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Title

5,5-dimethyl-3-(5-methyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)-1-phenyl-3-(trifluoromethyl)-3,5,6,7-tetrahydro-1H-indole-2,4-dione, A Potent Inhibitor for Mammalian ELOVL6: Examination of Its Potential Utility As A Pharmacological Tool

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Running Title: A potent inhibitor for ELOVL6

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Non-standard abbreviations:

HTS, high throughput screening; ER, endoplasmic reticulum; Elovl, elongase of very long chain fatty acid; CoA, Coenzyme A; LCFA, long chain fatty acid; FAS, fatty acid synthase; SREBP, sterol regulatory element-binding protein; Chrebp, carbohydrate response element-binding protein; ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase; SCD, stearoyl-CoA desaturase; NADPH, nicotinamide adenine dinucleotide phosphate; ACBP, acyl-CoA binding protein; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; LC-MS/MS, liquid chromatography mass spectrometry/mass spectrometry; EI, elongation index; 2-NPH, 2-nitrophenylhydrazine; DI, desaturation index;

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Abstract

Long chain fatty acid elongases reside in the endoplasmic reticulum (ER) and are responsible for the rate-limiting step of the elongation of long chain fatty acids. The elongase of long chain fatty acids (Elovl) family 6 (Elovl6) is involved in the elongation of saturated and monosaturated fatty acids. Increased expression of Elovl6 in *ob/ob* mice suggests a role for Elovl6 in metabolic disorders. Furthermore, Elovl6-deficient mice are protected from high-fat diet-induced insulin resistance, which suggests that Elovl6 might be a new therapeutic target for diabetes. As reported previously, we developed a high-throughput screening system for fatty acid elongases and discovered lead chemicals that possess inhibitory activities against ELOVL6. In the present study, we examined in detail the biochemical and pharmacological properties of Compound-A, a potent inhibitor of ELOVL6. In *in vitro* assays, Compound-A dose-dependently inhibited mouse and human ELOVL6 and displayed more than 30-fold greater selectivity for ELOVL6 over the other ELOVL family members. In addition, Compound-A effectively reduced the elongation index of fatty acids of hepatocytes, suggesting that Compound-A penetrates the cell wall and inhibits ELOVL6. More importantly, upon oral administration to mice, Compound-A showed high plasma and liver exposure and potently reduced the elongation index of the fatty acids of the liver. This is the first study to report a potent and selective inhibitor of mammalian elongases. Furthermore, Compound-A appears to be a useful tool to further understand the physiological roles of

ELOVL6 and to evaluate the therapeutic potential of an ELOVL6 inhibitor.

Introduction

The incidence of type 2 diabetes has dramatically increased over the past decade. Accumulated evidence suggests a strong correlation between insulin resistance and the development of type 2 diabetes mellitus. An increase in fat storage in non-adipose tissues, such as liver, leads to dysfunction of those tissues i.e. insulin resistance (Unger, 2003). Although the mechanism by which increased intracellular lipid content exacerbates tissue and whole body insulin sensitivity is unclear, it has been suggested that increased levels of long chain fatty acyl-CoA antagonizes the metabolic actions of insulin (Silveira, et al., 2008; Taylor, et al., 2005; van Herpen and Schrauwen-Hinderling, 2008).

Microsomal enzymes have been shown to be responsible for the elongation of long chain fatty acids (LCFAs) with chain length >C16 while fatty acid synthase is responsible for the synthesis of fatty acids with chain length <C16 (Barrett and Harwood, 1998; Nugteren, 1965). Fatty acid elongation in microsome occurs through four sequential steps: (1) condensation by ELOVL (elongase of long chain fatty acyl-CoA), (2) reduction by β-ketoacyl-CoA reductase, (3) dehydrogenation by β-hydroxyacyl-CoA dehydrogenase, and (4) reduction by *trans*-2,3-enoyl-CoA reductase (Barrett and Harwood, 1998; Nugteren, 1965). Given the NADPH-dependent activity of β-ketoacyl-CoA reductase, the condensation activities of ELOVLs can be monitored in the absence of NADPH (Moon, et al., 2001; Moon and Horton, 2003).

At present seven ELOVL enzymes have been identified in mammals, ELOVL1-7 (Matsuzaka, et al., 2002; Moon, et al., 2001; Tvrdik, et al., 2000; Tvrdik, et al., 1997; Zhang, et al., 2001; Leonard, et al., 2000). Human and mouse ELOVL enzymes display high homology in amino acids (**Supplemental Figure 1**), suggesting that physiological functions of ELOVL enzymes are well conserved across species. Each Elovl has a distinct tissue distribution and exhibits different fatty acid substrate preferences; the ELOVL enzymes can be divided into two groups: (a) enzymes which are elongases of saturated and monosaturated LCFAs (ELOVL1, 3, and 6) and (b) enzymes which are elongases of polyunsaturated LCFAs (ELOVL2, 4, and 5). ELOVL7 is the most recently indentified member of ELOVL family based on the primary structure information (Strausberg, et al., 2002). However, its elongation activity and substrate specificity have remained unknown. The ELOVL enzymes are expressed in distinct tissues and their expression is differently regulated, which suggests that these ELOVL enzymes have distinct physiological roles (Brolinson, et al., 2008; Matsuzaka, et al., 2002; Wang, et al., 2006b).

ELOVL6 (as known as LCE and FACE) was originally identified as a target of SREBP-1 by microarray analysis of SREBP-1 transgenic mice (Matsuzaka, et al., 2002; Moon, et al., 2001). Subsequently, investigators have revealed that ELOVL6 elongates palmitoyl-CoA (C16:0) and palmitoleoyl-CoA (C16:1) to stearoyl-CoA (C18:0) and cis-vaccicate-CoA (C18:1), respectively, using malonyl-CoA as a 2 carbon donor while having no capacity to

elongate beyond C18 (Moon, et al., 2001). Elovl6 is abundantly expressed in liver and white adipose tissue, the major tissues for lipid synthesis and storage (Matsuzaka, et al., 2002; Moon, et al., 2001). The Elovl6 expression in these tissues are up-regulated in obese rodents and by re-feeding after fasting, with a high carbohydrate diet (Matsuzaka, et al., 2002; Miyazaki, et al., 2004; Moon, et al., 2001). In addition, the expression levels of Elovl6 are also up-regulated in sterol regulatory element-binding protein (SREBP)-1 overexpressing transgenic mice, suggesting that the expression of Elovl6 is regulated by SREBP-1c, a lipogenic gene transcription factor (Moon, et al., 2001). In support of this suggestion, Elovl6 is directly regulated by SREBP-1c (Kumadaki, et al., 2008). In contrast, the expression levels of Elovl6 are down-regulated in transgenic mice lacking the carbohydrate response element-binding protein (Chrebp), a transcriptional factor that drives the expression of a series of lipogenic genes by responding to the carbohydrate diet (Iizuka, et al., 2004). Taken together, these observations suggest that ELOVL6 plays a key role in the regulation of de novo lipid synthesis. Moreover, recently, Matsuzaka and colleagues reported that Elovl6-deficient mice are protected from high-fat induced hyperinsulinemia, hyperglycemia and hyperleptinemia, the fundamental signs of obesity and diabetes, despite the development of obesity and hepatosteatosis (Matsuzaka, et al., 2007). These findings suggest that a ELOVL6 inhibitor might be a potential new therapeutic for diabetes.

The UHTS of our company chemical library resulted in the discovery of chemical

leads that inhibit ELOVL6 activity *in vitro* (Nagase et al., submitted for publication). In the present study, we report the biochemical and pharmacological properties of 5,5-dimethyl-3-(5-methyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)-1-phenyl-3-(trifluoromethyl)-3,5,6,7-tetrahydro-1H-indole-2,4-dione, an orally active ELOVL6 inhibitor. As far as we know, this is the first study to report a potent and selective inhibitor for mammalian fatty acid elongases.

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Methods

Reagents

Glucose-6-phosphate, β-nicotinamide-adenine dinucleotide phosphate and glucose-6-phosphate dehydrogenase were purchased from Oriental Yeast Co., Ltd (Tokyo, Japan). [2-^{14}C]-malonyl-CoA was purchased from GE Healthcare science (Little Chalfont, UK). [^{14}C] palmitoyl-CoA was purchased from PerkinElmer Japan (Kanagawa, Japan). [^{14}C] stearoyl-CoA was purchased from Muromachi-Yakuhin (Tokyo, Japan). The oligonucleotide primers were purchased from Hokkaido System Science (Hokkaido, Japan). Human and mouse liver microsomes were obtained from BD Biosciences (San Jose, CA) and XENOTECH (Lenexa, KS), respectively. Other reagents were obtained from Sigma (St. Louis, MO). Male C57BL/6J mice (5-7 weeks of age), male SD rats and male Wister rats (5-7 weeks of age) were purchased from CLEA Japan (Tokyo, Japan) and Charles River Japan, respectively (Kanagawa, Japan). Compound-A (5,5-dimethyl-3-(5-methyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)-1-phenyl-3-(trifluoromethyl)-3,5,6,7-tetrahydro-1H-indole-2,4-dione) was synthesized by the Chemistry Department of BANYU Pharmaceutical Co., Ltd (Ibaraki, Japan) (Takahashi et al., submitted for publication).

Cloning and Expression of Elovl Family Enzymes

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The hElovl6 coding sequence (accession number: NM_024090) was amplified by PCR using the following primers: 5' primer, 5'-GGATCCAACATGTCAGTGTTGACTT-3' and 3' primer, 5'-CTCGAGCTATTCAGCTTTCGTTGTT-3', which introduced BamHI and XhoI restriction sites at the 5' and 3' ends, respectively, of the hElovl6 coding sequence while deleting the initial methionine residue of hElovl6. The amplified fragment was then digested at the restriction sites and ligated with the double-digested pCMV-Tag2B vector (Stratagene, La Jolla, CA), yielding the expression vector for the N-terminally FLAG-tagged fusion protein (FLAG-rhElovl6). This FLAG-rhElovl6 construct was subsequently used as a template for construction of a yeast expression vector. The FLAG-rhElovl6 coding sequence was amplified using the following primers: 5' primer, 5'-CTGCAGATTACAAGGATGACGACGAT-3' and 3' primer, 5'-CTCGAGCTATTCAGCTTTCGTTGTT-3', which introduced the PstI restriction site at the 5' end of the FLAG-rhElovl6 coding sequence while deleting the initial methionine of FLAG. The amplified fragment was then digested at the restriction sites and ligated with the double-digested pPICZαB vector (Invitrogen, Carlsbad, CA). In terms of the other Elovl family members, each coding sequence was amplified by PCR using the following primers: human Elovl1 (accession number NM_022821) 5' primer, 5'-AAACCATGGATGGAGGCTGTTGTGAACTTG-3' and 3' primer, 5'-AAATCTAGATCAGTTGGCCTTGACCTTGG-3'; human Elovl2 (accession number

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NM_017770) 5' primer, 5'-AAACCATGGATGGAACATCTAAAGGCC-3' and 3' primer, 5'-AAATCTAGATTATTGTGCTTTCTTGTTC-3'; human Elovl3 (accession number NM_152310) 5' primer, 5'-AAACCATGGATGGTCACAGCCATGAATG-3' and 3' primer, 5'-AAATCTAGAGACATGAGGCCCTTTTTCGA-3'; human Elovl5 (accession number NM_021814) 5' primer, 5'-AAACCCGGGGATGGAACATTTTGATGCATC-3' and 3' primer, 5'-AAATCTAGATTCATCCTGCGCAAGAACAA-3'; mouse Elovl6 (accession number NM_130450) 5' primer, 5'-AAACCATGGATGAACATGTCAGTGTTGACT-3' and 3' primer, 5'-AAATCTAGAACTACTCAGCCTTCGTGGCTTTC-3', which introduced NcoI (hElovl1, 2, 3 and mElovol6) or SmaI (hElovl5) at the 5' end and XbaI restriction sites at the 3' ends. These PCR products were digested by each introduced restriction site. In addition, the sequence of 3xHA tag was synthesized as follows: AACTGCAGCAGCGGCCGCGATGTACCCATACGATGTTCCAGATTACGCTTACCCAT ACGATGTTCCAGATTACGCTTACCCATACGATGTTCCAGATTACGCTCCATGGCCCG GGAAA, and digested by NotI for the 5' ends and NcoI (for hElovl1, 2, 3 and mElovl6) or SmaI (for hElovl5) for the 3' ends. The fragments of the Elovls and the 3xHA fragment were ligated to double digested pPICZαB. The integrity of all PCR products and ligations was confirmed by DNA sequencing.

Each expression vector was linearized and transformed into the *Pichia pastoris* SMD1168 yeast strain using the Pichia EasyComp^{TM} Transformation Kit (Invitrogen). The

transformants were selected on YPDS plates (1% yeast extract, 2% peptone, 2% dextrose and 1 M sorbitol) containing 100 μg/mL of Zeocin (Invitrogen). The cells were grown in BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6.0, 1.34% yeast nitrogen base, 4×10^{-5}% biotin and 1% glycerol). Expression of fusion protein was induced in BMMY medium (the 1% glycerol in BMGY was replaced with 0.5% methanol) and the cells were cultured for 48 h at 30°C in a rotary shaker (180 rpm). Preparation of the microsome fraction was performed at 4°C. In brief, the yeast cells were harvested by centrifugation at 3 000 g for 10 min and washed with cold breaking buffer (50 mM potassium phosphate (pH 7.4), 1 mM EDTA, 5% glycerol and 1 tablet/50 mL of protease inhibitor cocktail (Roche, Mannheim, Germany)). The cells were then vigorously broken with glass beads in cold breaking buffer. The resultant homogenate was centrifuged at 10,000 g for 10 min, and the supernatant was further centrifuged at 100,000 g for 1 h at 4°C. The pellet was suspended in re-suspension buffer (50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 20% glycerol and 1 tablet/50 mL of protease inhibitor cocktail), and again centrifuged at 100,000 g for 1 h at 4°C. The pellet was suspended in the re-suspension buffer and used as the microsomal fraction for the elongase assay.

In Vitro Enzyme Elongation Assay

The long chain fatty acyl-CoA elongation assay was performed as described elsewhere

(Kitazawa, Miyamoto et al., submitted for publication). In brief, for elongation reactions, 30 µL of the reaction mixture (100 mM potassium phosphate buffer (pH 6.5), 200 µM BSA (fatty acid free), 500 µM NADPH, 1 µM rotenone, 20 µM malonyl-CoA, 833 kBq/mL [^{14}C] malonyl-CoA and acyl-CoA) was used as the substrate mixture. The following long-chain acyl-CoAs were used as the preferential substrate for each ELOVL; ELOVL1, 10 µM stearoyl-CoA; ELOVL2, 10 µM arachidonoyl-CoA; ELOVL3, 10 µM stearoyl-CoA; ELOVL5, 40 µM arachidonoyl-CoA; ELOVL6, 40 µM palmitoyl-CoA. To start the reaction, 20 µL of the ELOVL microsomal fraction was added to the substrate mixture, and then incubated for 1 h at 37°C with gentle shaking. This reaction step was performed in a 96-well plate. After the 1-h incubation, 100 µL of 5 M HCl was added for the hydrolysis of acyl-CoAs, and then the reaction mixture was filtered through a Unifilter-96, GF/C plate (PerkinElmer, Waltham, MA) using a FilterMate cell harvester (PerkinElmer). The 96-well GF/C filter plate was subsequently washed with distilled water to remove excess [^{14}C] malonyl-CoA and dried, after which 25 µL of MICROSCINT 0 was added to each well and radioactivity determined.

In Vitro Assays for ACC, FAS, and SCD

Courter assays for other lipid enzymes i.e. acetyl-carboxylase (ACC), fatty acid synthetase (FAS), and stearoyl-CoA desaturase (SCD) were conducted according to the assay methods

reported in the previous studies with slight modifications (**Supplemental Methods 1**).

Analysis of the Mode-of Action of the ELOVL6 Inhibitor

The kinetic parameters of inhibition, i.e. the inhibition constant (*Ki*) of the ELOVL6 inhibitor, and the Michaelis-Menten constant (*Km*) of the ELOVL6 substrates, were determined by Lineweaver-Burk plot analysis using various concentrations of the inhibitor (0-3 μM) with ELOVL6 microsomes. The assay conditions for measurement of ELOVL6 activity were identical to the method for the "ELOVL6 *In Vitro* Enzyme Assay" above except for the tested substrate concentration (2 - 20 μM of malonyl-CoA, 0 - 20 μM of palmitoyl-CoA). To determine the *Km* and *Ki* values for palmitoyl-CoA and malonyl-CoA, the concentration of the other substrate involved, i.e., malonyl-CoA and palmitoyl-CoA, respectively, was fixed at 20 μM. Lineweaver-Burk analysis was performed by plotting the reciprocal of the rate of the activity (v) against the reciprocal of the substrate concentration. The type of inhibition was determined based on the graphical views of the Lineweaver-Burk plots. The K_m and K_i values were calculated as described previously (Lineweaver and Burk, 1934) using GraphPad PRISM software version 4.00 (GraphPad Software Inc., San Diego, CA).

***In Vitro* Hepatocyte Assay**

In order to assess the effects of Compound-A in cells, we used mouse hepatocyte cell line

H2.35 and rat primary hepatocyte. H2.35 cells were originally developed to induce liver-specific gene transcription in a temperature-sensitive manner and were reported to express significant amounts of SREBP-1c (Hasty, et al., 2000) H2.35 were grown on 24-well plates in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 200 nM dexamethasone and 4% heat-inactivated fetal bovine serum (FBS) at 33°C under 5% CO_2 in a humidified incubator. Rat primary hepatocytes were prepared as described previously (Seglen, 1976) from a high-fructose-diet fed (1 day) rat and incubated in DMEM with 100 nM dexamethasone and 10% FBS at 37°C for 3 hours before use. The test compound dissolved in medium was incubated with subconfluent H2.35 cells or rat primary hepatocytes for 60 minutes at 33°C or 37°C, respectively. [1-^{14}C]-palmitic acid (16:0) was added to each well to a final concentration of 0.8 μCi/mL to detect elongase activity. After 4 hours incubation at 33°C for H2.35 cells or 2 hours at 37°C for rat primary hepatocytes, the culture medium was removed and the labeled cells were washed with chilled PBS (3 x 0.5 mL) and dissolved in 250 μL of 2N sodium hydroxide. The cell lysate was incubated at 70°C for 1 hour to hydrolyze radio labeled cellular lipids. After acidification with 100 μL of 5N hydrochloric acid, fatty acids were extracted with 300 μL of acetonitrile. Radio labeled palmitic acid (16:0), palmitoleic acid (16:1), stearic acid (18:0), and vaccenic acid and oleic acid (18:1) were quantified by reversed-phase radio- HPLC. Radio-HPLC analysis was performed with D-7000 interface (Hitachi, Tokyo, Japan), equipped with a radio-detector

(FSA515TR, Amersham Biosciences, Piscataway, NJ), a diode array detector (L-7455, Hitachi), pumps (L-7100, Hitachi) and an auto-sampler (L-7200, Hitachi). The mobile phase consisted of CH_3CN-water with 50 mM ammonium acetate (60:40 for 5 min, 80:20 for 2 min, 99:1 for 8 min, flow rate: 1.0 mL/min). The separation was performed with a CAPCELL PAK C18 MG (3.0 mm i.d. × 150 mm). The identity of the labeled fatty acids was determined by comparing the retention times with known fatty acid standards. Elongation activity was monitored as the elongation index (EI), which was the ratio of radio labeled C18 (C18:0 + C18:1) to C16 (C16:0 + C16:1) estimated from each peak area measured by RI-HPLC.

***In Vivo* [^{14}C] Palmitate Elongation Assay in Liver**

Male C57BL/6J mice (CLEA Japan) and SD rats (Charles River Japan) were individually housed in plastic cages with *ad libitum* access to normal rodent chow (CE2, CLEA Japan) （**Supplemental Methods 2**） and water. Mice were orally administered Compound-A (dissolved in 0.5% methylcellulose) and 1 hour later [1-^{14}C]-palmitic acid was interperitoneally administered at 10 μCi/body. At 2 hours post-dosing of Compound-A, animals were anesthetized with isoflurane (4%) and killed by blood collection from the vena cava. 50 mg of the liver was harvested, incubated in potassium hydroxide/ethanol (2 mL/1.4 mL) at 70°C for 1 hour. The non-acid-lipid was extracted by 4 mL of petroleum ether, and discarded. Fatty acids were extracted by 2 mL of petroleum ether following saponification by

2 mL of 6N hydrochloride. The ether phase containing fatty acids fraction was evaporated under nitrogen gas and reconstituted in methanol to measure the radioactivity by radio-HPLC as described above. The radioactivity corresponding to each fatty acid was quantified to calculate the EI as described above. All animal procedures were conducted according to protocols and guidelines approved by the Banyu Institutional Animal Care and Use Committee.

Fatty Acid Composition Assay in Liver

Mice were orally administered Compound-A (dissolved in 0.5% methylcellulose) twice daily (09:30, 18:30) for 10 days at 30 mg/kg or 2 days at 100 mg/kg dose. At 4 hours post final dosing of Compound-A, mice were anesthetized and tissues were immediately isolated, weighed, frozen in liquid nitrogen, and stored at -80 °C until use. The liver samples were incubated in 100-fold volume (w/v) of 5 M NaOH / ethanol (1:1) at 60°C. After a 2-h incubation, 500 µL of 5 M HCl and C17:0 (internal standard) were added to all hydrolysates. The fatty acids compositions were analyzed by a previously described method (Miwa, et al., 1985) with slight modifications. Briefly, the fatty acids in the tissue hydrolysate were derivatized with 2-nitrophenylhydrazine (2-NPH), and these derivatives were purified using an Oasis HLB column. An aliquot (10 µL) of the eluate was injected into the HPLC apparatus for analysis. HPLC analysis was performed with a Shimadzu 10Avp system (Shimadzu,

Kyoto, Japan), equipped with a UV detector (SPD-10Avp), two pumps (LC-10ADvp), an auto-sampler (SIL-10ADvp) and a column oven (CTO-10ACvp). The mobile phase consisted of CH_3CN-water (80:20, flow rate: 0.6 mL/min). The separation was performed with a CAPCELL PAK C18 MGII (2.0 mm i.d. x 150 mm, 5 μm) at 35°C and the UV absorbance was subsequently measured at 400 nm. The elongation index represented the ratio of C18 (C18:0 + C18:1) to C16 (C16:0 + C16:1) which was quantified from each fatty acid amount.

Pharmacokinetics

(1) Plasma and liver concentrations of Compound-A: Pharmacokinetic characterizations were conducted in male C57BL/6J mice following single oral administration of Compound-A. Single doses of Compound-A at 10 mg/kg body weight were administered orally by gavage in a vehicle of 0.5% methylcellulose aqueous suspension. Blood samples from the abdominal vein and liver samples were obtained 2 hours after administration. Blood samples were centrifuged to separate the plasma. Liver samples were homogenized with phosphate-buffered saline (pH 7.4). Each sample was deproteinized with ethanol containing an internal standard. Compound-A and the internal standard were detected by liquid chromatography mass spectrometry/mass spectrometry (LC-MS/MS). LC/MS analyses were performed on Quattro Ultima mass spectrometer (Waters, Milford, MA), operating under positive ion mode using an electrospray ionization probe, and connected to an Alliance 2790

Separations Module (Waters). Chromatographic separations were performed on a Waters Symmetry RP18 column (2.1 x 150-mm), and eluted with 65% acetonitrile containing 10 mM ammonium acetate at a flow rate of 0.2 mL/min for 6 min. Detection of Compound-A was carried out by Multiple Reaction Monitoring mode, whereby the precursor ion of m/z 496 ($[M+H]^+$) and the product ion of m/z 175 were selected.

(2) Metabolic stability in liver microsomes: 1 μM Compound-A was incubated at 37ºC in 0.25 mg/mL of human and mouse liver microsomes supplemented with 10 mM glucose-6-phospate, 1 mM β-nicotinamide-adenine dinucleotide phosphate, 1 U/mL glucose-6-phosphate dehydrogenase, 100 mM phosphate buffer and 3 mM magnesium chloride. Concentrations of Compound-A were determined by LC-MS/MS. LC/MS analyses were performed in the same way as described above except the chromatographic condition. Chromatographic separations were performed on a Shiseido CAPCELL PAK C8 (4 x 20-mm) at a flow rate of 1 mL/min under gradient conditions. The mobile phases consisted of 10% acetonitrile containing 10 mM ammonium acetate (solvent A) and 90% acetonitrile containing 10 mM ammonium acetate (solvent B). Solvent B was linearly increased from 0 to 100% over 1.2 min, maintained at 100% for 0.5 min, then decreased to 0% over 0.1 min. Metabolic stability was calculated from the ratio of Compound-A concentration at 0 min to that at 30 min after the initiation of incubation.

Results

Discovery of Indoledione Derivatives as ELOVL6 Inhibitors

Previous high-throughput screening led to the discovery of lead compounds that possess inhibitory activities against human ELOVL6 (Shimamura, et al., 2009, Nagase et al., submitted for publication). Among them, the indoledione derivatives were of interest given their intrinsic potency and chemical tractability for further derivatization. Compound-A, 5,5-dimethyl-3-(5-methyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)-1-phenyl-3-(trifluor omethyl)-3,5,6,7-tetrahydro-1H-indole-2,4-dione, was identified as one of the representative compounds of the indoledione derivatives **(Figure 1A)**. Compound-A dose-dependently inhibited human and mouse ELOVL6 activities with IC_{50} values of 0.169 μM and 0.350 μM, respectively **(Figure 1B, Table 1)**. In order to assess the specificity of Compound-A over the other ELOVL family enzymes, we expressed several recombinant human ELOVL family enzymes (ELOVL1, 2, 3, and 5) and examined the effects of Compound-A on these enzymes in the presence of the respective preferred substrate for each enzyme. Compound-A displayed greater than 30-fold selectivity for ELOVL6 over the other representative ELOVL family enzymes **(Table 1)**. In addition, Compound-A showed negligible or very weak inhibitory effects on rat microsomal stearoyl-CoA desaturase (SCD), human ACC1 and 2, and human FAS at 100 μM (6.5%, 0%, 14%, and 16% inhibition, respectively, suggesting that Compound-A is a potent and selective ELOVL6 inhibitor.

Mode-of-action of Compound-A

ELOVL6 catalyzes the first condensation step of LCFA elongation e.g. palmitoyl-CoA (C16:0) and malonyl-CoA, a 2 carbon donor, leading to the production of the corresponding β-ketoacyl-CoA (Moon, et al., 2001). In order to assess the inhibitory mode-of-action of Compound-A on ELOVL6, we examined the effects of increasing concentrations of Compound-A on ELOVL6 in the presence of various concentrations of palmitoyl-CoA or malonyl-CoA, as described in Methods. Human ELOVL6 exhibited standard Michaelis-Menten kinetics with a K_m of 11.1 μM for malonyl-CoA and 4.0 nM for palmitoyl-CoA. The Lineweaver-Burk plot analysis suggested that Compound-A inhibits ELOVL6 in a non-competitive manner for malonyl-CoA (K_i=994 nM) and in an un-competitive manner for palmitoyl-CoA **(Figures 2A, 2B)**.

***In Vitro* Cellular Assays**

Because ELOVL6 resides in the endoplasmic reticulum (ER), a specific intracellular compartment, compounds need to penetrate the intracellular space to interact with the target. The logD value of Compound-A is 2.7, suggesting that Compound-A is sufficiently lipophilic and has the potential to penetrate the intracellular space in a passive diffusion manner. In order to determine whether Compound-A penetrates the cells and inhibits cellular ELOVL6,

we examined the effects of Compound-A on the elongation index of the cellular lipids, as described in Methods. Given that ELOVL6 preferably elongates C16:0 and C16:1 as substrates, leading to the production of C18:0 and C18:1, the elongation index was based on the ratio of peak area[C18:0+C18:1]/peak area[C16:0+C16:1]. Compound-A effectively reduced the elongation index of the mouse hepatocyte cell line H2.35 and of the primary culture of mouse hepatocytes, with IC_{50} values of 0.427 μM and 0.200 μM, respectively **(Figure 3)**. These data suggest that Compound-A efficiently penetrates cells and inhibits the elongation activity of ELOVL6. There were no significant effects on the desaturation index (DI) i.e. peark area[C18:1+C16:1]/peak area[C18:0+C16:0] even at 30 μM : DI = 0.52±0.07 (Compound-A) vs. 0.58±0.03 (vehicle control) in H2.35 cells, DI = 0.12±0.02 (Compound-A) vs. 0.15±0.01 (vehicle control) in rat primary hepatocytes.

In Vivo Activity of Compound-A

Given the promising activities of Compound-A in the *in vitro* assays described above, we next examined the *in vivo* profiles of Compound-A. As shown in **Table 2**, Compound-A displayed appreciable plasma and liver exposure (5.22 μM, 13.7 nmol/g tissue) at 2 hours after dosing when orally administered at 10 mg/kg to C57BL/6J mice, which is consistent with the relatively stable metabolic profile in the *in vitro* human and mouse liver microsome assays. Subsequently, we examined the effects of Compound-A on the elongation index of the

liver lipids using the [1-^{14}C]-palmitic acid as a radiotracer. Orally administered Compound-A significantly and potently reduced the elongation index of the liver lipids at 10 and 30 mg/kg ($p < 0.01$, **Figures 4A, 4B**).

As next, we examined the effects of Compound-A on the elongation index of the unlabelled total fatty acids of the liver. As results, the subchronic-treatment with Compound-A at 30 mg/kg for 10 days tended to reduce the elongation index of the total fatty acids of the liver but did not reach the statistically significant difference vs. vehicle treated control (**Figure 5A**). Meanwhile, the treatment with a higher dose (i.e. 100 mg/kg) of Compound-A for 2 days significantly reduced the elongation index of the total fatty acids of the liver (**Figure 5B**).

Discussion

Accumulating evidences have revealed that increased tissue fatty acids levels are closely linked with metabolic disturbance such as insulin resistance and hence inhibitors for lipid enzymes have gained much attention as potential therapeutic agents for the treatment of diabetes and obesity (Silveira, et al., 2008; Taylor, et al., 2005; van Herpen and Schrauwen-Hinderling, 2008).With regard to inhibitors for de novo fatty acid synthesis, FAS inhibitors such as C75 have been reported and are shown to suppress lipid synthesis and feeding behavior *in vivo* (Loftus, et al., 2000). Meanwhile, Cafenstrole, Indanofan, and chloroacetamides have been reported as inhibitors of the long chain fatty acid elongases, with micromolar potency for plant very long chain fatty acid elongase (Takahashi, et al., 2002; Takahashi, et al., 2001; Gotz and Boger, 2004). However, no inhibitor for mammalian long chain fatty acid elongases has been identified. Despite the increasing interest in ELOVL6 as a therapeutic target for the treatment of metabolic disorder (Matsuzaka, et al., 2007), lack of a pharmacological tool has limited further assessment of ELOVL6 as the therapeutic target. Thus the identification of a specific and potent inhibitor is essential for further understanding of the physiological roles and therapeutic potential of ELOVL6.

Discovery and Characterization of the ELOVL6 Inhibitor

In order to identify inhibitors of mammalian fatty acid elongases, we established a high

throughput assay system for elongases using a homologous assay platform (Shimamura, et al., 2009). Intensive screening of our company chemical library identified chemical leads that possess significant inhibitory actions on ELOVL6 in the micromolar range, and derivation of these compounds resulted in the identification of Compound-A (Nagase et al., submitted for publication). In the present study, Compound-A potently inhibited the elongation activity of human and mouse ELOVL6 for palmitate in a dose dependent manner with almost equal potency. Importantly, Compound-A has more than 30-fold greater selectivity for ELOVL6 over the other ELOVL family enzymes i.e. ELOVL1, 2, 3, and 5 (**Table 1**). Although the selectivity for other ELOVL members (i.e. ELOVL4 and ELOVL7) can not be excluded at this time and needs to be addressed, the current data suggest that Compound-A is a potent and selective ELOVL6 inhibitor. Furthermore, 100 μM of Compound-A has no detectable effects on rat microsomal SCD. In keeping with these findings, Compound-A did not alter the desaturation index in either in the *in vitro* or *in vivo* studies. The selectivity over SCD is critical because ELOVL6 and SCD work closely together to modify long chain fatty acids e.g. elongation and desaturation of palmitate (Matsuzaka, et al., 2002; Moon, et al., 2001; Enoch and Strittmatter, 1978). Several small molecule SCD inhibitors have been reported to date (Liu, et al., 2007). The selectivity of Compound-A over SCD and other lipid synthesis enzymes such as FAS and ACC will enable assessment of the specific roles of ELOVL6.

From the point of view of drug discovery, the mode-of-action of a compound is

critical, especially for enzymes that constitute “a family” and share substrates. As shown in **Figure 3**, Compound-A inhibited ELOVL6 in an un-competitive manner for palmitoyl-CoA and in a non-competitive manner for malonyl-CoA. Although further studies are required to fully elucidate the mode-of-action of Compound-A, we speculate that one of two possibilities are at work: (1) Compound-A binds to the allosteric site of the palmitoyl-CoA and malonyl-CoA binding sites (i.e. the catalytic domain of the enzyme), resulting in enzymatic activity, possibly by causing a conformational change in the enzyme; (2) Compound-A recognizes the specific conformational change that occurs during the formation of acyl-enzyme intermediates. Regarding the latter, Wang et al., demonstrated that platensimycin, a potent inhibitor for β-ketoacyl-(acyl-carrier-protein) synthase I/II (FabF/B), specifically interacts with the acyl-enzyme intermediate of the target protein (Wang, et al., 2006a). As in their study, further analysis using radioactive compounds will help us further understand the mode-of-action of Compound-A. Regarding the selectivity of Compound-A, we speculate that the allosteric binding allows Compound-A to be a specific ELOVL6 inhibitor with good selectivity over the other ELOVL family enzymes that commonly use malonyl-CoA and long chain fatty acid as substrates. The selectivity over ELOVL3 is critical because ELOVL3 possesses the highest homology to ELOVL6 (**Supplemental Figure 1**) and is also responsible for the elongation of palmitoyl-CoA (Westerberg, et al., 2005).

In order to further evaluate the potential of Compound-A as a pharmacological tool,

we examined the effects of Compound-A on the elongation activities of hepatoma and liver primary cells (**Figure 3**). Compound-A has sufficient lipophilicity (logD7.4 = 2.7) and is thought to penetrate cells by passive infusion. Compound-A effectively reduced the elongation index of the fatty acids of the hepatocyte cell line, indicating that Compound-A penetrated cells well and suppressed elongation activity. Because immortalized hepatocyte cell lines often lose the intrinsic properties of the native liver cells i.e. transporters, enzymes etc., we next examined the effects of Compound-A on primary liver cells that retain intrinsic molecules such as transporters and metabolism enzymes. In the rat primary cells, we observed by immunoblot analysis that the expression levels of ELOVL6 were maintained up to 48 hours post preparation (data not shown). Thus Compound-A was similarly active in primary hepatocytes as in the cell line, suggesting that Compound-A penetrated the primary cells well and is resistant to metabolism by metabolic enzymes such as cytochrome pigments. Consistent with this, Compound-A was metabolically stable in the *in vitro* microsome assay (**Table 2**).

In vivo Activity of Compound-A

To determine whether oral administration of Compound-A can be effective, we measured the plasma and liver exposure after oral administration of Compound-A in mice. High plasma and liver exposure of Compound-A were seen at 2 hours post administration, suggesting that

Compound-A is a metabolically stable and orally available compound.

Because Compound-A showed appreciable exposure *in vivo*, we further examined the effects of Compound-A on the fatty acid profile of liver. Given that ELOVL6 is mainly responsible for the elongation of palmitic acid (C16:0) to stearic acid (C18:0), the elongation index (peak area) was used as the surrogate indicator of ELOVL6 activity in the liver using [^{14}C]-C16:0 as a radiotracer. Consistent with the high liver exposure and its potent intrinsic activity, when orally administered, Compound-A potently suppressed the elongation index of the fatty acids of the liver (**Figure 4**). Furthermore, consistent with the selectivity over SCD, Compound-A did not significantly affect the desaturation index (i.e. (C18:1+C16:1)/(C18:0+C16:0)) in both *in vitro* and *in vivo* experiments. The selectivity over SCD is critical because genetic modification of SCD has been reported to have profound impacts on lipid metabolism and glucose homeostasis, hence concomitant activity against SCD would make it difficult to assess the specific role and therapeutic potential of ELOVL6 (Ntambi, et al., 2002; Flowers, et al., 2006).

In order to further assess the potential utility of Compound-A as a pharmacological tool, we next examined the effects of Compound-A on unlabeled fatty acids of the liver. Given that the preliminary data suggested a half-life of Compound-A could be less than 12 hours, we used a twice daily dosing regimen in these studies in order to obtain higher and more prolonged

plasma exposures than single dosing (data not shown). As shown in **Figure 5**, 10-day dosing of Compound-A at 30 mg/kg tended to decrease the elongation index of unlabeled fatty acids of the liver. Moreover, 2-day dosing of Compound-A at 100 mg/kg significantly reduced the elongation index of the unlabelled fatty acids, demonstrating the potential utility of Compound-A as a pharmacological tool. Considering the more remarkable changes in the fatty acids composition of the liver from Elovl6 deficient mice (Matsuzaka, et al., 2007), current data have suggested that the treatment with Compound-A for more prolonged period and/or at a higher dose might give further impact on the elongation index of the total fatty acids of the liver as reported for Elovl6 deficient mice.

Accumulated evidence suggests that increased levels of intracellular LCFAs are closely related to the increased tissue lipid contents and dysfunction of insulin signaling i.e. suppression of gluconeogenesis in liver, and to the decrease in glucose intake into adipose and skeletal muscles. Intriguingly, beyond the levels of total lipids (e.g. existence of steatosis in liver), alternation of specific lipid components (e.g. C16:1) has been suggested to have significant impact on tissue and whole body lipid and glucose homeostasis (Cao, et al., 2008; Matsuzaka, et al., 2002). Given the substrate specificity, unique tissue distribution, and expression regulation of ELOVL6, examination of the impact of pharmacological blockade of ELOVL6 on lipid and glucose homeostasis in disease model animals is of great interest.

To the best of our knowledge, this is the first report to describe a potent and selective inhibitor for mammalian fatty acid elongases. In this study, we characterized the *in vitro* and *in vivo* profile of the potent and specific ELOVL6, inhibitor Compound-A, which will be useful as a pharmacological tool.

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Footnotes

K.S and H.K. contributed equally to this work.

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Legends for Figures

Figure 1: Structure and inhibition effect of Compound-A on ELOVL6 activity

(A) Structure of Compound-A: 5,5-dimethyl-3-(5-methyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)-1-phenyl-3-(trifluoromethyl)-3,5,6,7-tetrahydro-1H-indole-2,4-dione **(B)** Dose-dependent inhibition of recombinant human ELOVL6 by Compound-A. ELOVL6 activity was measured by the method described in Methods with various concentration of Compound-A. Each value is expressed as the percentage of control in the absence of Compound-A. The results are taken from at least three independent tests, and express mean ± S.E.D..

Figure 2: Kinetics of ELOVL6 inhibition by Compound-A

Inhibitory effects of increasing concentrations of Compound-A (0 (●), 0.01 (○), 0.03 (♦), 0.1 (◊), 0.3 (■), 1 (□), and 3 μM (▼)) on human ELOVL6 were examined in the presence of varying concentrations of malonyl-CoA **(A)** or palmitoyl-CoA **(B)** as outlined in Methods. The inhibitory mode-of-action of Compound-A for human ELOVL6 was analyzed by Lineweaver-Burk plot analysis. The results are taken from at least three independent tests, and express mean ± S.E.D.

Figure 3: Inhibition of mouse and rat hepatocyte LCFA elongation activity by

Compound-A

Dose-response inhibitory effect on LCFA elongation by Compound-A in the mouse hepatocyte cell line, H2.35 **(A)** and rat primary hepatocytes **(B)**. The elongation index was determined by the RI-HPLC as described in Methods with varying concentration of Compound-A, and defined as the ratio of peak area[C18:0+C18:1]/peak area[C16:0+C16:1]. Each value is the mean ± S.D. of three replications.

Figure 4: Effects of Compound-A on LCFA elongation index for radiolabled fatty acids of the liver in C57BL/6J mice

(A) Scheme for the experimental procedure **(B)** The elongation index of LCFAs of the liver after treatment with vehicle (open column), 10 mg/kg Compound-A (gray column), and 30 mg/kg Compound-A (black column). Four mice were used for each treatment. The asterisks represent a significant change ($p < 0.01$) from vehicle-treated controls, as measured using ANOVA with post hoc Dunnetts test.

Figure 5: Effects of Compound-A on LCFA elongation index for unlabeled fatty acids of the liver in C57BL/6J mice

The effects of Compound-A on the unlabelled lipids of the liver were examined. Compound-A was dosed twice daily for 10 days at 30 mg/kg (**A**) or for 2 days at 10 mg/kg

(**B**). The elongation index of LCFAs of unlabeled fatty acids of the liver after treatment with vehicle (open column) and Compound-A (gray column) are shown. 6-10 mice were used for each treatment. The asterisks represent a significant change ($p < 0.05$) from vehicle-treated controls, as measured using ANOVA with post hoc Dunnetts test.

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Table 1: IC_{50} values of Compound-A for representative ELOVL family enzymes

Enzyme assays were run as described in Methods in the presence of respective substrate for each elongase. ELOVL1, 10 μM stearoyl-CoA; ELOVL2, 10 μM arachidonoyl-CoA; ELOVL3, 10 μM stearoyl-CoA; ELOVL5, 40 μM arachidonoyl-CoA; ELOVL6, 40 μM palmitoyl-CoA. Results represent the mean of two replications.

	IC_{50} (μM)					
	hELOVL1	hELOVL2	hELOVL3	hELOVL5	hELOVL6	mELOVL6
Compound-A	> 10	> 10	> 5	> 10	0.169	0.350

Table 2: *In vivo* mouse exposure and *in vitro* stability in liver microsomes of Compound-A

Compound-A exposure *in-vivo* was measured in plasma and liver. Results represent the mean of the data from three mice. The l/p ratio was calculated as follows: liver concentration divided by plasma concentration.

In vitro metabolic stability in liver microsomes is expressed as the percentage of converted parent compound (1 μM) after 30 min.

Mouse exposure (10 mpk, po, 2hr)			Microsome metabolism (% remaining)	
plamsa	liver	l/p ratio	human	mouse
5.22 μM	13.7 nmol/g	2.6	83	81

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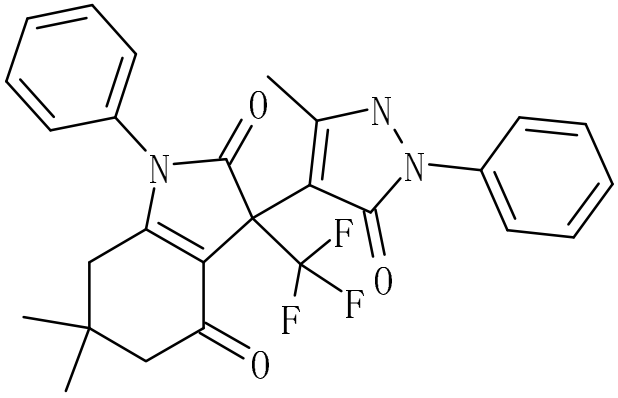
Fig. 1

A



Compound-A

B

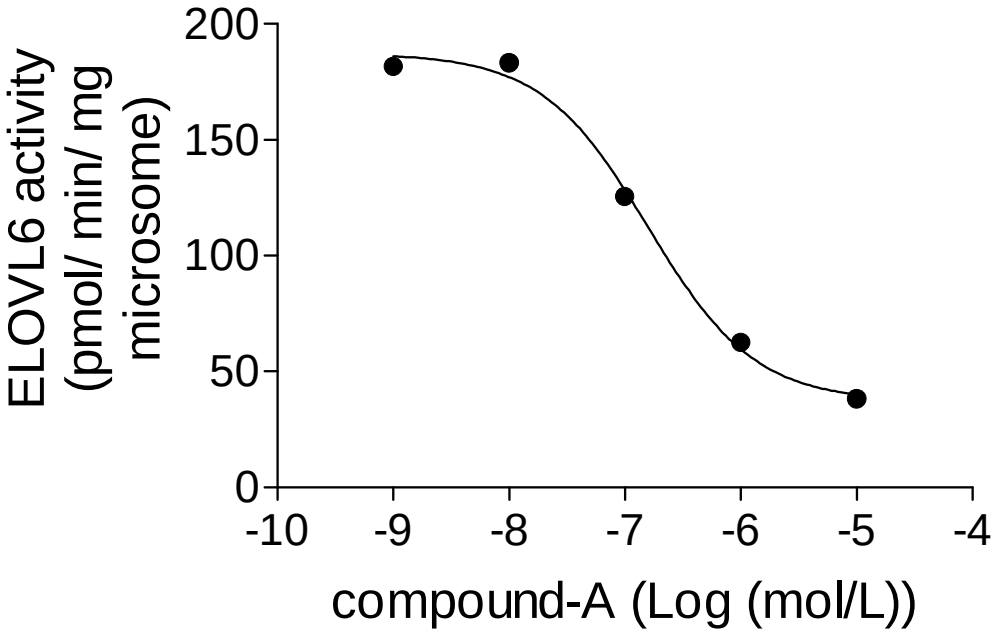


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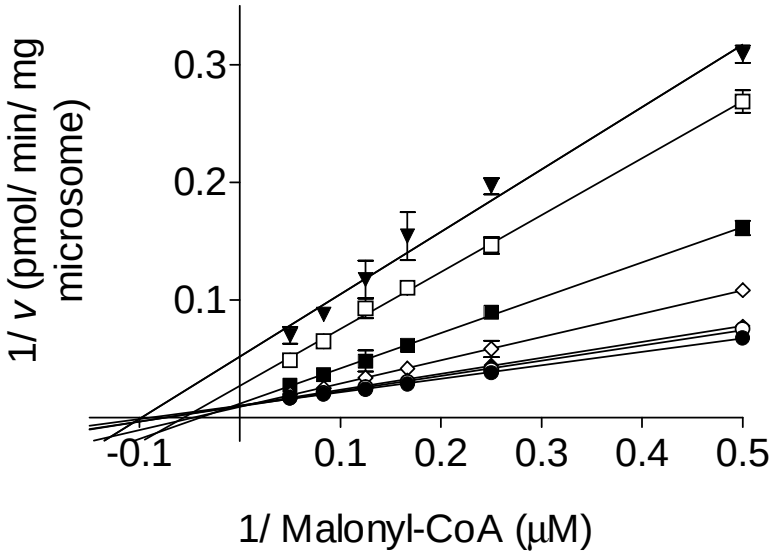
Fig. 2

A



B

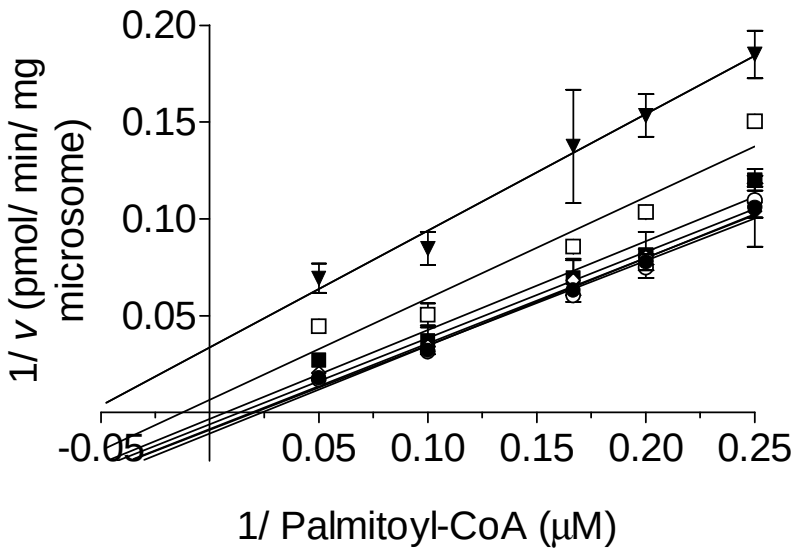


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Fig. 3

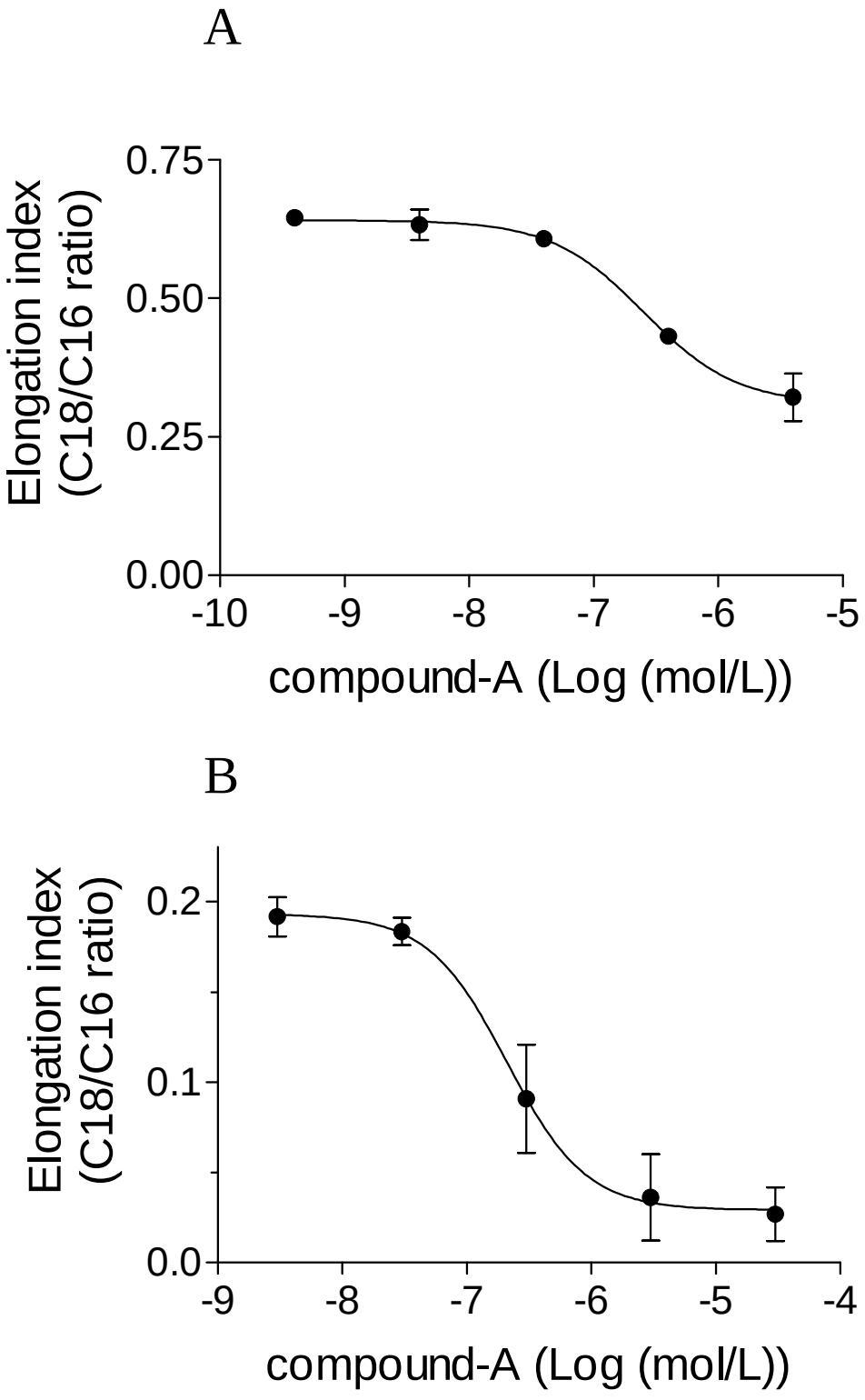

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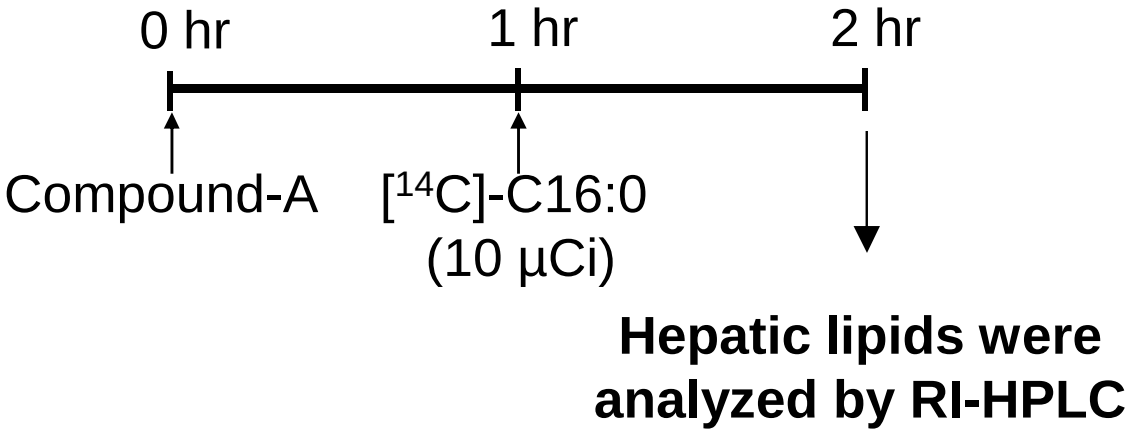
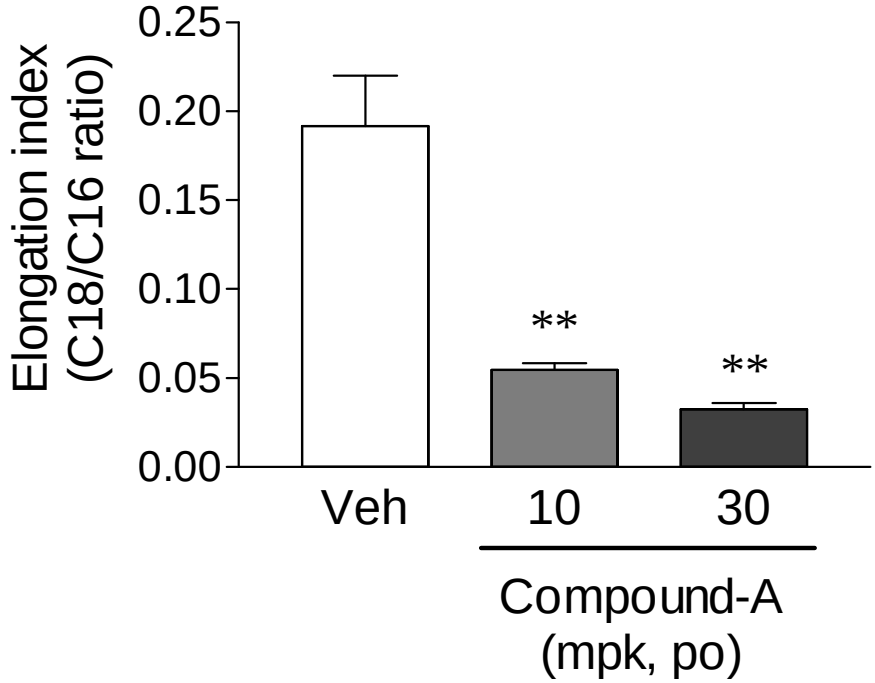
Fig. 4

A



Hepatic lipids were analyzed by RI-HPLC

B



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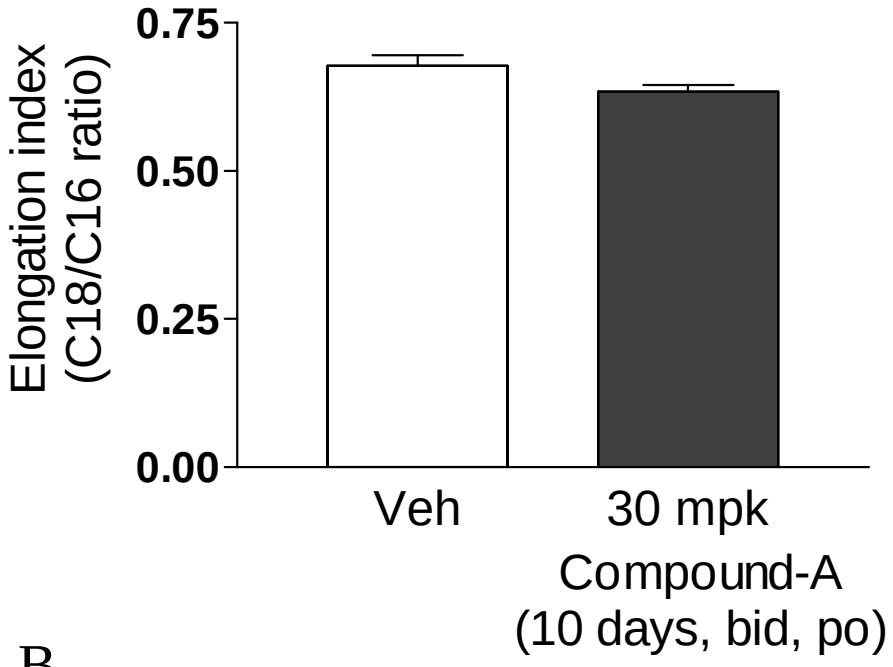
A Fig. 5



B