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A novel assay of cellular stearoyl-CoA desaturase activity of primary rat hepatocytes by HPLC

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ABSTRACT

The stearoyl-CoA desaturase (SCD) activity is involved in regulation of metabolism, energy storage, and membrane fluidity. However, only few cellular assays have been developed. We describe a simple and robust method to quantitate SCD activity and its inhibition in primary rat hepatocytes. Hepatocytes assimilate stearic acid, with or without modification by SCD, into its lipid pool. To measure the extent of this conversion primary rat hepatocytes were cultivated 4 h or overnight with [1-^{14}C]18:0 and extracellular fatty acids were washed out. Total cell lipids were then hydrolyzed and extracted. Recoveries of 18:0 were secured with a modified Folch method by addition of 0.1% Triton X-114 to the samples. The extracted fatty acids were dissolved in 85% ethanol and separated by reverse phase HPLC, which took 10 min including column recovery time. [1-^{14}C]18:0 and [1-^{14}C]18:1(n9) were detected and quantified by on-line flow scintillation analysis. Incubation of the cells with SCD inhibitors resulted in decreased ratios of 18:1/18:0 in dose-dependent manners. The improvements enabled us to establish a novel robust assay based solely on HPLC analysis of cellular SCD activity, which was developed in 12-well format.

1. Introduction

Stearoyl-CoA desaturase-1 (SCD1) has become a potential therapeutic target for metabolic disorders including obesity, diabetes, dyslipidemia and hepatic steatosis [1–4]. SCD1, also known as a Δ9 desaturase, is an integral membrane protein anchored in the endoplasmic reticulum [5], which is necessary for de novo formation of monounsaturated fatty acids. The substrates are mainly stearoyl-CoA (C18:0) and palmitoyl-CoA (C16:0), which are desaturated to oleoyl-CoA (C18:1) and palmitoleoyl-CoA (C16:1), respectively. SCD1 prefers to convert stearate (18:0) to oleate (18:1) rather than palmitate (16:0) to palmitoleate (16:1) [6], both of which make up the primary storage unsaturated fatty acids in human adipose tissue [7]. The ratio of monounsaturated to saturated fatty acids affects phospholipid composition and membrane fluidity. Changes in this ratio are implicated in numerous diseases [6]. Human SCD1 is ubiquitously expressed in most tissues, whereas rodent SCD1 is expressed in lipogenic tissues, such as the liver and adipose tissue [3,4]. SCD1 has been regarded as a switch between fatty acid storage and consumption, promoting or preventing lipid-induced disorders [4,8].

In vitro assays for measuring long-chain fatty acid (LCFA) desaturation activities conventionally involve several steps, such as lipid saponification, extraction and chromatography. Thin-layer chromatography (TLC) with $AgNO_3$-impregnated silica-gel-plates is widely used for SCD activity assays [8–10] due to its simplicity. However, this type of TLC is often semi-quantitative and scraping the peak area from the plate for further quantification can be a bottleneck for accuracy and throughput, unless a scanner (time consuming) or an image facility (often expensive) are implemented [11]. Gas chromatography (GC) or TLC coupled with GC is classically used in desaturation index measurement [11–13], whereas GC/mass spectrometry (MS) usually provides more decisive determination, but GC requires derivatization and is often time consuming. On the other hand, HPLC offers the advantages of speed, resolution, sensitivity and specificity for quantitation of LCFAs [14] and also a direct separation of long-chain acyl-CoA species [15]. Liquid chromatography (LC)/MS is yet another well-suited powerful technology for quantitative analysis of multiple lipid mixtures [16] and it was recently developed as a robotic high-throughput screening (HTS) platform [17]. Besides chromatographic methodology, a filter assay based on detection of tritiated water released from tritium-labeled C18:0 was established by Talamo and Bloch [18] and has been preferably adapted as an HTS-compatible format for drug screening [19,20].

Abbreviations: SCD, stearoyl-CoA desaturase; C18:0, stearoyl-CoA; C16:0, palmitoyl-CoA; C18:1, oleoyl-CoA; C16:1, palmitoleoyl-CoA; LCFA, long-chain fatty acid; HTS, high-throughput screening; DAG, diacylglycerides; SPE, solid-phase extraction; PEST, penicillin streptomycin solution; FBS, fetal bovine serum; PBS, phosphate-buffered saline solution; BSA, bovine serum albumin.

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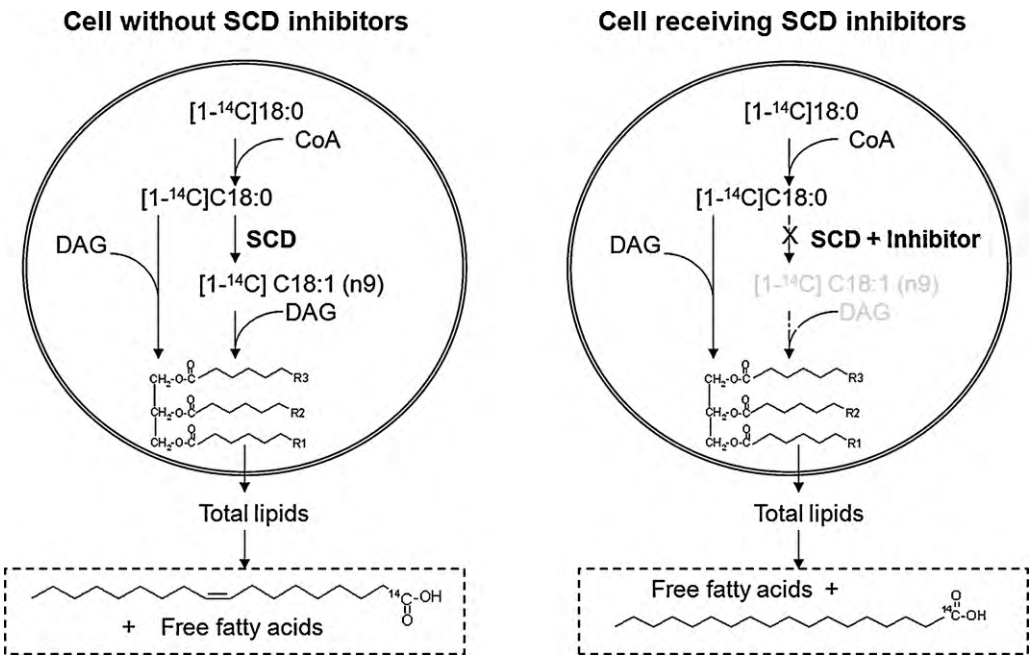

Fig. 1. Cellular assimilation of stearic acid and cellular inhibition of SCD. In this model, cells are incubated with [1-^{14}C]18:0. Hydrolysis of total lipids will include both radiolabeled 18:0 and 18:1, and the formation of the latter is blocked by the SCD inhibitor in the right panel. The extra structure in the rectangle highlights an increased readout of oleate (left panel) and of stearate (right panel), respectively. The ratio 18:1/(18:0 + 18:1) was used as an index of SDC activity. DAG, diacylglycerides; SCD, stearoyl-CoA desaturase.

Cell-based assays often become more complex in nature compared to straightforward enzymatic assays. When cells are cultivated in a medium supplemented with an LCFA, they assimilate the fatty acid into their lipid pool and part of this assimilation naturally goes through desaturation pathways. The extent of this involvement can be defined by extracting the total lipids of the cells and analyze the identities and quantities of the assimilated products. If the desaturation is perturbed by desaturase antagonists, the LCFAs of the lipid pool will be more saturated. A term referred to as the "fatty acid desaturation index" is employed to describe the ratio of products over substrates for the SCD enzyme activity and also as a predictor of metabolic disease [21]. With growing interests for targeting SCD1, LC/MS-based methods to analyze SCD1 inhibition in HepG2 cells emerged recently from Liu et al. [19] and Dillon et al. [22], respectively. The first paper [19] was on chemistry and the assay was just a part of the experimental results. In the second paper [22], the cells are scraped from the plates to allow for a saponification step and thereafter free LCFA species are extracted for LC/MC analysis. Sterculic acid, a substrate mimic inhibitor, is used to validate the assay for measuring SCD inhibition, giving an EC_{50} at 247 nM under the test conditions [22].

A simple and robust cellular SCD assay directly based on HPLC methods could not be found in the literature. To establish such an assay we chose rat primary hepatocytes and [1-^{14}C]18:0 as the fatty acid substrate in an effort to evaluate the potency of SCD antagonists in cells. Fig. 1 shows a schematic illustration of the setup, focused here on the assimilation of 18:0 into storage triglycerides. [1-^{14}C]18:0 is taken up by the cells and subsequently becomes assimilated into the cell pool of lipids. In the absence of intracellularly available SCD inhibitors (left cycle), C18:0 can be either desaturated to C18:1 by SCD or esterified directly into diacylglycerides (DAG) and subsequently triglycerides. The C18:1 formed by SCD can also be esterified and incorporated into triglycerides. In the presence of a SCD inhibitor (right cycle), the amount of 18:1 in the triglycerides is attenuated. After treatment with controls or inhibitors, the total cell lipids are saponified and the total free fatty acids are subjected to radiometric HPLC analysis of [1-^{14}C]18:0 and [1-^{14}C]18:1.

The goal of this paper was to develop of a simple cellular SCD assay in terms of improved sample preparation and subsequent robust HPLC analysis of cellular desaturation. We found that the recovery of the saturated LCFA from cellular samples was low and irreproducible, both by solid-phase extraction (SPE) and conventional liquid–liquid partition. A reliable lipid extraction procedure based on Folch extraction [23] with 0.1% Triton X-114 in the samples was established. This enabled us to develop a novel cellular assay based on radiometric HPLC analysis for the measurement of SCD activity and inhibition.

2. Materials and methods

2.1. Reagents and chemicals

Stearic acid (> 99%) was obtained from Aldrich (Steinheim, Germany). [1-^{14}C]Stearic acid (57 Ci mol^{-1}) and [1-^{14}C]oleic acid (53 Ci mol^{-1}) were purchased from Amersham (Buckinghamshire, UK). Williams' medium E with Glutamax I, penicillin streptomycin solution (PEST), fetal bovine serum (FBS) and phosphate-buffered saline solution (PBS) were purchased from Gibco (Paisley, UK). Insulin, fatty acid free bovine serum albumin (BSA) and Triton X-114 (polyethylene glycol *tert*-octylphenyl ether) were from Sigma (St. Louis, MO). Ultima-Flo M was from Perkin Elmer (Shelton, CT). Other chemicals and solvents of analytical or HPLC or higher quality were acquired from Fluka (Buchs, Switzerland) or Merck KGaA (Darmstadt, Germany). Sterculic acid [8-(2-octylcyclopropenyl)octanoic acid] and BVT168149 (a property compound of Biovitrum) were prepared in-house; BVT168149 has been optimized from a lead series of an in-house HTS with a tritium-release method (18, 20), with an apparent IC_{50} value of 15 nM against human SCD1. The SCD1 inhibitor N-(2-phenylethyl)-6-(4-{[2-(trifluoromethyl)phenyl]carbonyl}piperazin-1-yl)pyridazine-3-carboxamide [19] was also synthesized in-house, designated BVT153212, and found to inhibit rat SCD1 with an apparent IC_{50}

value of 6 nM in our hands. All internally synthesized compounds were controlled for their structural authenticity by LC/MS and had a purity >95% by HPLC.

2.2. Isolation of rat primary hepatocytes

Experiments were carried out in accordance to national and international guidelines for the care and use of laboratory animals, approved by the Local Committee on Ethics of Animal Experimentation. One male Sprague–Dawley rat (200–300 g) was anaesthetized by inhalation of isoflurane and perfused with collagenase prior to removal of the liver. The isolation was described by Neufeld [24].

2.3. Cell cultivation and treatment

Cells with viability not less than 70% as determined by the trypan blue exclusion method were typically used for the experiments. A number of 0.5×10^6 cells, suspended in 1 mL Williams medium E with Glutamax I containing 20 nM insulin, 1% PEST and 2% FBS (medium B), were added into each well of 12-well collagen I coated plates (BD Biosciences, Bedford, MA). The plates were incubated in the presence of 5% CO_2 at 37 °C for 2 h for cell attachment. Before addition of 0.90 mL medium B to each well, the cells were washed twice with medium A. Thereafter, 50 μL test compound solution (diluted in medium B containing 4% dimethyl sulfoxide) was added to each well and the plates were incubated in the presence of 5% CO_2 at 37 °C for 1 h. Next, 50 μL substrate solution (0.15 μCi [1-^{14}C]18:0 at 0.257 μCi μmol^{-1}, 2.3 mM BSA and 3% ethanol in medium B) was added. The incubation was continued for 4 h or overnight (18–22 h). Finally, the cells were washed three times with PBS (1–1.5 mL well^{-1}), and the remaining liquid was carefully aspirated. In the end, 300 μL alkaline alcohol solution (1 M KOH in 50% ethanol) was added to each well. The plates were stored at 2–8 °C overnight and thereafter the lysate from each well was transferred into a 1.5-mL screw-cap tube. The tubes were stored at −20 °C before analysis.

2.4. Saponification and total lipid extraction

Portions of 300 μL samples, in tubes with the cap tightly screwed, were heated for 20 min at 98 °C in the heating block of an Eppendorf Thermomixer with 500 rpm stirring and thereafter cooled down to room temperature. To each sample the following was added: 80 μL of 5 M HCl, 40 μL of 1% Triton X-114 and 0.8 mL Folch reagent ($CHCl_3$/methanol, 2:1, by vol)[2]. The resulting solution was vigorously vortexed for 30 s. The lower organic phase was transferred into a conic glass tube and evaporated to dryness at 40 °C under a flow of nitrogen. The residues in the tube were reconstituted with 50 μL sample phase (85% ethanol, 14% water and 1% acetic acid) and the sample tubes were placed on ice. A brief centrifuge (~200 g, 3 min) at 4 °C was performed before transferring the supernatant into a 96-well round bottom plate. The plate with samples was sealed with a cap pad and loaded in the thermostat sample tray for HPLC analysis or temporarily stored in a refrigerator before loading.

2.5. Chromatographic and instrumental methods

HPLC was performed with the column XBridge 2.5 C_{18} (2.1 mm × 50 mm, Waters) and a guard column 2.5 C_{18} (2.1 mm × 10 mm, Waters), which were held at 20 °C in a thermostat column compartment (Agilent G1316A). The solvent system consisted of 0.1% trifluoric acid in water (solvent A) and acetonitrile (solvent B). The HPLC effluent at a rate of 0.3 mL min^{-1} was mixed with a scintillant Ultima-Flo M (Perkin Elmer) at a rate of 1 mL min^{-1} before entering a Flo-One/Beta detector (Radiomatic 500TR Series, Packard) for radioactivity determination. The elution was performed by a binary HPLC pump (Agilent G1312A) and the column was equilibrated with 85% solvent B at a flow rate of 0.3 mL min^{-1}. The elution gradient started at 85% solvent B, and then solvent B was increased to 95% over a time period of 5 min, and thereafter solvent B was decreased to 85% in 0.1 min. The gradient cycle time was 10 min. Samples (10 μL) were injected by a well plate autosampler (Agilent G1367A) equipped with a sample thermostat (Agilent G1330A) that kept the sample chamber at 4 °C. All the related parts of the chromatographic system were operated via the on-line Agilent ChemStation software (GC ChemStation Rev. B.01.03, Agilent). To confirm the authenticity of the analytes, the effluent was collected directly after the XBridge column for mass determination.

LC/MS was carried out with a gradient mobile phase consisted of water/methanol, 95:5, containing 20 mM ammonium acetate (solvent C) and methanol/isopropanol, 80:20, containing 5 mM ammonium acetate (solvent D), at 0.2 mL min^{-1}. A column Discovery BIO Wide Pore C5 (5 μm, 2.1 mm × 100 mm, Supelco) was connected to a triple quadrupole mass spectrometer (TQD, Waters) with electrospray ionization in negative ion mode and unit mass resolution. The elution gradient started at 50% solvent D and increased to 100% solvent D over 5 min.

2.6. Data processing and analysis

The amount of each fatty acid was calculated based on the peak areas. An off-line Agilent ChemStation software (GC ChemStation Rev. B.01.03, Agilent) was used to both capture the raw data and for analysis of peak areas. The peak areas were used to calculate how many percent of oleic acid that had been formed according to the following equation:

$$\text{Oleic formation}\,(\%) = 100 \times A^{18:1}/(A^{18:0} + A^{18:1})$$

Herein $A^{18:1}$ is the peak area of [1-^{14}C]18:1 and $A^{18:0}$ the peak area of [1-^{14}C]18:0. Dose–response curve fitting and EC_{50} calculation were made by using Prism (Graph Pad Software) or XLfit (ID Business Solutions) programs. Apart from the standard deviation (SD) and coefficient of variation (CV) that reflect data precision, the Hill coefficient (h), correlation coefficient squared (r^2) and Z' parameter [25] were also determined to evaluate the quality of the data and the assay system whenever possible.

3. Results

3.1. HPLC method development

Two narrow bore reverse phase columns (2.1 mm × 50 mm, Waters), XBridge 2.5 Phenyl and XBridge 2.5 C_{18} were tested for their ability to separate [1-^{14}C]18:0 and [1-^{14}C]18:1. The latter showed superior ability to separate the two peaks of interest and the chromatographic conditions were thus optimized as described in the method. The retention times were typically 4.8 min and 7.2 min for 18:1 and 18:0, respectively. The resolution (R_s) was beyond baseline-separation ($R_s > 1$). Increasing the column temperature from 20 to 45 °C did not shorten the cycle time in a useful way.

To further verify the HPLC analysis, the effluent peaks for 18:1 (4.0–5.5 min) and 18:0 (6.5–8.0 min) from the column were collected and analyzed by MS respectively. The mass spectrum of the first eluting material yielded a strong anion signal at m/z 281 (100%)

[2] Be aware of the hazard of $CHCl_3$ vapour and when uncovered, handle it in a fuming hood.

and the expected weaker signals at m/z 282 (20%) and m/z 283 (4%) characteristic of 18:1, whereas the mass spectrum of the second eluting material was characteristic of 18:0 [m/z 283 (100%); m/z 284 (20%); m/z 285 (4%)]. The ^{14}C-label could not be detected due to the low specific activity.

3.2. Optimization of cell lysis and lipid saponification

These procedures were optimized as follows. The hepatocytes were scraped off from the bottom of the wells of the culture plate and collected by centrifugation for lipid saponification. We used 1 M KOH in 50% ethanol to digest the cells and to saponify the esters of the cellular lipids, which were transferred into screw-cap tubes and allowed to react under nitrogen or normal atmosphere. For nitrogen controls, the sample tubes were purged under N_2 flow 2 min and sealed. For non-nitrogen controls, the sample tubes were sealed directly by screwing the caps. We found that alkaline hydrolysis could be completed at 98 °C with vortexing at 500 rpm by 20 min (data not shown). We also found that no significant difference could be observed when heating the samples with or without nitrogen saturation. Oxidized or incompletely hydrolytic species, if any, would be expected to elute before 18:1 because of increased polarity. These species appeared as two small peaks at 1.3 min and 2.9 min. The total areas of the two peaks were 0.58% with and 0.88% without nitrogen protection, respectively. The difference was 0.3% and below the assay precision limit. The nitrogen treatment step could thus be omitted to simplify the procedure.

Cell harvest by scraping was laborious, and we studied an alternative. The cells, which were confluent and attached to the bottom in the medium with BSA (0.76%) and FBS (2%), were thoroughly washed with PBS to remove fatty acids; this was not improved by including 0.1% BSA in the PBS wash. As controls, cells were harvested by conventional scraping, and transferred to tubes for digestion in the alkaline alcohol solution. In parallel samples, the cells were lysed *in situ* by adding the alkaline alcohol solution directly in the wells and the plates were covered and incubated in a refrigerator overnight. We found that this *in situ* alkaline digest prevented sample loss and largely simplified the procedure and the sample could be stored at −20 °C for a few weeks without visible change in the fatty acid profile (data not shown).

3.3. Optimization of the lipid extraction method

In the early stages of assay development, a hydrophilic–lipophilic balance cartridge (Oasis HLB, Waters) was chosen. However, when cellular materials were extracted the recovery of added [1-^{14}C]18:0 was significantly decreased and varied between cartridges. This in turn affected the assay CV significantly. We next evaluated liquid–liquid extraction.

Three extraction reagents were screened, namely chloroform/methanol (Folch reagent [23]), hexane/isopropanol and heptane/isopropanol. Folch reagent was chosen because of higher recovery and convenience than the other reagents. It was found that extraction of the cellular samples after addition of 0.1% Triton X-114 yielded a near complete recovery of both 18:0 and 18:1, as illustrated in Fig. 2. Extractions with vigorous vortexing or ultrasonic treatment were compared, and the former yielded the best recovery. However, the recovery of 18:0 sometimes varied. This variation likely resulted from co-precipitation of the 18:0 with other components of the extracts. Tiny white particles were observed when the sample in 85% acetonitrile stayed on ice and this sample was accompanied with a lower recovery of 18:0 (HPLC analysis) after a few hours in the cooling tray. A further study showed that the sample leftover, when reconstituted in 85% ethanol and loaded into the column, gave a burst of 18:0 signal (data not shown). Extract dissolved in 85% ethanol rather than 85% acetonitrile prevented the loss of 18:0 due to co-precipitation. Although white precipitates were also formed in 85% ethanol when stored in cold, they did not affect the yield of 18:0. Ethanol (85%) secured 97–102% recovery of 18:1 and 93–97% of 18:0 (CV < 5% in triplicate). With these improvements, we found that the assay was reliable with at least in a cell count of 0.1–3 × 10^6 cells per well of the 12-well plates.

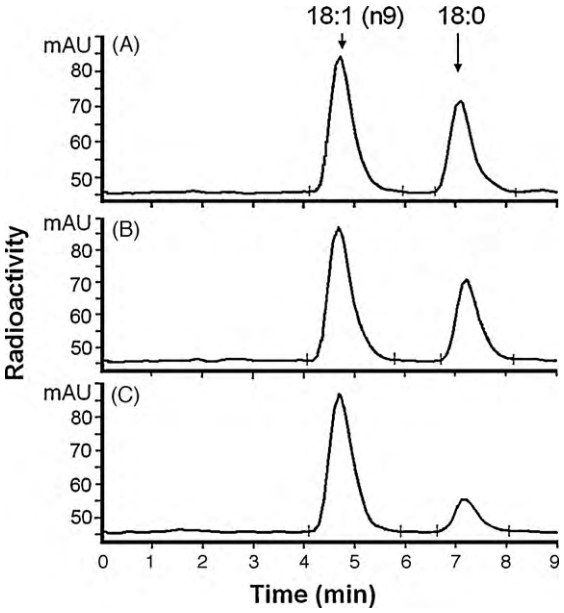

Fig. 2. Recovery of the fatty acids using Folch extraction supplemented with 0.1% Triton X-114 in the samples. The study was conducted with rat primary hepatocytes supplied with a medium without [1-^{14}C]18:0. A known amount of [1-^{14}C]18:1 (15 nCi) and [1-^{14}C]18:0 (15 nCi) was subsequently added into 300 μL (equal to one well sample in 12-well format) of the cell lysate and heated to achieve alkaline hydrolysis. Total lipids were extracted and residues were reconstituted in 50 μL sample phase (85% ACN, 14% H_2O, and 1% HAc). All samples were applied in 10 μL in duplicate and only one of the duplicate was shown. Chart A, sample (3 nCi each of the ^{14}C fatty acids) injected to column directly without extraction as 100% control ($A^{18:1}$ 1242 ± 30, CV 2.4%; $A^{18:0}$ 818 ± 3, CV 0.4%); Chart B, sample loaded from the extraction with Triton X-114 ($A^{18:1}$ = 1324 ± 13, CV 1.0%, oleic acid recovery 107%; $A^{18:0}$ = 750 ± 50, CV 6.7%, stearic acid recovery 92%); Chart C, sample from the extraction without Triton X-114 ($A^{18:1}$ = 1332 ± 39, CV 2.9%, oleic acid recovery 107%; $A^{18:0}$ = 345 ± 35, CV 10.3%, stearic acid recovery 42%).

3.4. Incubation time with stearic acid for the intracellular assimilation

Preliminary experiments showed that rat primary hepatocytes incubated with [1-^{14}C]18:0 at 0.6 mM (0.257 μCi μmol^{-1}) for 2 h yielded significant signals for both 18:0 and 18:1 in vehicle controls. Sterculic acid blocked the18:1 signal in a dose–response manner and down to a minimal level at concentrations of 20 μM and above (data not shown). In order to optimize the incubation time for stearate with the cells, three different co-incubation times (2 h, 4 h, and overnight) were tested, as illustrated in Fig. 3. This study indicates that the 4 h incubation gave the most significant assay window, while the overnight incubation ranked second. The overnight incubation with substrate was routinely used due to the convenience in terms of working hours. The data also indicate that BVT168149 at 0.2 μM suppressed the SCD1 activity even below the activity with 20 μM sterculic acid.

The blockage of SCD1 results in a significant attenuation of the intracellular assimilation or lipid accumulation, compared the total signal areas of the vehicle controls with that of the sterculic controls and the BVT1688149 tests, as shown in Fig. 3. This observation agrees well with the lipogenic role of SCD1 [8]. To reduce the animal consumption for preparation of hepatocytes, we studied cells culti-

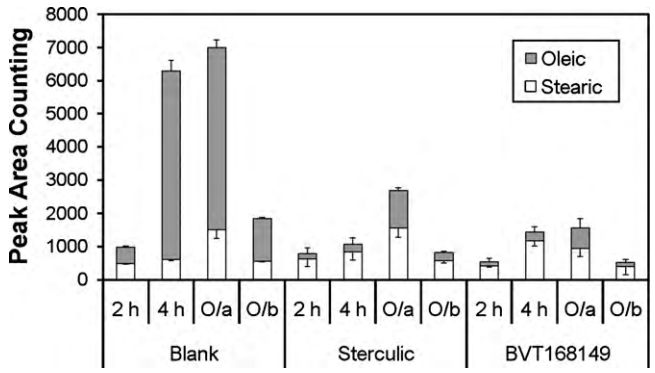

Fig. 3. Co-incubation time with substrate and cells in the hepatocyte assay. The study was performed in 6-well format and each incubation was made in duplicate with the samples from separate plates. The cells were preincubated with a compound for 1 h before receiving the substrate as described in Section 2.3. Peak area counting plot for both oleic and stearic acids. Blank (vehicle only control), Sterculic (20 μM sterculic acid) and BVT168149 (0.2 μM) were the three compound groups. Each group contains four substrate feeding experiments, 2 h, 4 h, O/a (overnight feeding) and O/b (4-h feeding, but prior to the experiment the cells were cultured overnight) respectively. The columns show the mean values with SD bars for the two fatty acids.

vated overnight before treatment with radiolabeled fatty acids, but these cells were less sensitive to changes in the 18:1/18:0 ratios (O/b, Fig. 3).

3.5. Dose–response characterization of inhibitors

Given the progress of the assay development a stable and robust assay window had now been established in a 12-well assay format. We subsequently characterized BVT168149 for the dose-dependent inhibition of SCD1 in primary rat hepatocytes as shown in Fig. 4. Since the primary rat hepatocytes are metabolically active, it is worthwhile to point out that the actual contact time between compound and cell will also depend on the metabolic stability of the tested compounds. BVT168149 was found to be stable under these conditions and its potency was consistent with a 4 h or an overnight incubation. To confirm this prediction, a less metabolically stable compound BVT153212 was assayed for SCD1 inhibition in the hepatocytes. The EC_{50} values were 51 nM using a contact

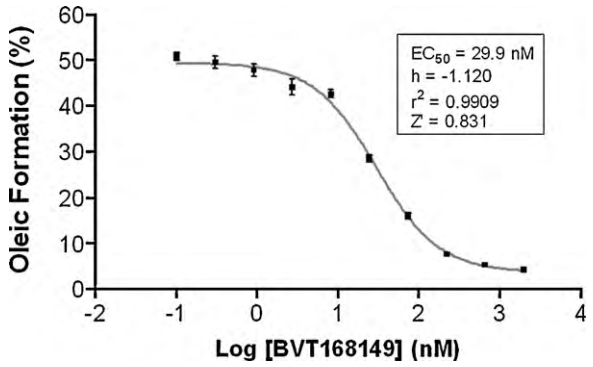

Fig. 4. Dose–response characterization of BVT168149 for SCD1 inhibition in rat primary hepatocytes. The cells received the stearic acid overnight. The oleic acid formation was calculated and used for the curve fitting. Each value was given as a mean of three independent experiments in separate 12-well plates. Each plate contained 10 dilutions of BVT168149 and a pair of vehicle control and full inhibition control wells. The means of the vehicle controls and the full inhibition controls were 49.48 ± 1.25% (fully active SCD1) and 4.68 ± 1.26% (fully inhibited SCD1), respectively. The insert legend shows the apparent EC_{50} value with 95% confidence intervals (25.2 ~ 35.5 nM) and the data quality parameters as described in Section 2.6. The Z' parameter calculation was based on the 3 pairs of the independent control data. The vertical bars illustrate the standard deviations.

time of 2 h and this shifted to 120 nM with an overnight incubation.

4. Discussion

Using [1-^{14}C]18:0 as the substrate for rat primary hepatocytes we have shown that radiolabeled 18:0 and 18:1 in the cells could be completely separated within a few minutes with reversed phase HPLC. We chose to use 18:0 rather than 16:0 for two important reasons. Firstly, 18:0 is not elongated in the cell and only two analytes, 18:0 and 18:1, need to be separated. With 16:0 four products (16:0, 16:1, 18:0 and 18:1) would have to be analyzed. The presence of one double bond reduces the retention time by the equivalent of two carbon atoms in the chain length [14], which means 18:1 and 16:0 may not be separated. Secondly, SCD1 prefers 18:0 over 16:0 as a substrate for the desaturation reaction [6]. In addition, palmitate is commonly used to induce cellular insulin resistance [26] and an excess leads to subsequent apoptosis [27], which may affect the cell physiology and thereby entangle the measured SCD1 activity.

We have simplified the cell harvesting and lipid saponification procedures because the strong alkali ethanol solution is also an ideal solution for cell lysis, digestion *in situ* and de-assembling fats from triglyceride-rich lipoproteins that would be produced by the hepatocytes. We have demonstrated that the 20-min heating at 98 °C without nitrogen purge for saponification did not affect the quantification. We have placed a significant effort in optimizing the lipid extraction procedures. SPE with modern cartridges prevails over the liquid–liquid extraction due to its throughput. However, the problem of the cartridges for extraction of 18:0 from cellular materials was unexpected. The Folch extraction yielded poor recovery of 18:0 until 0.1% Triton X-114 was added to the samples. This finding allowed us to assure the 18:0 recovery from the cell materials. Furthermore, 85% ethanol prevents 18:0 loss from precipitate. Because of the reliable extraction procedure, an additional internal standard for 18:0 was not required, which simplified the analysis. With the improved sample preparation approach, the signals became robust and stable, and the assay was highly reproducible and sensitive. As a consequence the amount of the cells required for each data point could be reduced dramatically. We used the assay in a 12-well format, but it seems fully feasible to adopt the method to 24- or 48-well microplates.

Saturated fat strongly induces *Scd1* gene expression and the cellular SCD1 enzyme is rapidly degraded via the ubiquitin-proteasome-dependent mechanism with a half-life of ~3 h [28]. We have examined the incubation time for 18:0 assimilation. Under the investigated conditions the rat primary hepatocytes were able to assimilate the substrate rather quickly. Already after 2 h the cells gave a well detectable signal of SCD1 activity as shown in Fig. 3. The study of the assimilation showed that the best assay window for the desaturation index was after 4 h co-incubation of the substrate and the cells, a window that remained pronounced for measuring after overnight cultivation (Fig. 3, panel O/a). This long lasting span of a reading window is especially advantageous to capturing a metabolically stable inhibitor, which is the interest in a lead optimization phase of drug discovery. An overnight feeding was also due to a practical reason in our lab. However, if a more sensitive performance is desired, 3–5 h co-incubation is shown here to result in most robust window and in addition will likely be more suitable for testing less stable compounds in the presence of the metabolically active hepatocytes. A right shift of the dose–response curve was seen with overnight incubation, when the less stable compound BVT153212 was assayed. However, some caution should be exer-

cised with regards to the apparent potency, since this could be influenced to some extent by P450-mediated degradation of the tested compounds and by other enzymatic actions on the LCFA in the hepatocytes.

The rat primary hepatocytes primarily express the SCD1 isoform and thus represent an excellent source of the specific enzyme. Clearly the vehicle (0.2% dimethyl sulfoxide) treated cells have a great capacity for fatty acid desaturation (Fig. 3, see blank). For dose–response analysis, the normalization offered by the desaturation index is effective in minimizing the impact of cell number variation and total lipids extracted between wells. This shall also minimize the influence by extracellular lipids, such as lipoproteins that may have been secreted from the cells into the medium and contribute the count loss under the SCD1 inhibition conditions (ref. Fig. 3). Although this index can be designated from different pairs of SCD substrates and products we chose to base on our working equation, 18:1/(18:0 + 18:1), which we also refer to as "oleic formation (%)", for reasons discussed above. The potency determination of BVT168149 was extremely consistent both in 6-well and in 12-well formats, which demonstrated an EC_{50} value of 29.9 nM in this cell assay. In addition, there is a high serum albumin content (115 μM BSA plus 2% FBS) of cell culture medium and the cells are metabolically active which are in agreement with the excellent bioavailability of this compound (data not shown). We believe that our version of the hepatocyte assay is superior in terms of simplicity and usefulness with regard to the limitations of the MS equipment, and hence should be a value for experimentalists interested in measuring cellular SCD activity.

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References

[1] A. Dobrzyn, J. Ntambi, Stearoyl-CoA desaturase: a therapeutic target of insulin resistance and diabetes, Drug Discov. Today 2 (2005) 125.
[2] M.T. Flowers, J.M. Ntambi, Stearoyl-CoA desaturase and its relation to high-carbohydrate diets and obesity, Biochim. Biophys. Acta 1791 (2009) 85.
[3] G. Liu, Recent advances in stearoyl-CoA desaturase 1 inhibitors for dyslipidemia and obesity, Curr. Top. Med. Chem. 10 (2010) 419.
[4] P. Dobrzyn, M. Jazurek, A. Dobrzyn, Stearoyl-CoA desaturase and insulin signaling – What is the molecular switch? Biochim. Biophys. Acta 1791 (2010) 1189.
[5] W.C. Man, M. Miyazaki, K. Chu, J.M. Ntambi, Membrane topology of mouse stearoyl-CoA desaturase 1, J. Biol. Chem. 281 (2005) 1251.
[6] J.M. Ntambi, M. Miyazaki, Regulation of stearoyl-CoA desaturases and role in metabolism, Prog. Lipid Res. 43 (2004) 91.
[7] W. Insull Jr., G.E. Bartsch, Fatty acid composition of human adipose tissue related to age, sex and race, Am. J. Clin. Nutr. 20 (1967) 13.
[8] H. Sampath, M. Miyazaki, A. Dobrzyn, J.M. Ntambi, Stearoyl-CoA desaturase-1 mediates the pro-lipogenic effects of dietary saturated fat, J. Biol. Chem. 282 (2007) 2483.
[9] P.D. Jones, P.W. Holloway, R.O. Peluffo, S.J. Wakil, A requirement for lipids by the microsomal stearyl coenzyme A desaturase, J. Biol. Chem. 244 (1969) 744.
[10] V.C. Joshi, A.C. Wilson, S.J. Wakil, Assay for the terminal enzyme of the stearoyl coenzyme A desaturase system using chick embryo liver microsomes, J. Lipid Res. 18 (1977) 32.
[11] M. Miyazaki, F.E. Gomez, J.M. Ntambi, Lack of stearoyl-CoA desaturase-1 function induces a palmitoyl-CoA Δ6 desaturase and represses the stearoyl-CoA desaturase-3 gene in the preputial glands of the mouse, J. Lipid Res. 43 (2002) 2146.
[12] G. Jiang, Z. Li, F. Liu, K. Ellsworth, Q. Dallas-Yang, M. Wu, J. Ronan, C. Esau, C. Murphy, D. Szalkowski, et al., Prevention of obesity in mice by antisense oligonucleotide inhibitors of stearoyl-CoA desaturase-1, J. Clin. Invest. 115 (2005) 1030.
[13] E. Warensjö, M. Öhrvall, B. Vessby, Fatty acid composition and estimated desaturase activities are associated with obesity and lifestyle variables in man and women, Nutr. Metab. Cardiovasc. Dis. 16 (2005) 128.
[14] A. Mehta, A.M. Oeser, M.G. Carlson, Rapid quantitation of free fatty acids in human plasma by high-performance liquid chromatography, J. Chromatogr. B. 719 (1998) 9.
[15] M.Y. Golovko, E.J. Murphy, An improved method for tissue long-chain acyl-CoA extraction and analysis, J. Lipid Res. 45 (2004) 1777.
[16] U. Sommer, H. Herscovitz, F.K. Welty, C.E. Costello, LC–MS-based method for the qualitative and quantitative analysis of complex lipid mixtures, J. Lipid Res. 47 (2006) 804.
[17] P. Soulard, M. McLaughlin, J. Stevens, B. Connolly, R. Coli, L. Wang, J. Moore, M.S. Kuo, W.A. LaMarr, C.C. Ozbal, et al., Development of a high-throughput screening assay for stearoyl-CoA desaturase using rat liver microsomes, deuterium labeled stearoyl-CoA and mass spectrometry, Anal. Chim. Acta 627 (2008) 105.
[18] B.R. Talamo, K. Bloch, A new assay for fatty acid desaturation, Anal. Biochem. 29 (1969) 300.
[19] G. Liu, J.K. Lynch, J. Freeman, B. Liu, Z. Xin, H. Zhao, M.D. Serby, P.R. Kym, T.S. Suhar, H.T. Smith, et al., Discovery of potent, selective, orally bioavailable stearoyl-CoA desaturase 1 inhibitors, J. Med. Chem. 50 (2007) 3086.
[20] T. Lundbäck, I. Climent-Johansson, J. Vågberg, U. Bremberg, A. Lindén, J. Nilsson, M. Wiik, Pyrazolo [1,5-a] pyrimidine analogs for use as inhibitors of stearoyl-CoA desaturase (SCD) activity, Internatl Patent WO 2008/003753.
[21] M.T. Flowers, The Δ9 fatty acid desaturation index as a predictor of metabolic disease, Clin. Chem. 55 (2009) 2071.
[22] R. Dillon, M.J. Greig, B.G. Bhat, Development of a novel LC/MS method to quantitate cellular stearoyl-CoA desaturase activity, Anal. Chim. Acta 627 (2009) 99.
[23] J. Folch, M. Lees, G.H. SloaneStanley, A simple method for the isolation and purification of total lipids from animal tissues, J. Biol. Chem. 226 (1957) 497.
[24] D.S. Neufeld, Isolation of rat liver hepatocytes, in: J.W. Pollard, J.M. Walker (Eds.), Basic Cell Culture Protocols, Humana Press, Totowa, 1997, p. 145.
[25] J.H. Zhang, T.D. Chung, K.R. Oldenburg, A simple statistical parameter for use in evaluation and validation of high throughput screening assay, J. Biomol. Screen. 4 (1999) 67.
[26] J.J. Senn, Toll-like receptor-2 is essential for the development of palmitate-induced insulin resistance in myotubes, J. Biol. Chem. 281 (2006) 26865.
[27] L.L. Listenberger, X. Han, S.E. Lewis, S. Cases, R.V. Farese Jr., D.S. Ory, J.E. Schaffer, Triglyceride accumulation protests against fatty acid-induced lipotoxicity, Proc. Natl. Acad. Sci. U.S.A. 100 (2003) 3077.
[28] H. Kato, K. Sakaki, K. Mihara, Ubiquitin-proteasome-dependent degradation of mammalian ER stearoyl-CoA desaturase, J. Cell Sci. 119 (2006) 2342.