J. Biochem. 2009;146(3)429–437
doi:10.1093/jb/mvp088

Identification and Characterization of a Selective Radioligand for ELOVL6

Ken Shimamura[1], Hidekazu Takahashi[2], Hidefumi Kitazawa[1], Yasuhisa Miyamoto[1], Akira Nagumo[1], Cheng Tang[3], Dennis Dean[3], Tsuyoshi Nagase[2], Nagaaki Sato[2] and Shigeru Tokita[1,*]

[1]Department of Metabolic Disorder; [2]Department of Medicinal Chemistry, Tsukuba Research Institute, Banyu Pharmaceutical Co., Ltd., Okubo 3, Tsukuba, Ibaraki 300-2611, Japan; and [3]Merck Research Laboratories, Rahway, NJ 07065, USA

Received May 1, 2009; accepted June 1, 2009; published online June 8, 2009

ELOVL6, a member of the elongation of very long-chain fatty acids (ELOVL) family, has recently been identified as the rate-limiting enzyme for the elongation of palmitoyl-CoA. ELOVL6 deficient mice are protected from high-fat diet induced insulin resistance, suggesting that ELOVL6 might be a promising target for the treatment of metabolic disorders. Despite the increasing interest in Elovl6 as a therapeutic target, the lack of chemical tools for this enzyme has limited further elucidation of the biochemical and pharmacological properties of ELOVL6. We have identified Compound-A, a potent inhibitor for ELOVL6, by screening our company library and subsequently optimizing hit compounds. Compound-A potently inhibited human and mouse ELOVL6 and displayed >100-fold greater selectivity for ELOVL6 over other ELOVL family members. Consistent with its potent and selective inhibitory activity toward ELOVL6, [^{3}H]Compound-A bound to ELOVL6 with high affinity while showing no specific binding to other ELOVL enzymes. The observation that [^{3}H]Compound-A bound to ELOVL6 in a palmitoyl-CoA-dependent manner in the absence of malonyl-CoA and NADPH suggests that Compound-A might recognize an enzyme–substrate complex, e.g. an acyl–enzyme intermediate. Collectively, these observations demonstrate that Compound-A and its tritiated form are useful tools for biochemical and pharmacological characterization of ELOVL6.

Key words: elongases, ELOVL6, inhibitor, radioligand, ligand binding.

Abbreviations: ELOVL, elongation of very long-chain fatty acids.

The incidence of type 2 diabetes has dramatically increased over the past decade. Accumulating 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 tissue dysfunction such as insulin resistance (*1*). 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 antagonize the metabolic actions of insulin. Interestingly, recent reports have suggested that alternation of a specific fatty acid component (palmitoleate) has a significant impact on insulin sensitivity of the liver and the body as a whole (*2*, *3*). These observations suggest that modulation of fatty acid composition may lead to amelioration of insulin resistance.

Microsomal enzymes have been shown to be responsible for the elongation of long-chain fatty acids (LCFAs) with a chain length of >C16 while fatty acid synthase (FAS), a cytosolic enzyme, is responsible for the *de novo* synthesis of fatty acids with chain lengths up to C16 (*4*, *5*). Fatty acid elongation occurs through four sequential steps: (i) condensation by ELOVL (elongase of long-chain fatty acyl-CoA), (ii) reduction by β-ketoacyl-CoA reductase, (iii) dehydrogenation by β-hydroxyacyl-CoA dehydrogenase and (iv) reduction by *trans*-2,3-enoyl-CoA reductase (Supplementary data) (*4*, *5*). Given the NADPH-dependent activity of β-ketoacyl-CoA reductase, the condensation activities of ELOVLs can be monitored in the absence of NADPH (*6*, *7*).

Seven subtypes of ELOVL enzymes have been identified in mammals and are designated ELOVL1-7 (*6*, *8–12*). ELOVL enzymes can be divided into two major groups in terms of substrate preference: (i) elongases of saturated and monosaturated LCFAs (ELOVL1, 3 and 6) and (ii) elongases of polyunsaturated fatty acids (ELOVL2, 4 and 5). ELOVL7 is the most recently identified member of the ELOVL family enzymes based on primary structure information (*13*). However, its elongation activity and substrate specificity remain unknown.

In addition to these different fatty acid substrate preferences, each Elovl enzyme exhibits a different tissue distribution, suggesting that they play different physiological roles *in vivo* (*9*, *14*, *15*).

*To whom correspondence should be addressed.
Tel: +81-29-877-2000, Fax: +81-29-877-2027,
E-mail: shigeru.tokita@po.rd.taisho.co.jp

ELOVL6 (as known as LCE or FACE) was originally identified as a target gene of sterol regulatory element binding protein-1c (SREBP-1c) and subsequent reports revealed that ELOVL6 is responsible for the elongation of palmitoyl-CoA and is abundantly expressed in lipogenic tissues such as liver and adipose tissue (*6*, *9*). ELOVL6 is a multi-transmembrane protein that resides in the endoplasmic reticulum (ER) (*6*, *9*). Human and mouse ELOVL6 enzymes display 96% amino acid identity suggesting that ELOVL enzymes are well conserved across species (*6*, *9*).

Recent investigations have revealed potential physiological and pathological roles of ELOVL6. Matsuzaka and co-workers (*3*) reported that ELOVL6 deficient mice display improved insulin sensitivity and glucose homeostasis compared with wild-type mice when fed on a high-fat diet. Moreover, mRNA levels of ELOVL6 are up-regulated in obese rodents, by re-feeding after fasting, and by exposure to a high carbohydrate diet (*6*, *9*, *16*). These observations suggest that ELOVL6 might be a new therapeutic target for diabetes and obesity. In spite of increasing information on the physiological and pathological roles of ELOVL6, a lack of useful chemical tools makes it difficult to further address the pharmacological roles of ELOVL6 and its therapeutic potential.

We previously reported the establishment of a homogenous enzyme assay for Elovl6 that is applicable to ultra high-throughput screening (UHTS) and uses a recombinant histidine-tagged acyl-CoA binding protein (ACBP) as a molecular probe to monitor the enzymatic activity of ELOVL6 (*17*). UHTS of our company chemical library and the subsequent chemical derivatization of hit compounds resulted in the identification of Compound-A (Fig. 1) as a potent and selective inhibitor of Elovl6.

In this study, we report the biochemical properties of Compound-A and its tritiated form ([^{3}H]Compound-A) that may be useful tools for biochemical and pharmacological characterization of Elovl6. To our best knowledge, this is the first report of a selective radioligand for ELOVL6.

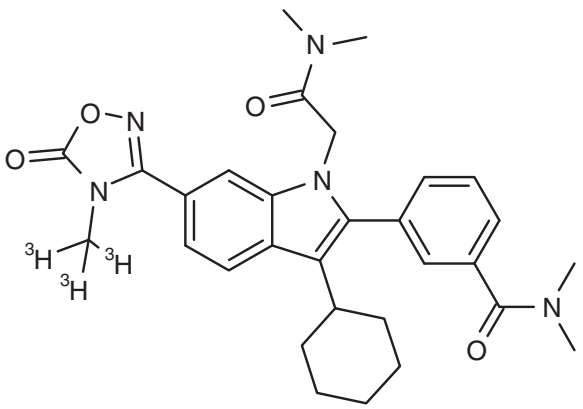

Fig. 1. **Structure of [^{3}H]Compound-A.** The structure and position of the tritiated moiety of [^{3}H]Compound-A is shown. Schemes for the synthesis of Compound-A and its tritiated form are provided in Supplementary data.

MATERIALS AND METHODS

Reagents—Glucose-6-phosphate, β-nicotinamide-adenine dinucleotide phosphate and glucose-6-phosphate dehydrogenase were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). [2-14C]-Malonyl-CoA was purchased from GE Healthcare science (Little Chalfont, UK). [14C] Palmitoyl-CoA was purchased from PerkinElmer Japan (Kanagawa, Japan). [14C] Stearoyl-CoA was purchased from Muromachi-Yakuhin (Tokyo, Japan). Oligonucleotide primers were purchased from Hokkaido System Science (Hokkaido, Japan). Human and mouse liver microsomes were obtained from BD Biosciences (San Jose, CA, USA) and Xenotech (Lenexa, KS, USA), respectively. Other reagents were obtained from Sigma (St Louis, MO, USA).

Compound-A and its tritiated form ([^{3}H]Compound-A) were synthesized by the Chemistry Department of BANYU Pharmaceutical Co., Ltd. (Ibaraki, Japan) and Merck Research Laboratories (Rahway, NJ, USA) (Fig. 1, Supplementary data).

Cloning and Expression of Elovl Family Enzymes—Full-length cDNA encoding hElovl6 (accession number: NM_024090) was amplified by PCR using the forward and reverse primers: 5′ primer, 5′-GGATCCAACATGTC AGTGTTGACTT-3′ and 3′ primer, 5′-CTCGAGCTATTCA GCTTTCGTTGTT-3′, which introduced *Bam*HI and *Xho*I restriction sites (underlined) at the 5′- and 3′-ends, respectively, of the hElovl6 coding sequence and deleted the initial methionine residue of hElovl6. The amplified fragment was then digested at these restriction sites and ligated with the double-digested pCMV-Tag2B vector (Stratagene), yielding an expression vector for an N-terminally FLAG-tagged rhElovl6-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 forward and reverse primers: 5′ primer, 5′-CTGCAGATTACAAGGATGACGA CGAT-3′ and 3′ primer, 5′-CTCGAGCTATTCAGCTTTC GTTGTT-3′, which introduced the *Pst*I restriction site (underlined) at the 5′-end of the FLAG-rhElovl6 coding sequence and deleted the initial methionine of FLAG. The amplified fragment was then digested at the designated restriction sites and ligated with the double-digested pPICZαB vector (Invitrogen, Carlsbad, CA, USA). For construction of N-terminally HA-tagged fusion proteins of other ELOVL family members, their coding sequences were individually amplified by PCR using the following forward and reverse primer pairs: human Elovl1 (accession number NM_022821) 5′ primer, 5′-AAACCATGGATGGAGGCTGTTGTGAAC TTG-3′ and 3′ primer, 5′-AAATCTAGATCAGTTGGCCT TGACCTTGG-3′; human Elovl2 (accession number NM_017770) 5′ primer, 5′-AAACCATGGATGGAACATC TAAAGGCC-3′ and 3′ primer, 5′-AAATCTAGATTATTG TGCTTTCTTGTTC-3′; human Elovl3 (accession number NM_152310) 5′ primer, 5′-AAACCATGGATGGTCACAGC CATGAATG-3′ and 3′ primer, 5′-AAATCTAGAGACATGA GGCCCTTTTTCGA-3′; human Elovl5 (accession number NM_021814) 5′ primer, 5′-AAACCCGGGGATGGAACATT TTGATGCATC-3′ and 3′ primer, 5′-AAATCTAGATTCAT CCTGCGCAAGAACAA-3′; mouse Elovl6 (accession

number NM_130450) 5′ primer, 5′-AAACCATGGATGAA CATGTCAGTGTTGACT-3′ and 3′ primer, 5′-AAATCTAG AACTACTCAGCCTTCGTGGCTTTC-3′, which introduced the underlined restriction sites of *Nco*I (hElovl1, 2, 3 and mElovol6) or *Sma*I (hElovl5) at the 5′-end and *Xba*I at the 3′-ends. These PCR products were digested by enzymes specific for the introduced restriction sites. For construction of HA-tagged fusion proteins, the sequence of an 3xHA tag was synthesized as follows: AACTGCAGCAGCGGCCGCGATGTACCCATACGATGT TCCAGATTACGCTTACCCATACGATGTTCCAGATTAC GCTTACCCATACGATGTTCCAGATTACGCTCCATGGC CCGGGAAA, and digested at the underlined sites by *Not*I at the 5′-end and *Nco*I (for hElovl1, 2, 3 and mElovl6) or *Sma*I (for hElovl5) at the 3′-ends. The Elovls and 3xHA fragments 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™ 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 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 the fusion proteins was induced in BMMY medium (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 r.p.m.). The microsome fraction was prepared 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 protease inhibitor cocktail (Roche)]. 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 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, the substrate mixture for elongation reactions consisted of 30 μl reaction mixture of (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. The following long-chain acyl-CoAs were used as the preferred substrate for each ELOVL; ELOVL1, 10 μM stearoyl-CoA; ELOVL2, 10 μM arachidonoyl-CoA; ELOVL3, 10 μM stearoyl-CoA; ELOVL5, 40 μM arachidonoyl-CoA, and ELOVL6, 40 μM palmitoyl-CoA. To start the reaction, 20 μL of the ELOVL microsomal fraction was added to the substrate mixture in a 96-well plate, and the mixture was then incubated for 1 h at 37°C with gentle shaking. After 1 h incubation, 100 μl of 5 M HCl was added to hydrolyze acyl-CoAs, and the reaction mixture was then filtered through a Unifilter-96, GF/C plate (PerkinElmer, Waltham, MA, USA) using a FilterMate cell harvester (PerkinElmer). The 96-well GF/C filter plate was subsequently washed three times with distilled water to remove excess [^{14}C] malonyl-CoA and then dried, after which 25 μl MICROSCINT 0 was added to each well and the radioactivity was determined.

In Vitro *Hepatocyte Assay using [^{14}C] Palmitate*—The mouse hepatocyte cell line H2.35 was used to assess the activity of Compound-A in cells. H2.35 cells were originally developed to induce liver-specific gene transcription in a temperature-sensitive manner and have been reported to express significant amounts of SREBP-1c (*18*). 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. The test compound, dissolved in medium, was incubated with subconfluent H2.35 cells for 60 min at 33°C following which [1-^{14}C]-palmitic acid (16:0) was added to each well to a final concentration of 0.8 μCi/ml for detection of elongase activity. After 4 h incubation at 33°C, the culture medium was removed and the labeled cells were washed with chilled PBS (3×0.5 ml) and dissolved in 250 μl of 2 N sodium hydroxide. The cell lysate was incubated at 70°C for 1 h to hydrolyze radiolabeled cellular lipids. After acidification with 100 μl of 5 N hydrochloric acid, fatty acids were extracted with 300 μl acetonitrile. Radiolabeled 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, USA), 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 column (3.0 mm i.d. × 150 mm). The identity of the labeled fatty acids was determined by comparison of the retention times with known fatty acid standards. Elongation activity was monitored as the elongation index (EI), which is 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.

Effects on Fatty Acid Composition of Hepatocytes—H2.35 cells (2×10^5 cells/well) were maintained and treated with Compound-A for 24 h as described above except the addition of [1-^{14}C]-palmitic acid. After three times washing with cold PBS, cells were incubated in 200 μl of 5 M NaOH/ethanol (1:1) at 70°C. After a 2 h incubation, 100 μl of 5 M HCl and C17:0 (internal standard) were added to all hydrolysates. The fatty acids compositions were analysed by a previously described method (Miwa, et al., 1985) with slight modifications. Briefly, the fatty acids in the cell 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 (73:27, flow rate: 0.6 ml/min). The separation was performed with a CAPCELL PAK C18 MGII (2.0 mm i.d. × 50 mm, 3 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.

Analysis of the Mode of Action of the ELOVL6 Inhibitor—The kinetic parameters of inhibition, i.e. the inhibition constant (*K*i) of Compound-A, and the Michaelis–Menten constant (*K*m) of the ELOVL6 substrates, were determined by Lineweaver–Burk plot analysis using various concentrations of Compound-A (0–3 μM) with ELOVL6 microsomes. The assay conditions for measurement of ELOVL6 activity were identical to the *in vitro* enzyme elongation assay except for the concentration of substrate tested (2–20 μM malonyl-CoA and 0–20 μM palmitoyl-CoA). To determine the *K*m and *K*i values for palmitoyl-CoA and malonyl-CoA, the concentration of the second substrate, 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 *Km* and *Ki* values were calculated as described previously (*19*) using the GraphPad PRISM software version 4.00 (GraphPad Software, Inc., San Diego, CA, USA).

Ligand-binding Assay—Microsome fractions from *Pichia Pastris* overexpressing human ELOVL enzymes were prepared as described above. The microsome fractions (1 μg/assay) were incubated at 37°C for 10 min with 20 nM [^{3}H]-Compound A in 100 mM potassium phosphate buffer (pH 6.5) containing 1 μM rotenone, 20 μM fatty acid free-bovine serum albumin and 40 μM LCFA-CoA (ELOVL1 and 3; stearoyl-CoA, ELOVL2 and 4; arachidonoyl-CoA, ELOVL6; palmitoyl-CoA) unless otherwise indicated in the figure legends. Bound and free radioligands were separated by filtration using a GF/C glass filter presoaked with 50 mM Tris buffer (pH 7.4). Following three washes with 50 mM Tris buffer (pH 7.4), the remaining radioactivity on the filter was quantified using TopCount-HTS (PerkinElmer). Non-specific binding (NS) was determined in the presence of 10 μM unlabeled Compound-A. All data were analysed by nonlinear regression using GraphPad Prism version 4.00 (GraphPad Software, Inc.).

RESULTS

Discovery of Compound-A as a Potent ELOVL6 Inhibitor—We previously reported the establishment of a high-throughput screening system for identification of inhibitors of Elovl6 using an ACBP as a molecular probe to monitor the fatty acid elongation activity of Elovl6 (*17*). This UHTS led to the discovery of several chemical leads with distinct structures including an indole class of hits. Subsequent medicinal chemistry-based research on this indole class of leads resulted in the identification of Compound-A, a potent inhibitor for Elovl6 (Fig. 1). Compound-A dose-dependently inhibited palmitoyl-CoA elongation mediated by human and mouse ELOVL6 with IC_{50} values of 26 nM and 75 nM, respectively, suggesting that Compound-A is a potent Elovl6 inhibitor (Fig. 2A, Table 1). Furthermore, Compound-A displayed greater than 100-fold selectivity for human ELOVL6 over other representative human ELOVL family enzymes (Table 1). Since ELOVL6 functions as a rate limiting enzyme for the elongation of C16 fatty acids such as palmitoyl-CoA (C16:0) to stearoyl-CoA (C18:0), (Supplementary data), we examined the effect of Compound-A on the elongation index and LCFA composition of the mouse hepatocyte cell line, H2.35. As results, Compound-A reduced the elongation index of H2.35 cells in a concentration-dependent manner when [1-^{14}C]-palmitic acid (16:0) was used as a radioactive tracer for elongation of LCFAs (Fig. 2B). Furthermore, as expected, we observed that Compound-A increased

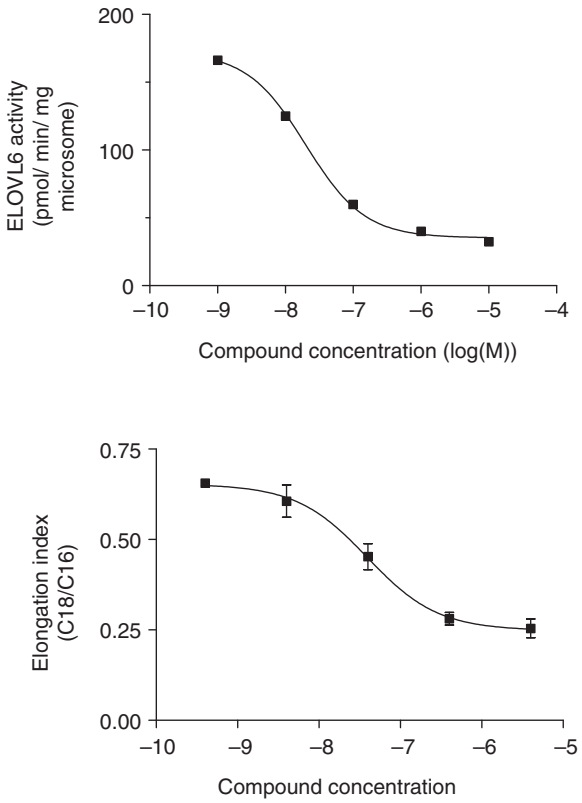

Fig. 2. **Effects of Compound-A on ELOVL6 activity and hepatocyte LCFA elongation activity.** The inhibitory effect of Compound-A on human ELOVL6 activity (A) and on the LCFA elongation index (EI) in mouse hepatocyte H2.35 cells (B) was examined as described in MATERIALS AND METHODS section. The results represent three independent tests, and are expressed as the mean ± SD.

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

	Enzyme activity (IC_{50}) (nM)					
	hElovl1 (μM)	hElovl2 (μM)	hElovl3 (μM)	hElovl5 (μM)	hElovl6	mElovl6
Compound A	>10	>10	>10	>10	26 ± 7.1	75 ± 9.2

Enzyme assays were carried out as described in MATERIALS AND METHODS section in the presence of the 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. The results represent the mean ± SD of three independent experiments.

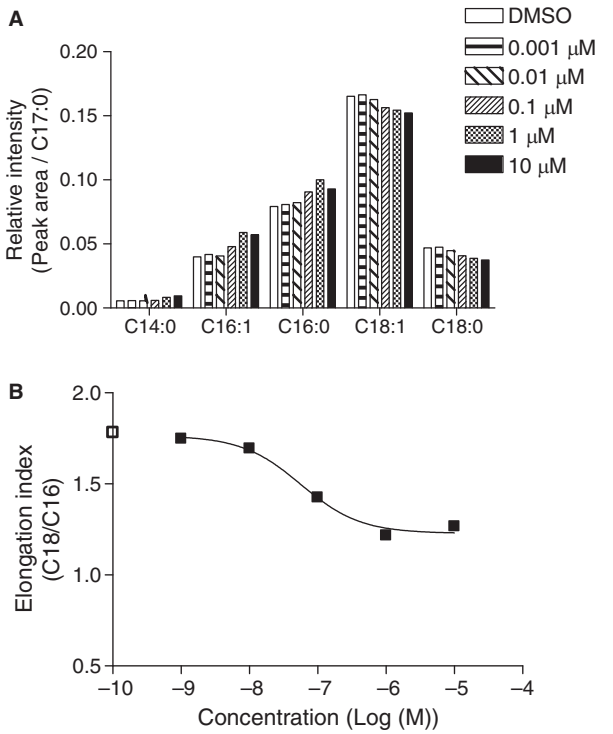

Fig. 3. **Effects of Compound-A on LCFA composition in hepatocytes.** The effects of Compound-A on the LCFA composition (A) and on the EI (B) in mouse hepatocyte H2.35 cells were examined as described in MATERIALS AND METHODS section. Open column or square indicate the control cells treated with DMSO. Hatched or closed columns and closed square indicated the cells treated with various concentrations of Compound-A. The results shown are from two independent experiments in duplicates.

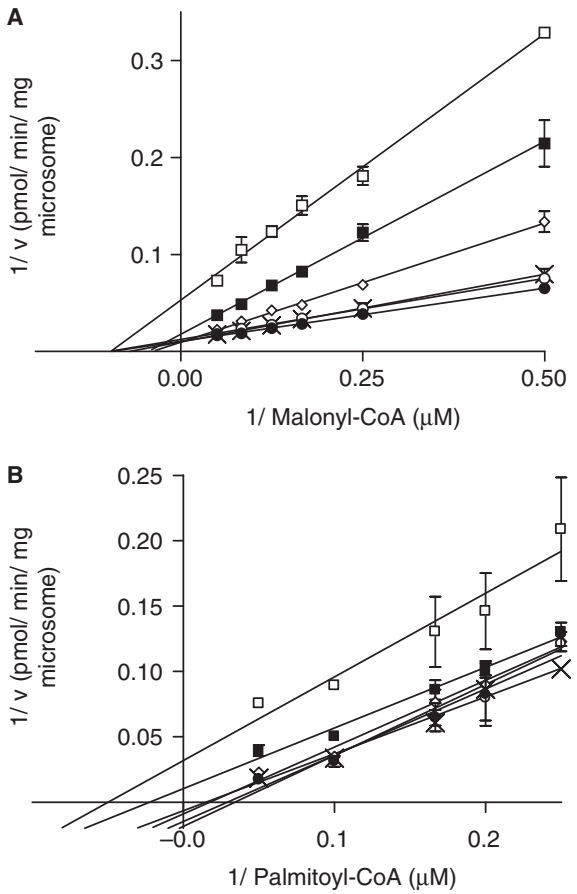

Fig. 4. **Kinetics of ELOVL6 inhibition by Compound-A.** The inhibitory effect of increasing concentrations of Compound-A [0 (filled circle), 0.01 (open circle), 0.03 (cross), 0.1 (open diamond), 0.3 (filled square) and 1 μM (open square)] on human ELOVL6 were examined in the presence of varying concentrations of malonyl-CoA (A) or palmitoyl-CoA (B) as outlined in MATERIALS AND METHODS section. The inhibitory mode-of-action of Compound-A for human ELOVL6 was analysed by Lineweaver–Burk plot analysis. The results represent at least three independent tests, and are expressed as the mean ± SD.

C16:0 and C16:1 fatty acid levels, while decreasing C18:0 and C18:1 fatty acid levels in a dose-dependent manner, leading to the reduction of EI of the total cellular LCFA (Figure 3A, 3B).

Mode-of-action of Compound-A—ELOVL6 catalyses the first condensation step of LCFA elongation such as condensation of palmitoyl-CoA (C16:0) and a 2-carbon donor malonyl-CoA, leading to the production of the corresponding β-ketoacyl-CoA (*6*). In order to address the mechanism by which Compound-A inhibits ELOVL6, we examined the effect of increasing concentrations of Compound-A on ELOVL6 activity in the presence of various concentrations of palmitoyl-CoA or malonyl-CoA by analysis of a Lineweaver–Burk plot of the data. Human ELOVL6 exhibited standard Michaelis–Menten kinetics with a *K*m of 3.1 μM for malonyl-CoA and 8.6 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 = 167 nM) and in an un-competitive manner for palmitoyl-CoA (Fig. 4A and B).

Synthesis and Characterization of [^{3}H]Compound-A—Given the potent and selective activity of Compound-A toward ELOVL6, we synthesized [^{3}H]Compound-A (Fig. 1, Supplementary data) and examined the utility of [^{3}H]Compound-A as a radiotracer for ELOVL6.

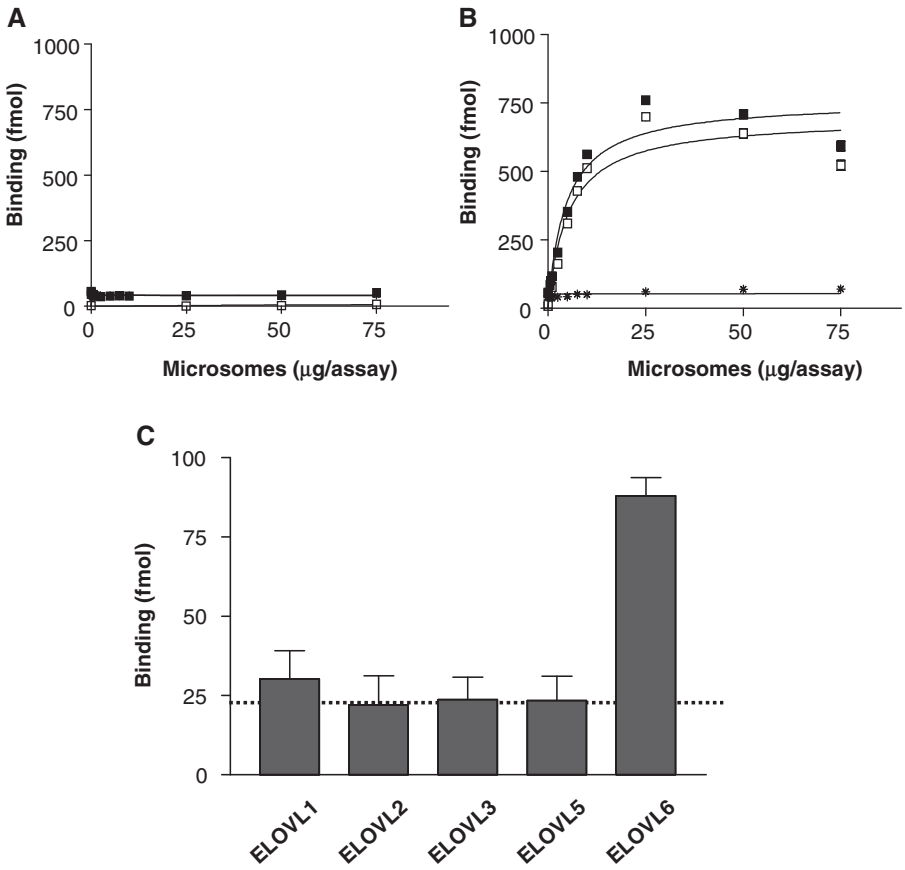

Fig. 5. **Binding profiles of [3H] Compound-A to yeast microsomal membranes expressing human ELOVL enzymes.** Binding of [3H] Compound-A (20 nM) to various concentrations of microsomes from *Pichia Pastris* yeast expressing mock vector (A) or human ELOVL6 (B) in the presence of DMSO (filled square, total binding) or 10 μM cold Compound-A (asterisk, non-specific binding). Specific binding (open square) was determined by subtraction of the non-specific binding from the total binding. The results represent the means of two independent tests. (C) The total binding of [3H] Compound-A (20 nM) to microsomes from *Pichia Pastris* yeast expressing human ELOVL enzymes (1 μg/assay) is shown. The binding assays were conducted in the presence of 40 μM of the respective ELOVL enzyme acyl-CoA substrate (ELOVL1 and 3; stearoyl-CoA, ELOVLl2 and 4; arachidonoyl-CoA, ELOVL6; palmitoyl-CoA). The dotted line indicates the level of background noise for ELOVL6 that was obtained in the presence of 10 μM cold Compound-A. The results represent three independent tests, and are expressed as the mean ± SD.

This [^{3}H]Compound-A displayed specific binding to microsomes overexpressing human ELOVL6 (1μg/assay) in the presence of palmitoyl-CoA, with an appreciable specific window (signal-to-background noise ratio of ∼3.0), while showing low-specific binding to the mock-transfected membranes (Fig. 5A and B). In order to address the binding selectivity preference of [^{3}H]Compound-A over other ELOVL enzymes, we examined the binding activity of [^{3}H]Compound-A to microsomal fractions overexpressing other ELOVL enzymes in the presence of their respective acyl-CoA substrates. This assay showed that, the total binding signals observed for other ELOVL enzymes (∼25 fmol) are comparable to those for mock-transfected membranes or to the background noise for ELOVL6 (signals in the presence of an excess of cold Compound-A) (Fig. 5C). These observations suggest that, consistent with its functional selectivity for ELOVL6 over the other ELOVL enzymes, [^{3}H]Compound-A specifically bound to ELOVL6.

Since Compound-A inhibited ELOVL6 in a non-competitive manner for malonyl-CoA and in an un-competitive manner for palmitoyl-CoA as described above (Fig. 4), we next examined the effect of acyl-CoAs on the specific binding of [^{3}H]Compound-A to ELOVL6. The results found that palmitoyl-CoA dose-dependently increases the specific binding of [^{3}H]Compound-A to ELOVL6 (Fig. 6A). In contrast, increasing concentrations of either stearoyl-CoA or malonyl-CoA showed no effect on the binding activity of [^{3}H]Compound-A to ELOVL6 (Fig. 6A and B).

Finally, we determined the binding affinity of [^{3}H]Compound-A to ELOVL6 in a saturation binding study (Fig. 7). In this assay, [^{3}H]Compound-A bound to ELOVL6 with a *K*d value of 10.4 ± 0.7 nM (Bmax: 98.8 ± 0.7 2.3 pmol/mg microsome) in the presence of 40 μM palmitoyl-CoA, suggesting that Compound-A has a high binding affinity for ELOVL6.

DISCUSSION

In this study, we have further characterized the utility of Compound-A, identified in a HTS system, as a potent

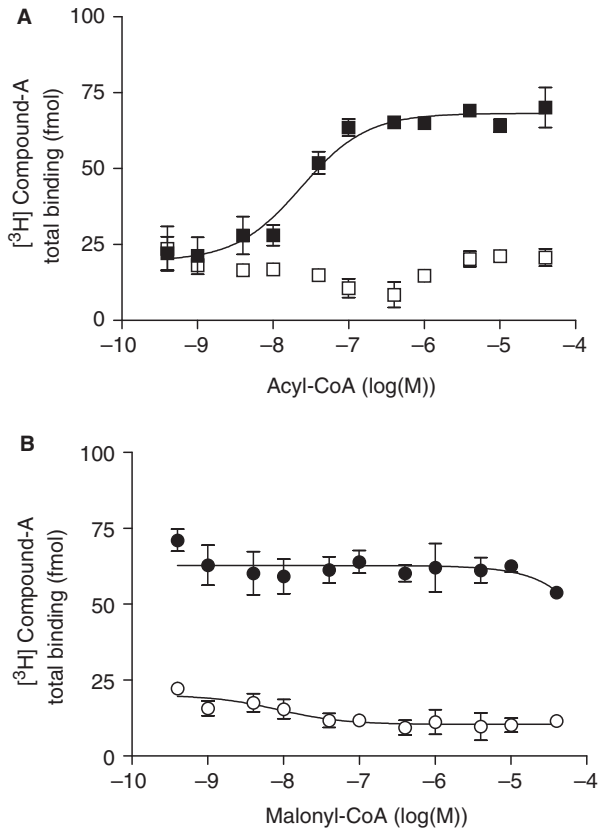

Fig. 6. **Binding of [^{3}H]Compound-A to yeast microsomal membranes overexpressing human ELOVL6 in the presence of various concentrations of acyl-CoAs.** (A) Binding of [^{3}H]Compound-A (20 nM) to microsomes from *Pichia Pastris* yeast expressing human ELOVL6 (1 μg/assay) in the presence of increasing concentrations of palmitoyl-CoA (filled square) or stearoyl-CoA (open square) (total binding). (B) The effect of increasing concentrations of malonyl-CoA on the binding of [^{3}H]Compound-A (20 nM) to microsomes from *Pichia pastris* yeast expressing human ELOVL6 (1 μg/assay) in the presence (filled circle) or absence (open circle) of 20 μM palmitoyl-CoA. The results represent three independent tests, and are expressed as the mean ± SD.

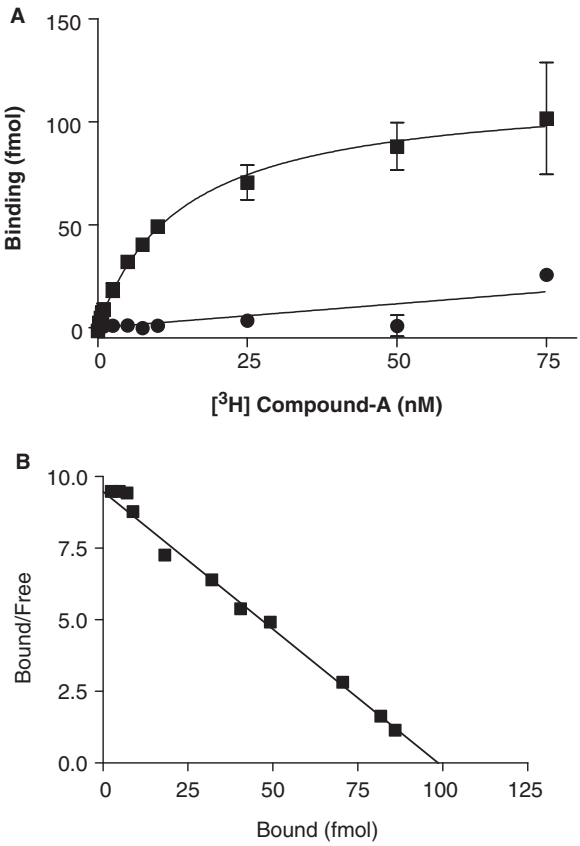

Fig. 7. **Saturation binding of [^{3}H]Compound-A to human ELOVL6 expressed on yeast microsomal fractions.** (A) Microsomes (1 μg/assay) from *Pichia pastris* yeast expressing human ELOVL6 (filled square) or mock transfected (filled circle) were incubated with the indicated concentrations of [^{3}H] Compound-A and the total binding level was determined. (B) Scatchard plot analysis of the specific binding of [^{3}H] Compound-A to ELOVL6 that was determined by subtraction of the non-specific binding from the total binding. The results represent three independent tests, and are expressed as the mean ± SD.

and selective inhibitor of ELOVL6 activity. Compound-A displayed >100-fold greater selectivity for ELOVL6 over other ELOVL family members: ELOVL1, 2, 3 and 5 (Fig. 2, Table 1). Although selectivity for other ELOVL members (i.e. ELOVL4 and ELOVL7) cannot be ruled out at this point, our current data suggest that Compound-A is a potent ELOVL6 inhibitor with appreciable selectivity. Moreover, Compound-A effectively altered the cellular LCFA composition (i.e. increase in C16:0 and C16:1 fatty acids, decrease in C18:0 and C18:1 fatty acids) (Fig. 3A) and significantly reduced the elongation index of LCFA of H2.35 mouse hepatocyte cells (Figs 2B and 3B), suggesting that Compound-A penetrates cell membranes and inhibits the elongation activity of ELOVL6 that resides on the ER.

From a drug discovery point of view, the mode-of-action of a compound is critical, especially for enzymes that constitute 'a family' and share substrates. As shown in Fig. 4, Compound-A inhibited ELOVL6 in a non-competitive manner for malonyl-CoA and in an un-competitive manner for palmitoyl-CoA. Although further studies remain to be conducted, we speculate that two possible mechanisms can explain these data: (i) Compound-A binds to an allosteric site within 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; (ii) Compound-A recognizes a specific conformational change that occurs during the formation of acyl–enzyme intermediates. The latter mechanism has been demonstrated for platensimycin, a potent inhibitor for β-ketoacyl-(acyl-carrier-protein) synthase I/II (FabF/B) that specifically interacts with the acyl–enzyme intermediate of the target protein (*20*). Regarding the selectivity of Compound-A, we speculate that the allosteric binding site would confer selectivity to Compound-A for preferential inhibition of ELOVL6 over other ELOVL family enzymes that commonly use malonyl-CoA and LCFAs as substrates. Among the ELOVL enzymes, ELOVL3 possesses the highest homology to ELOVL6 (44% amino acid identity). Despite the high homology between

ELOVL3 and ELOVL6, Compound-A showed a much higher selectivity for ELOVL6 over ELOVL3 (Table 1).

Synthesis and Characterization of a Radiotracer for ELOVL6—As exemplified by a variety of experiments, a radioligand (i.e. radiotracer) is a powerful research tool that allows investigators to further characterize and understand biochemical and pharmacological properties of target molecules. Based on its primary structure, ELOVL6 is predicted to have multiple transmembrane domains that are reminiscent of membrane proteins such as G protein-coupled receptors, transporters and channels for which selective radioligands have been developed that have been used for detailed characterization of target molecules. Therefore, taking advantage of the fact that Compound-A is amenable to tritiation, we synthesized [^{3}H]Compound-A (Fig. 1, Supplementary data) and examined its utility as a radioligand for ELOVL6. [^{3}H]Compound-A showed appreciable binding to membranes overexpressing human ELOVL6, while showing lower binding to membranes overexpressing other ELOVL enzymes or mock-transfected membranes, strongly demonstrating the utility of [^{3}H]Compound-A as a specific radiotracer for ELOVL6 (Fig. 5). Residual binding of [^{3}H]Compound-A to ELOVL6-overexpressing membranes in the presence of cold Compound-A (Fig. 5A) and its binding to mock-transfected membranes (Fig. 5C) (~25 fmol/μg microsome) might represent non-specific binding to off-target molecules.

Intriguingly, as shown in Fig. 6, palmitoyl-CoA dose-dependently enhanced specific binding of [^{3}H]Compound-A to ELOVL6. In contrast, neither malonyl-CoA nor stearoyl-CoA affected the specific binding of [^{3}H]Compound-A to ELOVL6. Since the absence of NADPH in the assay prevented subsequent enzymatic reactions (e.g. reduction by β-ketoacyl-CoA reductase after the initial condensation step) (Supplementary data, Fig. 4), these observations suggest that Compound-A might bind to an allosteric site exposed on the acyl-CoA–enzyme complex (e.g. a palmitoyl-CoA-ELOVL6 intermediate). If this is the case, then [^{3}H]Compound-A might be a useful tool to monitor the conformational changes of ELOVL6 that occur during its enzymatic reactions. In summary, [^{3}H]Compound-A is a useful radiotracer for ELOVL6, which might allow investigators to further characterize the biochemical and pharmacological properties of ELOVL6.

To the best of our knowledge, this is the first report to describe a potent and selective radiotracer for mammalian elongase. In this study, we characterized the biochemical profile of the potent and specific ELOVL6 inhibitor Compound-A, and demonstrated the utility of its tritiated form as a radiotracer. We believe that these materials will help investigators to further examine the biochemistry and pharmacology of ELOVL6.

SUPPLEMENTARY DATA

Supplementary data are available at *JB* online.

CONFLICT OF INTEREST

None declared.

REFERENCES

1. Unger, R.H. (2003) Minireview: weapons of lean body mass destruction: the role of ectopic lipids in the metabolic syndrome. *Endocrinology* **144**, 5159–5165
2. Cao, H., Gerhold, K., Mayers, J.R., Wiest, M.M., Watkins, S.M., and Hotamisligil, G.S. (2008) Identification of a lipokine, a lipid hormone linking adipose tissue to systemic metabolism. *Cell* **134**, 933–944
3. Matsuzaka, T., Shimano, H., Yahagi, N., Kato, T., Atsumi, A., Yamamoto, T., Inoue, N., Ishikawa, M., Okada, S., Ishigaki, N., Iwasaki, H., Iwasaki, Y., Karasawa, T., Kumadaki, S., Matsui, T., Sekiya, M., Ohashi, K., Hasty, A.H., Nakagawa, Y., Takahashi, A., Suzuki, H., Yatoh, S., Sone, H., Toyoshima, H., Osuga, J., and Yamada, N. (2007) Crucial role of a long-chain fatty acid elongase, Elovl6, in obesity-induced insulin resistance. *Nat. Med.* **13**, 1193–1202
4. Barrett, P.B. and Harwood, J. L. (1998) Characterization of fatty acid elongase enzymes from germinating pea seeds. *Phytochemistry* **48**, 1295–1304
5. Nugteren, D.H. (1965) The enzymic chain elongation of fatty acids by rat-liver microsomes. *Biochim. Biophys. Acta* **106**, 280–290
6. Moon, Y.A., Shah, N.A., Mohapatra, S., Warrington, J.A., and Horton, J.D. (2001) Identification of a mammalian long chain fatty acyl elongase regulated by sterol regulatory element-binding proteins. *J. Biol. Chem.* **276**, 45358–45366
7. Moon, Y.A. and Horton, J.D. (2003) Identification of two mammalian reductases involved in the two-carbon fatty acyl elongation cascade. *J. Biol. Chem.* **278**, 7335–7343
8. Leonard, A.E., Bobik, E.G., Dorado, J., Kroeger, P.E., Chuang, L.T., Thurmond, J.M., Parker-Barnes, J.M., Das, T., Huang, Y.S., and Mukerji, P. (2000) Cloning of a human cDNA encoding a novel enzyme involved in the elongation of long-chain polyunsaturated fatty acids. *Biochem. J.* **350**(Pt 3), 765–770
9. Matsuzaka, T., Shimano, H., Yahagi, N., Yoshikawa, T., Amemiya-Kudo, M., Hasty, A.H., Okazaki, H., Tamura, Y., Iizuka, Y., Ohashi, K., Osuga, J., Takahashi, A., Yato, S., Sone, H., Ishibashi, S., and Yamada, N. (2002) Cloning and characterization of a mammalian fatty acyl-CoA elongase as a lipogenic enzyme regulated by SREBPs. *J. Lipid Res.* **43**, 911–920
10. Tvrdik, P., Westerberg, R., Silve, S., Asadi, A., Jakobsson, A., Cannon, B., Loison, G., and Jacobsson, A. (2000) Role of a new mammalian gene family in the biosynthesis of very long chain fatty acids and sphingolipids. *J. Cell Biol.* **149**, 707–718
11. Tvrdik, P., Asadi, A., Kozak, L.P., Nedergaard, J., Cannon, B., and Jacobsson, A. (1997) Cig30, a mouse member of a novel membrane protein gene family, is involved in the recruitment of brown adipose tissue. *J. Biol. Chem.* **272**, 31738–31746
12. Zhang, K., Kniazeva, M., Han, M., Li, W., Yu, Z., Yang, Z., Li, Y., Metzker, M.L., Allikmets, R., Zack, D.J., Kakuk, L.E., Lagali, P.S., Wong, P.W., MacDonald, I.M., Sieving, P.A., Figueroa, D.J., Austin, C.P., Gould, R.J., Ayyagari, R., and Petrukhin, K. (2001) A 5-bp deletion in ELOVL4 is associated with two related forms of autosomal dominant macular dystrophy. *Nat. Genet.* **27**, 89–93
13. Strausberg, R.L., Feingold, E.A., Grouse, L.H., Derge, J.G., Klausner, R.D., Collins, F.S., Wagner, L., Shenmen, C.M., Schuler, G.D., Altschul, S.F., Zeeberg, B., Buetow, K.H., Schaefer, C.F., Bhat, N.K., Hopkins, R.F., Jordan, H., Moore, T., Max, S.I., Wang, J., Hsieh, F., Diatchenko, L., Marusina, K., Farmer, A.A., Rubin, G.M., Hong, L., Stapleton, M., Soares, M.B., Bonaldo, M.F., Casavant, T.L., Scheetz, T.E., Brownstein, M.J., Usdin, T.B., Toshiyuki, S., Carninci, P., Prange, C., Raha, S.S., Loquellano, N.A., Peters, G.J., Abramson, R.D., Mullahy, S.J., Bosak, S.A.,

McEwan, P.J., McKernan, K.J., Malek, J.A., Gunaratne, P.H., Richards, S., Worley, K.C., Hale, S., Garcia, A.M., Gay, L.J., Hulyk, S. W., Villalon, D.K., Muzny, D.M., Sodergren, E.J., Lu, X., Gibbs, R.A., Fahey, J., Helton, E., Ketteman, M., Madan, A., Rodrigues, S., Sanchez, A., Whiting, M., Madan, A., Young, A.C., Shevchenko, Y., Bouffard, G.G., Blakesley, R.W., Touchman, J.W., Green, E.D., Dickson, M.C., Rodriguez, A.C., Grimwood, J., Schmutz, J., Myers, R.M., Butterfield, Y. S., Krzywinski, M.I., Skalska, U., Smailus, D.E., Schnerch, A., Schein, J.E., Jones, S.J., and Marra, M.A. (2002) Generation and initial analysis of more than 15,000 full-length human and mouse cDNA sequences. *Proc. Natl Acad. Sci. USA* **99**, 16899–16903

14. Brolinson, A., Fourcade, S., Jakobsson, A., Pujol, A., and Jacobsson, A. (2008) Steroid hormones control circadian Elovl3 expression in mouse liver. *Endocrinology* **149**, 3158–3166
15. Wang, Y., Botolin, D., Xu, J., Christian, B., Mitchell, E., Jayaprakasam, B., Nair, M., Peters, J.M., Busik, J., Olson, L.K., and Jump, D.B. (2006) Regulation of hepatic fatty acid elongase and desaturase expression in diabetes and obesity. *J. Lipid Res.* **47**, 2028–2041
16. Miyazaki, M., Dobrzyn, A., Man, W.C., Chu, K., Sampath, H., Kim, H.J., and Ntambi, J. M. (2004) Stearoyl-CoA desaturase 1 gene expression is necessary for fructose-mediated induction of lipogenic gene expression by sterol regulatory element-binding protein-1c-dependent and -independent mechanisms. *J. Biol. Chem.* **279**, 25164–25171
17. Shimamura, K., Miyamoto, Y., Kitazawa, H., Kobayashi, T., Kotani, H., and Tokita, S. (2009) High-throughput assay for long chain fatty acyl-CoA elongase using homogeneous scintillation proximity format. *Assay Drug Dev. Technol.* **7**, 124–132
18. Hasty, A.H., Shimano, H., Yahagi, N., Amemiya-Kudo, M., Perrey, S., Yoshikawa, T., Osuga, J., Okazaki, H., Tamura, Y., Iizuka, Y., Shionoiri, F., Ohashi, K., Harada, K., Gotoda, T., Nagai, R., Ishibashi, S., and Yamada, N. (2000) Sterol regulatory element-binding protein-1 is regulated by glucose at the transcriptional level. *J. Biol. Chem.* **275**, 31069–31077
19. Lineweaver, H. and Burk, D. (1934) The determination of enzyme dissociation constants. *J. Am. Chem. Soc.* **56**, 658–666
20. Wang, J., Soisson, S.M., Young, K., Shoop, W., Kodali, S., Galgoci, A., Painter, R., Parthasarathy, G., Tang, Y.S., Cummings, R., Ha, S., Dorso, K., Motyl, M., Jayasuriya, H., Ondeyka, J., Herath, K., Zhang, C., Hernandez, L., Allocco, J., Basilio, A., Tormo, J.R., Genilloud, O., Vicente, F., Pelaez, F., Colwell, L., Lee, S. H., Michael, B., Felcetto, T., Gill, C., Silver, L.L., Hermes, J.D., Bartizal, K., Barrett, J., Schmatz, D., Becker, J.W., Cully, D., and Singh, S.B. (2006) Platensimycin is a selective FabF inhibitor with potent antibiotic properties. *Nature* **441**, 358–361