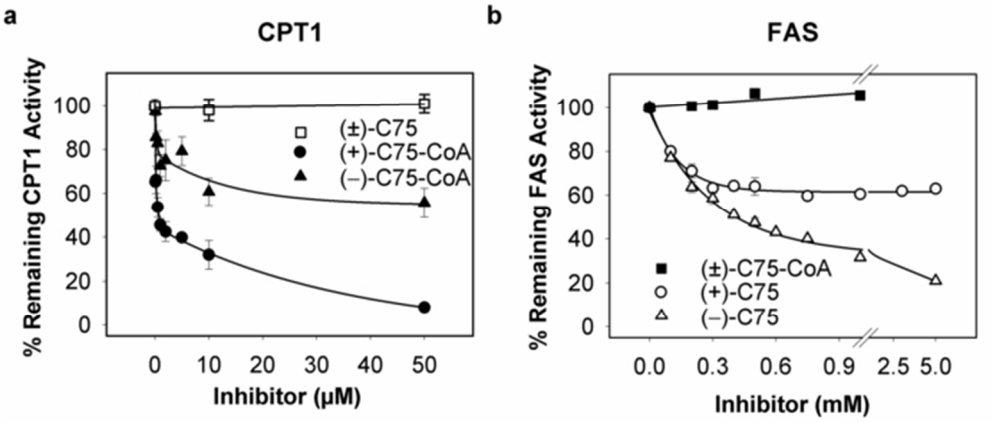

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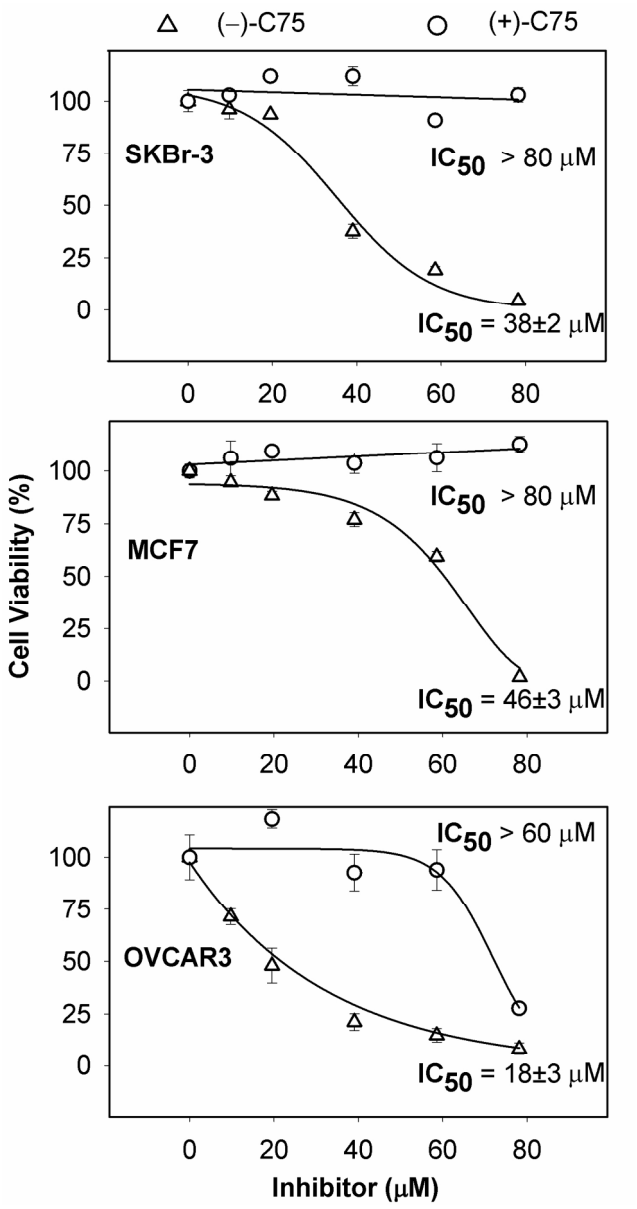

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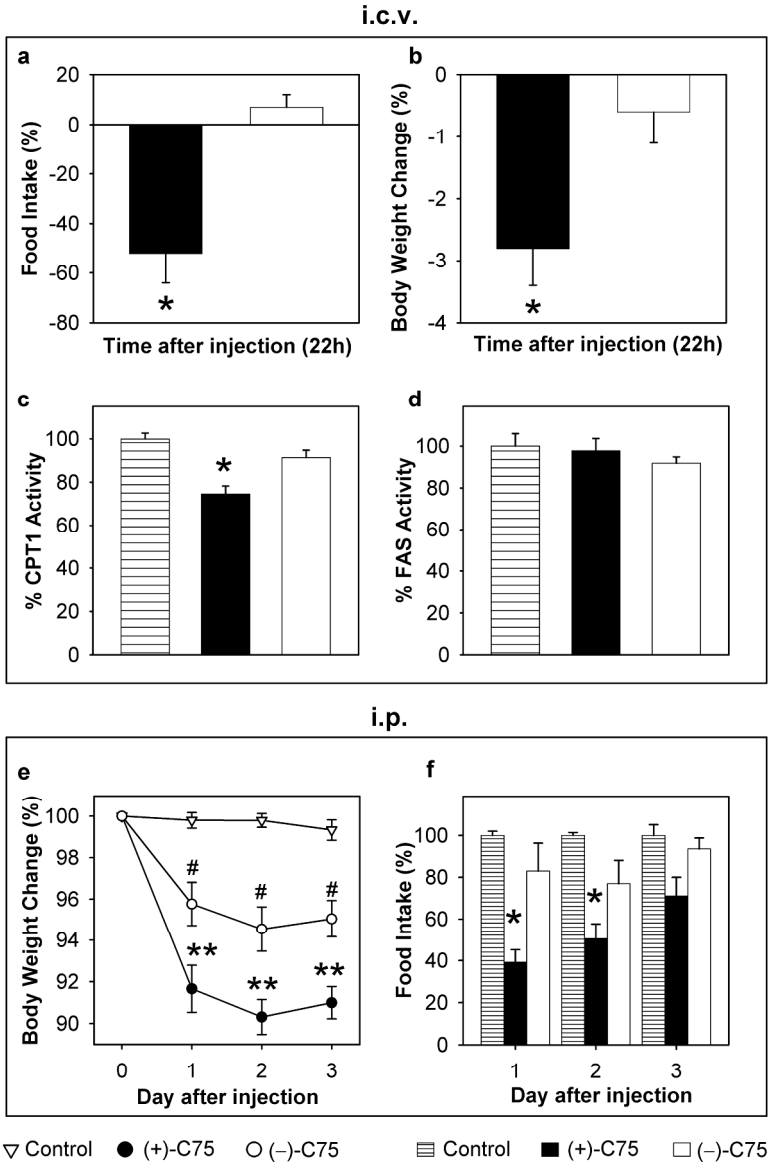

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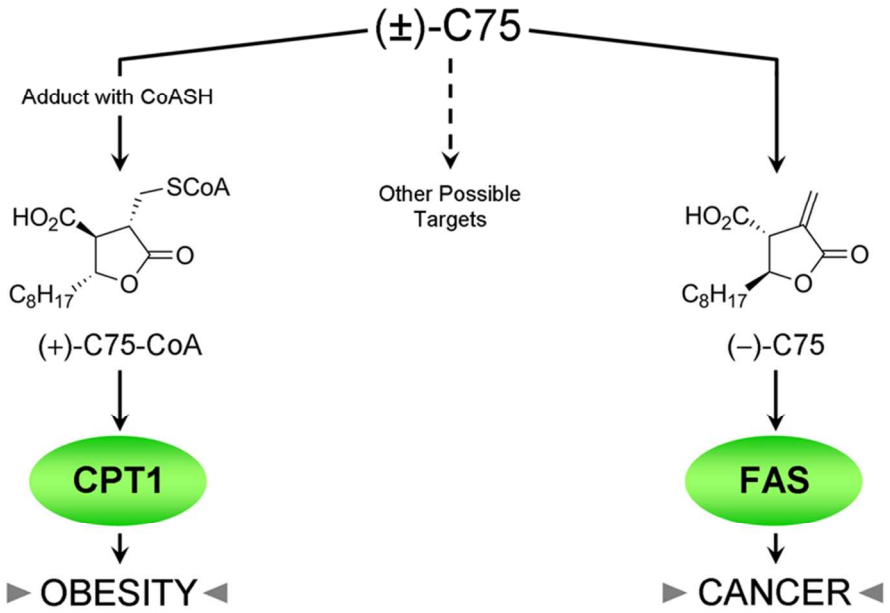

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