C75, an inhibitor of fatty acid synthase, suppresses the mitochondrial fatty acid synthesis pathway and impairs mitochondrial function

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\textsuperscript{*}Running title: C75 on mitochondrial function

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\textbf{Key words:} Antioxidants; Fatty acid synthase; Mitochondrial diseases; Oxidative stress; Reactive oxygen species (ROS)

\textbf{Background:} C75 is a fatty acid synthase inhibitor and potential anticancer drug.

\textbf{Results:} C75 treatment leads to mitochondrial dysfunction which is rescued by overexpression of β-ketoacyl-acyl carrier protein synthase or lipoic acid.

\textbf{Conclusion:} The effect of C75 on mitochondria is caused by inhibition of β-ketoacyl-acyl carrier protein synthase.

\textbf{Significance:} The mitochondrial fatty acid synthesis pathway plays an important role in mitochondrial function.

\textbf{ABSTRACT}

\textbf{C75} (4-methylene-2-octyl-5-octotetrahydrofuran-3-carboxylic acid) is a synthetic fatty acid synthase (FASN) inhibitor with potential therapeutic effects in several cancer models. Mitochondrial β-ketoacyl-acyl carrier protein synthase (HsmtKAS) is a key enzyme in the newly discovered mitochondrial fatty acid synthesis pathway (mtFAS II) that can produce the substrate for lipoic acid (LA) synthesis. HsmtKAS shares conserved catalytic domains with FASN, which are responsible for binding to C75. In our study, we explored the possible effect of C75 on HsmtKAS and mitochondrial function. C75 treatment decreased LA content, impaired mitochondrial function, increased content of reactive oxygen species (ROS) and reduced cell viability. HsmtKAS but not FASN knockdown had an effect that was similar to C75 treatment. Meanwhile, an LA supplement efficiently inhibited C75-induced mitochondrial dysfunction and oxidative stress.
stress. Overexpression of HsmtKAS showed cellular protection against low dose C75 addition, while no protective effect on high dose C75 addition. In summary, the mtFAS II pathway has a vital role in mitochondrial function. Besides FASN, C75 might also inhibit HsmtKAS, thereby reducing LA production, impairing mitochondrial function, and potentially having toxic effects. LA supplements sufficiently ameliorated the toxicity of C75, showing that a combination of C75 and LA may be a reliable cancer treatment.

Fatty acid synthase (FASN) is a key lipogenic enzyme located in the eukaryote cytoplasm that produces long-chain fatty acids (1,2). Several human cancer cells have high FASN expression relative to non-cancer cells, including prostate, breast and colon cancer cells (3,4). Abnormal FASN expression is associated with tumor cell progression and metabolism. Characterization of FASN has made it as an attractive target for drug development. To date, many potent small molecule compounds that inhibit FASN have been explored for cancer therapy. Among these inhibitors, C75 (4-methylene-2-octyl-5-oxotetrahydrofuran-3-carboxylic acid), an analogue of chemically unstable cerulenin, has significant antitumor activity. C75 interacts with FASN domains and induces apoptosis using the fatty acid oxidation pathway in breast cancer, prostate cancer, and human lung carcinoma cells (5). It also exhibited cytostatic and chemopreventive actions in a neu-N transgenic mouse model of mammary cancer (6). Thus, C75 is a strong candidate for further clinical development.

In addition to “classic” cytoplasmic FASN, a novel system for fatty acid synthesis, the mtFAS II pathway, was recently identified in the mitochondria and includes a number of enzymes (7-10). To date, most of the characterization of mtFAS II has been performed in yeast. The mtFAS II system is closely associated with the physiological and biochemical functions of the mitochondria, including mitochondrial fusion and fission, mitochondrial DNA replication and the antioxidant system (7). A deficiency in any of the mtFAS II genes in yeast leads to RNA processing defects, loss of mitochondrial cytochromes a and b and defects in cellular lipoic acid (LA) (11-13). The main biological function of the mtFAS II pathway is the production of the octanoic acid precursor for LA synthesis (12,14,15). LA is a potent antioxidant that improves mitochondrial function, reduces organ dysfunction and provides beneficial effects for the prevention of several diseases, such as diabetes, cardiovascular disease and liver disease (16). However, little is known about the mtFAS II genes in mammals. Recent research has shown that defects in mammalian mtFAS II genes result in mitochondrial dysfunction. Transgenic mice for mitochondrial malonyl-CoA acyl carrier protein transacylase (Mcat) have disrupted energy equilibrium and protein lipoylation (17). The overexpression of 2-enoyl thioester reductase (Mecr) causes myocardial dysfunction in mice (18).

Over the past few years, screening of FASN inhibitors to design antitumor drugs has ignored the effects on mtFAS II. Whether these FASN inhibitors have impact on mtFAS II remains unclear. Human mitochondrial β-ketoacyl-acyl carrier protein synthase (HsmtKAS; OXSM) is the key enzyme of the mtFAS II pathway, which catalyzes the chain-elongating reaction of the fatty acid synthesis cycle. The catalytic domains are quite conserved between FASN and HsmtKAS. The structure of HsmtKAS shows that it has a highly conserved malonyl-binding pocket for C75, which inhibits enzyme activity (19). These observations prompted the question whether C75 produces side effects in normal cells through HsmtKAS during cancer therapy.

In the present study, we have investigated the underlying mechanisms of the toxic effects of C75 on the mtFAS II pathway and
mitochondrial function in HEK293T cells. In addition, we explored agents that effectively reduce toxic side effects on non-cancer cells, which may contribute to future therapeutic cancer treatments.

EXPERIMENTAL PROCEDURES

Materials-C75 (4-methylene-2-octyl-5-oxotetrahydrofuran-3-carboxylic acid) was purchased from Sigma (St. Louis, MO, USA). The α-Lipoic acid (R-LA) was gift from Dr. Davis Carlson (GeroNova Research Inc., California, USA). H2DCFDA (2‘,7‘-dichlorodihydrofluorescein diacetate), JC-1, TRIzol, NAC (N-acetylcysteine), and the transfection reagent Lipofectamine 2000 were from Invitrogen (Carlsbad, CA, USA). Anti-NQO1 (NADH quinone oxidoreductase) was from Cell Signaling Technology. Anti-HsmtKAS, Nrf2 (nuclear factor erythroid 2 related factor 2) and HO-1 (Heme oxygenase 1) were from Santa Cruz Biotechnology. Anti-lipoic acid was from Millipore (Billerica, MA, USA). Anti-complexes I, II, III, IV, and V were from Sigma. SYBR green was from TaKaRa (Otsu, Shiga, Japan).

Cell culture-Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum at 37 °C in a 5% CO2 atmosphere. The cells were cultured for up to 10 generations, and the medium was changed every 2 days. For C75 treatment, cells were initially treated with 10, 50 or 100 μM C75 for 2, 6, 12, 24 and 48 h. For NAC treatment, cells were initially treated with 50 μM C75 and 10 mM NAC for 2, 6, 12, 24 and 48 h. For the LA supplement experiment, cells were pretreated with 50 μM C75 for 24 h, after which the medium was discarded and replaced with new medium containing R-LA at 20 or 100 μM for another 24 h. To further investigate whether LA would protect cells from C75-induced damage, HEK293T cells were treated with 50 μM C75 for 24 h, followed by the addition of 100 μM R-LA for another 24 h.

Transfection-The transfections were performed using Lipofectamine 2000 according to the supplier’s instructions. For the transfection of cells in 6-well plates, HEK293T cells were seeded at 6×10^4 cells per well. Lipofectamine 2000 (5 μl) was incubated in 250 μl serum-free medium for 5 min. An appropriate amount of siRNA (HsmtKAS siRNA, FASN siRNA or a combination of both) and the Lipofectamine 2000/medium were combined and incubated for another 20 min. The final mixture was added to each well. After 4-6 h, the medium in each well was exchanged with fresh H-DMEM medium. For transfection of cells in 96-well plates, HEK293T cells were seeded at 3×10^3 cells per well. An appropriate amount of siRNA, 0.5 μl Lipofectamine 2000 and 25 μl medium were combined and then applied to cells as described above. For the HsmtKAS knockdown experiment, cells were transfected with HsmtKAS siRNA for 24 h, followed by the addition of 100 μM R-LA for another 24 h. For the NAC experiment, cells were transfected with siRNA for 24 h followed by 10 mM NAC for another 24 h. For the HsmtKAS overexpression experiment, cells were transfected with HsmtKAS siRNA for 24 h, followed by the addition of 100 μM R-LA for another 24 h. For the HsmtKAS overexpression construct (pcDNA3.1-HsmtKAS) and then treated with 50 or 150 μM C75 for another 24 h.

MTT assay for cell viability-The cells were treated with different concentrations of C75 or R-LA for cell viability assays. MTT (3-[4, 5-dimethylthiazol-2-yl] -2,5-diphenyltetrazolium bromide; 0.5 mg/ml final concentration) was added for 1 h and then exchanged with DMSO overnight at room temperature. The cell viability was detected at 570 nm using a microplate spectrophotometer (Multiskan Ascent; Thermo Fisher Scientific Inc. Waltham, MA, USA).

JC-1 assay for mitochondrial membrane...
potential (MMP)- The MMP was measured with JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazoly1-carbocyanine iodide; 5 mg/ml stock concentration), a lipophilic, cationic dye that exhibit potential-dependent accumulation in mitochondria, indicated by a fluorescence mission shift from green to red. The red/green fluorescence intensity ratio reflects mitochondrial membrane potential. Cells were stained with JC-1 solution at a 1:1000 dilution for 30 min. Cells were rinsed twice with PBS after JC-1 staining and scanned with a microplate fluorometer (Fluoroskan Ascent; Thermo Fisher Scientific Inc.). The MMP was determined at an excitation wavelength of 485 nm and emission wavelengths of 538 nm and 585 nm to measure green and red JC-1 fluorescence respectively. Each well was scanned by measuring the intensity of each of 25 squares (of 1-mm² area) arranged in a 5×5 rectangular array. Data were analyzed with GraphPad Prism using semi-log concentration response analysis.

Cellular reactive oxygen species (ROS) determination-Cellular ROS were incubated with H2DCFDA for 30 min and assayed following the manufacturer’s instructions. Cellular ROS were measured at an excitation wavelength of 485 nm and an emission wavelength of 538 nm using a microplate fluorometer (Fluoroskan Ascent; Thermo Fisher Scientific Inc.). The relative H2DCFDA fluorescence was normalized to the protein concentration.

Protein carbonyl detection-Protein carbonyls were detected by western blot analysis using the Oxyblot protein oxidation detection kit (Cell Biolabs, San Diego, CA, USA) according to the manufacturer’s instructions. The carbonyl groups in the protein side chains were derivatized to DNP-hydrazone (2,4-dinitrophenylhydrazine). After the protein samples were incubated with DNPH for 15-20 min, they were subjected to western blot analysis. As a control, the same amount of protein for each sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Brilliant Blue R250. The quantification was calculated by a total densitometry of oxyblots over a total densitometry of blue-stained gels.

Quantitative real-time PCR-Total RNA was isolated using TRIzol reagent according to the manufacturer’s protocol. The RNA (1 μg) was reverse transcribed using the PrimeScript RT-PCR kit (TaKaRa, DaLian, China). The cycling conditions of quantitative real-time PCR were as follows: 50 °C for 2 min, initial denaturation at 95 °C for 10 min, and 40 cycles of 95 °C for 30 s, 55 °C for 1 min and 72 °C for 30 s. The specific primers used are as follows: HsmtKAS: CTGATGTGATGGTGGCTGGAG (forward), ACTTCTCGATAGGATCGCGGG (reverse); FASN: AGCTCGTGTTGACTTCTGCC (forward), ACTCTGGGGTCTGGTTCTCC (reverse); Nrf2: TTCAGCAGCATCCTCTCAAG (forward), GCA TGCTGTTGCTGA TACTGG (reverse); NQO1: TGGCTAGGTA TCATTCAACTC (forward), CCTTAGGGCAGGTAGATT CAG (reverse); HO-1: GCCAGCAACAAAGTGCAAGAT (forward), GGTAAGGAAGCCAGCCAAGAG (reverse); β-actin: CCACACCTTCT CAAATGAGC (forward), GGTCTCAAACAT GTCTGGG (reverse).

Western blot analysis-Cells were suspended in western blot and IP lysis buffer (Beyotime, Jiangsu, China). The lysates were incubated for 30 min on ice and then centrifuged at 13,000 g for 15 min at 4 °C. The supernatants were collected, and their protein concentrations were measured using the BCA Protein Assay Kit (Pierce 23225). The purification of nuclear and
cytoplasmic proteins is followed the manufacturer’ instructions (Beyotime, Jiangsu, China). Next, 20 μg of each protein sample was separated on 10% SDS-PAGE gel and then transferred to a pure nitrocellulose membrane (PerkinElmer Life Science, Boston, MA, USA). The membranes were blocked with 5% nonfat milk for 1 h at room temperature, washed three times with TBST for 15 min each, and finally incubated with anti-HsmtKAS, anti-lipoic acid, anti-Nrf2, anti-HO-1, anti-NQO1 (1:1000), or anti-β-actin (1:5000) antibodies at 4 ºC overnight. The membrane was then incubated with the appropriate anti-rabbit, anti-mouse or anti-goat secondary antibody at room temperature for 1 h. Chemiluminescent detection was performed using an ECL western blotting detection kit (Pierce).

Statistical analysis—The data are shown as the mean ± S.E.M. of at least three independent experiments. Statistical significance was evaluated using one-way ANOVA followed by a post hoc test to analyze differences. Statistical significance was set at p< 0.05.

RESULTS

C75 induces mitochondrial dysfunction in HEK293T cells—HEK293T cells were dose-dependently treated with C75 for 2, 6, 12, 24 or 48 h to evaluate its effects on mitochondrial function. The MMP is an essential factor for maintaining mitochondrial function and cellular viability. At 6 h, C75 at 50 or 100 μM doses concentration began to induce MMP loss, while cell viability was not affected (Fig. 1B). After 12 h, a serious mitochondrial dysfunction was observed accompanied by increased ROS overproduction (Fig. 1C) and cell viability loss (Fig. 1A). Taken together, MMP loss was assumed an early response to C75 addition. And a 50 μM C75 treatment concentration was used as a toxicity dose for the following assays.

To further determine whether ROS was the major factor to induce cell death, free radical scavenger NAC was applied in the study. As expected, NAC efficiently removed excess ROS (Fig. 1F). Meanwhile, both MMP loss (Fig. 1E) and cell viability (Fig. 1D) decrease were inhibited by NAC, suggesting that ROS was a major contributor to C75-induced cell death.

R-LA supplement ameliorates C75 toxicity—Our data showed that 100 μM R-LA treatments significantly increased cell viability as well as the MMP after C75 challenge (Fig. 2A, B). ROS overproduction induced by C75, a major cause of mitochondrial dysfunction, was also efficiently eliminated by R-LA (Fig. 3A). R-LA also significantly attenuated the protein carbonyl levels that were generated by C75 (Fig. 3B). More importantly, western blot analysis demonstrated that C75 mainly reduced mitochondrial complex I and spared other complexes. In contrast, the expression level of mitochondrial complex I recovered when R-LA was added (Fig. 3C).

R-LA normalized the phase II antioxidant enzyme system—Nrf2 is a transcription factor that binds to antioxidant response elements (ARE) and regulates the antioxidant response (20,21). It is tethered in the cytoplasm by Keap1 protein under normal or unstressed conditions (22). Through the activation process, Nrf2 can translocate into the nucleus and activates transcription of target genes known as phase II enzymes such as Heme oxygenase-1 (HO-1) (23) and NAD(P)H:quinone oxidoreductase (NQO-1) (24). As shown in Figure 4A-C, the mRNA levels of the transcription factor Nrf2, NQO1 and HO-1 were induced to a statistically significant degree after C75 treatment. And these increased levels were returned to normal after R-LA supplementation. Similar observations were also confirmed by western blot analysis (Fig. 4D). As the key regulator of phase II enzymes...
enzymes, Nrf2 nuclear translocation was increased by C75 and restored to normal level by R-LA treatment (Fig. 4E).

**HsmtKAS knockdown impairs mitochondrial function**-To clarify whether FASN or HsmtKAS plays a role in mitochondrial dysfunction, we developed specific siRNA for both FASN and HsmtKAS. As shown in Fig. 5A, FASN with specific siRNA lead to 80% knockdown of FASN without significant effect on HsmtKAS mRNA and protein contents. And HsmtKAS siRNA resulted in 70% decrease of both mRNA and protein expression without significant effects on FASN (Fig. 5B). Interestingly, FASN knockdown had no significant effects on mitochondrial function in HEK293T cells, while HsmtKAS knockdown had toxic effects on mitochondrial function, including a decrease in cell viability (Fig. 5D) and MMP (Fig. 5E) and an increase in ROS production (Fig. 5C). However, no synergetic effects were observed when both FASN and HsmtKAS were down-regulated simultaneously.

We then applied NAC to further investigate which event is the major factor in HsmtKAS-knockdown cells. 10 mM NAC efficiently scavenged overproduced ROS (Fig. 5F), recovered MMP (Fig. 5H) and cell viability (Fig. 5G). These results indicated that excess ROS induced by HsmtKAS knockdown might contribute to oxidative stress in damage and death.

**HsmtKAS knockdown and C75 treatment reduces protein lipoylation**-In addition to generating longer fatty acids, the mtFAS II pathway can produce the octanoyl-acyl carrier protein substrate for endogenous LA synthesis. To investigate the role of C75 on cellular LA synthesis, an anti-lipoic acid antibody was used in western blot analysis to detect LA binding to two key mitochondrial enzymes, the E2 subunits of the pyruvate dehydrogenase complex (PDC) and α-keto glutarate dehydrogenase (KDH). Both PDC and KDH use LA as a cofactor to form LA-PDC-E2 and LA-KDH-E2, respectively. The LA-PDC-E2 and LA-KDH-E2 were recognized based on their size that the predicted size of PDC-E2 was 63 kDa and that of KDH-E2 was 50 kDa. Our results showed that knockdown of HsmtKAS led to a decrease in protein lipoylation (Fig. 6A) that LA associated with PDC and KDH decrease to ~70%. C75 also affected protein lipoylation; obvious effects were evident after the 50 and 100 μM treatments (Fig. 6B).

**R-LA attenuates mitochondrial dysfunction induced by HsmtKAS knockdown**-Data showed that R-LA could increase mRNA level of HsmtKAS, especially at doses of 100 and 200 μM (Fig. 7A). Consistently, protein expression was also increased by R-LA treatment (Fig. 7B). Meanwhile, HsmtKAS knockdown-induced ROS overproduction was normalized by R-LA (Fig. 7C). In addition, western blot analysis revealed that the expression level of mitochondrial complex I was significantly decreased by HsmtKAS knockdown (Fig. 7D), which was similar to the results of C75 treatment. As expected, R-LA supplementation efficiently restored the expression level of complex I.

**HsmtKAS overexpression protects cells against C75-induced damage**-We developed a pcDNA3.1-HsmtKAS overexpression plasmid. After increasing the HsmtKAS expression level, MMP (Fig. 8A) and ROS (Fig. 8B) levels partially recovered. At 6 h, HsmtKAS protected cells against C75-induced MMP loss. For long-term treatment, C75 caused a more serious damage in MMP loss and ROS overproduction, while HsmtKAS provided a protective effect on mitochondrial function. Meanwhile, mitochondrial complex I was also protected by HsmtKAS overexpression in C75-injured cells (Fig. 8C). In addition, the activation of Nrf2 and...
other phase II enzymes induced by C75 was normalized through HsmtKAS overexpression (Fig. 8C). It’s interesting that C75 treatment could also decrease the protein expression of HsmtKAS (Fig. 8C), suggesting a regulation effect of C75 on protein expression besides working as an inhibitor. Moreover, overexpression of HsmtKAS didn’t show protection against higher dose C75 treatment which induced nearly 100% cell death (Fig. 8D).

DISCUSSION

The action of C75 is not attributed to a single enzyme since C75 was reported to inhibit FASN, stimulate carnitine palmitoyl-transferase-1 (CPT-1) (25), activate AMP-activated protein kinase (AMPK) (26,27), and induce peroxisome proliferator-activated receptor-α (PPARα) (28). In the present study, C75 dramatically produced excessive ROS in HEK293T cells. Similar ROS production occurred after HsmtKAS knockdown. The induced ROS might be produced by inhibition of HsmtKAS, because HsmtKAS overexpression can attenuate ROS. The role of HsmtKAS is distinguished with FASN, which is another of the most important C75 targets. HEK293T cells treated with FASN siRNA had relatively unaffected cellular ROS content. These results suggest that the excessive ROS caused by C75 was mainly due to inhibition of HsmtKAS but not FASN. This finding was consistent with down-regulation of mitochondrial acyl carrier protein in HEK293T cells, which generates excessive ROS by compromising the mtFAS II pathway (29). The release of ROS is thought to occur in the mitochondria to regulate cellular signaling and impair biological macromolecules. As the major ROS producer, mitochondria are also a vulnerable target of ROS, mitochondrial dysfunction induced ROS overproduction may further damage mitochondria to create a vicious cycle. In response to cellular oxidative stress, one of the antioxidant systems, phase II enzyme is usually activated to counteract the oxidative stress and protect cell health. In current study, the mRNA levels of phase II antioxidant enzymes were significantly increased by oxidative stress. It is well known that Nrf2 is a key regulator of phase II antioxidant enzyme expression (30,31). As shown by the results, Nrf2 nuclear translocation was significantly increased in C75-treated cells. Expression of other well-known Nrf2 targets enzymes, NQO1 and HO-1, also followed this trend.

Mitochondria are the primary source of ROS. ROS production can be ascribed to different factors, such as the expression levels of mitochondrial complex I (32,33). Mitochondrial respiratory complexes are one of the important factors that affect cellular ROS abundance. Increasing evidence suggests that the mtFAS II pathway is essential for mitochondrial respiratory function (7,34). Interestingly, one of the mtFAS II genes, 3-hydroxymyristoyl-acyl carrier protein, is a component of the bovine mitochondrial complex I (35). The role of HsmtKAS in mitochondria was further proved in the present study. MMP is one critical factor for maintaining the mitochondrial respiratory chain that is used to assess mitochondrial function. The loss of MMP is associated with cell depletion. In the present study, both C75 and HsmtKAS knockdown significantly affected MMP loss. Importantly, C75 treatment compromised the expression of respiratory complex I. A lesser effect was observed for complex II, but complexes III-V were not affected. The same result occurred when HsmtKAS was knocked down. In contrast, HsmtKAS overexpression was shown to protect mitochondria against C75-induced damage. Therefore, it appears likely that excessive ROS levels induced by C75 are released because mitochondrial complex I is compromised. A similar phenomenon was observed during RNA interference experiments on the mitochondrial acyl carrier protein, in which its knockdown
caused an approximately 60% reduction in complex I activity. The activity of complex II was decreased, but complexes III-V were not significantly altered (29). These events increase our understanding of the link between the mtFAS II genes and the respiratory chain. Defects in mtFAS II genes might mainly compromise complex I and consequently lead to excessive ROS generation and loss of MMP.

Many studies have established that deficiency in any of the yeast mtFAS II genes leads to a decrease in the endogenous LA content (7,36). Generally, endogenous LA covalently attaches to two key mitochondrial enzymes PDC-E2 and KDH-E2, which participate in the oxidative decarboxylation of α-keto acids. Down-regulation of HsmtKAS reduces protein lipoylation, which is also observed after C75 treatment, supporting our assumption that C75 could target HsmtKAS in the mtFAS II pathway. Interestingly, R-LA supplementation was able to recover mitochondrial function and eliminated the oxidative response after C75 treatment or HsmtKAS knockdown. As a redox regulator, R-LA is a well-known powerful mitochondrial antioxidant (37,38). In addition, we found that R-LA could activate HsmtKAS expression, suggesting that additional lipoic acid stimulated its own production, which is consistent with previous studies that exogenously administered lipoic acid increases lipoic acid synthase (LASY) expression (39). Likewise, C75 treatment affected HsmtKAS expression might due to its decreased R-LA content. Therefore, we assumed that the protection by R-LA might be due to its antioxidant activity, or an indirect regulation of HsmtKAS or possibly to a combination of the two mechanisms. Many enzymes have been reported involving in lipoic acid synthesis, however, the regulations of lipoic acid on those enzymes are limited and need to be further investigated.

To better understand whether HsmtKAS is the major target of C75, HsmtKAS expression was manipulated in the cells. It’s interesting to notice that additional HsmtKAS is not sensitive to C75 like the endogenous one and could protect cell survival against C75 toxicity. C75 is derivative from cerulenin, which inhibited HsmtKAS with IC50 value of 300 μM (40). In addition, C75 has been reported to inhibit purified human FASN with IC50 value of over 100 μM (41). Taken together, we assumed the main reason for non-sensitive of additional HsmtKAS to C75 might be the high efficiency of HsmtKAS overexpression together with high Km value of HsmtKAS for C75. Therefore, a high dose of C75 (150 μM) treatment was performed. As expected, C75 induced nearly 100% cell death, which was not prevented by HsmtKAS. Anyway, lack of direct evidence on Km value of HsmtKAS for C75 is a limitation in this study, which requires investigation further.

With the experimental design and methodology used in the present study, we observed that C75 has an adverse effect on mitochondrial function, compromising mitochondrial complex I and protein lipoylation and generating excessive ROS. R-LA supplementation plays a protective role against C75-induced damage. Regardless, these data reveal that HsmtKAS is a new potential target of C75 and a novel regulator of oxidative stress. More attention should be focused on mtFAS II genes during the identification and design of antitumor drugs, especially FASN inhibitors. A combination of R-LA and C75 may provide promising prospects for therapeutic treatments.

COMPETING INTERESTS
All of the authors declare that they have no competing interests.
REFERENCES

1. Heath, R. J., and Rock, C. O. (2004) Fatty acid biosynthesis as a target for novel antibacterials. *Curr Opin Investig Drugs* 5, 146-153

2. Flavin, R., Peluso, S., Nguyen, P. L., and Loda, M. (2010) Fatty acid synthase as a potential therapeutic target in cancer. *Future Oncol* 6, 551-562

3. Yoshii, Y., Furukawa, T., Oyama, N., Hasegawa, Y., Kiyono, Y., Nishii, R., Waki, A., Tsuji, A. B., Sogawa, C., Wakisaka, H., Fukumura, T., Yoshii, H., Fujibayashi, Y., Lewis, J. S., and Saga, T. (2013) Fatty Acid synthase is a key target in multiple essential tumor functions of prostate cancer: uptake of radiolabeled acetate as a predictor of the targeted therapy outcome. *PLoS One* 8, e64570

4. Berndt, J., Kovacs, P., Ruschke, K., Kloting, N., Fasshauer, M., Schon, M. R., Korner, A., Stumvoll, M., and Bluher, M. (2007) Fatty acid synthase gene expression in human adipose tissue: association with obesity and type 2 diabetes. *Diabetologia* 50, 1472-1480

5. Puig, T., Vazquez-Martin, A., Relat, J., Petriz, J., Menendez, J. A., Porta, R., Casals, G., Marrero, P. F., Haro, D., Brunet, J., and Colomer, R. (2008) Fatty acid metabolism in breast cancer cells: differential inhibitory effects of epigallocatechin gallate (EGCG) and C75. *Breast Cancer Res Treat* 109, 471-479

6. Alli, P. M., Pinn, M. L., Jaffee, E. M., McFadden, J. M., and Kuhajda, F. P. (2005) Fatty acid synthase inhibitors are chemopreventive for mammary cancer in neu-N transgenic mice. *Oncogene* 24, 39-46

7. Hiltunen, J. K., Autio, K. J., Schonauer, M. S., Kursu, V. A., Dieckmann, C. L., and Kastaniotis, A. J. (2010) Mitochondrial fatty acid synthesis and respiration. *Biochim Biophys Acta* 1797, 1195-1202

8. Hiltunen, J. K., Chen, Z., Haapalainen, A. M., Wierenga, R. K., and Kastaniotis, A. J. (2010) Mitochondrial fatty acid synthesis--an adopted set of enzymes making a pathway of major importance for the cellular metabolism. *Prog Lipid Res* 49, 27-45

9. Cronan, J. E., Fearnley, I. M., and Walker, J. E. (2005) Mammalian mitochondria contain a soluble acyl carrier protein. *FEBS Lett* 579, 4892-4896

10. Jordan, S. W., and Cronan, J. E., Jr. (1997) A new metabolic link. The acyl carrier protein of lipid synthesis donates lipoic acid to the pyruvate dehydrogenase complex in Escherichia coli and mitochondria. *J Biol Chem* 272, 17903-17906

11. Sulo, P., and Martin, N. C. (1993) Isolation and characterization of LIP5. A lipoate biosynthetic locus of Saccharomyces cerevisiae. *J Biol Chem* 268, 17634-17639

12. Schonauer, M. S., Kastaniotis, A. J., Hiltunen, J. K., and Dieckmann, C. L. (2008) Intersection of RNA processing and the type II fatty acid synthesis pathway in yeast mitochondria. *Mol Cell Biol* 28, 6646-6657

13. Hiltunen, J. K., Schonauer, M. S., Autio, K. J., Mittelmeier, T. M., Kastaniotis, A. J., and Dieckmann, C. L. (2009) Mitochondrial fatty acid synthesis type II: more than just fatty acids. *J Biol Chem* 284, 9011-9015

14. Cicchillo, R. M., Iwig, D. F., Jones, A. D., Nesbitt, N. M., Baleanu-Gogonea, C., Souder, M. G., Tu, L., and Booker, S. J. (2004) Lipoyl synthase requires two equivalents of S-adenosyl-L-methionine to synthesize one equivalent of lipoic acid. *Biochemistry* 43, 6378-6386

15. Nesbitt, N. M., Baleanu-Gogonea, C., Cicchillo, R. M., Goodson, K., Iwig, D. F., Broadwater, J. A., Haas, J. A., Fox, B. G., and Booker, S. J. (2005) Expression, purification, and physical characterization of Escherichia coli lipooyl(octanoyl)transferase. *Protein Expr Purif* 39, 269-282
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16. Liu, J., Shen, W., Zhao, B., Wang, Y., Wertz, K., Weber, P., and Zhang, P. (2009) Targeting mitochondrial biogenesis for preventing and treating insulin resistance in diabetes and obesity: Hope from natural mitochondrial nutrients. Adv Drug Deliv Rev 61, 1343-1352

17. Smith, S., Witkowski, A., Moghul, A., Yoshinaga, Y., Nefedov, M., de Jong, P., Feng, D., Fong, L., Tu, Y., Hu, Y., Young, S. G., Pham, T., Cheung, C., Katzman, S. M., Brand, M. D., Quinlan, C. L., Fens, M., Kuypers, F., Misquitta, S., Griffey, S. M., Tran, S., Gharib, A., Knudsen, J., Hannibal-Bach, H. K., Wang, G., Larkin, S., Thweatt, J., and Pasta, S. (2012) Compromised mitochondrial fatty acid synthesis in transgenic mice results in defective protein lipoylation and energy disequilibrium. PLoS One 7, e47196

18. Chen, Z., Leskinen, H., Liimatta, E., Sormunen, R. T., Miinalainen, I. J., Hassinen, I. E., and Hiltunen, J. K. (2009) Myocardial overexpression of Mecr, a gene of mitochondrial FAS II leads to cardiac dysfunction in mouse. PLoS One 4, e5589

19. Christensen, C. E., Kragelund, B. B., von Wettstein-Knowles, P., and Henriksen, A. (2007) Structure of the human beta-ketoacyl [ACP] synthase from the mitochondrial type II fatty acid synthase. Protein Sci 16, 261-272

20. Li, W., and Kong, A. N. (2009) Molecular mechanisms of Nrf2-mediated antioxidant response. Molecular carcinogenesis 48, 91-104

21. Nguyen, T., Nioi, P., and Pickett, C. B. (2009) The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress. The Journal of biological chemistry 284, 13291-13295

22. Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., Igarashi, K., Engel, J. D., and Yamamoto, M. (1999) Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. Genes & development 13, 76-86

23. Alam, J., Stewart, D., Touchard, C., Boinapally, S., Choi, A. M., and Cook, J. L. (1999) Nrf2, a Cap'n'Collar transcription factor, regulates induction of the heme oxygenase-1 gene. The Journal of biological chemistry 274, 26071-26078

24. Venugopal, R., and Jaiswal, A. K. (1996) Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H:quinone oxidoreductase1 gene. Proceedings of the National Academy of Sciences of the United States of America 93, 14960-14965

25. Bentebibel, A., Sebastian, D., Herrero, L., Lopez-Vinas, E., Serra, D., Asins, G., Gomez-Puertas, P., and Hegardt, F. G. (2006) Novel effect of C75 on carnitine palmitoyltransferase I activity and palmitate oxidation. Biochemistry 45, 4339-4350

26. Landree, L. E., Hanlon, A. L., Strong, D. W., Rumbaugh, G., Miller, I. M., Thupari, J. N., Connolly, E. C., Huganir, R. L., Richardson, C., Witters, L. A., Kuhajda, F. P., and Ronnett, G. V. (2004) C75, a fatty acid synthase inhibitor, modulates AMP-activated protein kinase to alter neuronal energy metabolism. J Biol Chem 279, 3817-3827

27. Kim, E. K., Miller, I., Aja, S., Landree, L. E., Pinn, M., McFadden, J., Kuhajda, F. P., Moran, T. H., and Ronnett, G. V. (2004) C75, a fatty acid synthase inhibitor, reduces food intake via hypothalamic AMP-activated protein kinase. J Biol Chem 279, 19970-19976

28. Huang, H., McIntosh, A. L., Martin, G. G., Petrescu, A. D., Landrock, K. K., Landrock, D., Kier, A. B., and Schroeder, F. (2013) Inhibitors of Fatty Acid Synthesis Induce PPAR alpha -Regulated Fatty Acid beta -Oxidative Genes: Synergistic Roles of L-FABP and Glucose. PPAR Res 2013, 865604
Feng, D., Witkowski, A., and Smith, S. (2009) Down-regulation of mitochondrial acyl carrier protein in mammalian cells compromises protein lipoylation and respiratory complex I and results in cell death. *J Biol Chem* **284**, 11436-11445

Jung, K. A., and Kwak, M. K. (2010) The Nrf2 system as a potential target for the development of indirect antioxidants. *Molecules* **15**, 7266-7291

Florczyk, U., Loboda, A., Stachurska, A., Jozkowicz, A., and Dulak, J. (2010) [Role of Nrf2 transcription factor in cellular response to oxidative stress]. *Postepy Biochem* **56**, 147-145

Kushnareva, Y., Murphy, A. N., and Andreyev, A. (2002) Complex I-mediated reactive oxygen species generation: modulation by cytochrome c and NAD(P)+ oxidation-reduction state. *Biochem J* **368**, 545-553

He, Y., Leung, K. W., Zhang, Y. H., Duan, S., Zhong, X. F., Jiang, R. Z., Peng, Z., Tombran-Tink, J., and Ge, J. (2008) Mitochondrial complex I defect induces ROS release and degeneration in trabecular meshwork cells of POAG patients: protection by antioxidants. *Invest Ophthalmol Vis Sci* **49**, 1447-1458

Kastaniotis, A. J., Autio, K. J., Sormunen, R. T., and Hiltunen, J. K. (2004) Htd2p/Yhr067p is a yeast 3-hydroxyacyl-ACP dehydratase essential for mitochondrial function and morphology. *Mol Microbiol* **53**, 1407-1421

Carroll, J., Fearnley, I. M., Shannon, R. J., Hirst, J., and Walker, J. E. (2003) Analysis of the subunit composition of complex I from bovine heart mitochondria. *Mol Cell Proteomics* **2**, 117-126

Brody, S., Oh, C., Hoja, U., and Schweizer, E. (1997) Mitochondrial acyl carrier protein is involved in lipoic acid synthesis in Saccharomyces cerevisiae. *FEBS Lett* **408**, 217-220

Packer, L., Roy, S., and Sen, C. K. (1997) Alpha-lipoic acid: a metabolic antioxidant and potential redox modulator of transcription. *Adv Pharmacol* **38**, 79-101

Moini, H., Packer, L., and Saris, N. E. (2002) Antioxidant and prooxidant activities of alpha-lipoic acid and dihydrolipoic acid. *Toxicol Appl Pharmacol* **182**, 84-90

Padmalayam, I., Hasham, S., Saxena, U., and Pillarissetti, S. (2009) Lipoic acid synthase (LASY): a novel role in inflammation, mitochondrial function, and insulin resistance. *Diabetes* **58**, 600-608

Zhang, L., Joshi, A. K., Hofmann, J., Schweizer, E., and Smith, S. (2005) Cloning, expression, and characterization of the human mitochondrial beta-ketoacyl synthase. Complementation of the yeast CEM1 knock-out strain. *J Biol Chem* **280**, 12422-12429

Wu, M., Singh, S. B., Wang, J., Chung, C. C., Salituro, G., Karanam, B. V., Lee, S. H., Powles, M., Ellsworth, K. P., Lassman, M. E., Miller, C., Myers, R. W., Tota, M. R., Zhang, B. B., and Li, C. (2011) Antidiabetic and antisteatotic effects of the selective fatty acid synthase (FAS) inhibitor platensimycin in mouse models of diabetes. *Proc Natl Acad Sci U S A* **108**, 5378-5383
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FOOTNOTES
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FIGURE LEGENDS

FIGURE 1. C75 induces mitochondrial dysfunction in HEK293 cells. HEK293T cells cultured in 96-well plates were treated with 10, 50 or 100 μM C75 for 2, 6, 12, 24 or 48 h, and then cell viability was analyzed by MTT assay (A). The MMP was determined using JC-1 staining (B). Reactive oxygen species (ROS) content was measured using H2DCFDA staining (C). Cells were treated with 50 μM C75 and 10 mM NAC for 2, 6, 12, 24 48 h, and then were examined by cell viability (D), MMP level (E) and ROS content (F). Values are the means ± S.E.M. of at least three independent experiments. *P< 0.05, **P< 0.01 relative to the control.

FIGURE 2. Effects of R-LA on C75 toxicity. HEK293T cells were treated with 50 μM C75 for 24 h and then exposed to 20 or 100 μM R-LA for another 24 h. A. Cell viability was analyzed by MTT assay. B. The MMP was measured by JC-1 staining. Values are the means ± S.E.M. of at least three independent experiments. *P<0.05 relative to the control.

FIGURE 3. Effects of R-LA on C75-induced mitochondrial dysfunction. HEK293T cells were treated with 50 μM C75 for 24 h and then exposed to 100 μM R-LA for another 24 h. A. The ROS level was analyzed by H2DCFDA staining. B. Protein carbonyl levels were detected by western blotting (left panel), and total protein was used as a loading control. The quantitation of the bands is shown (right panel). C. Protein expression levels of mitochondrial complex subunits were measured by western blotting (left panel), and quantitation of the bands is shown (right panel). The predicted protein size has been marked in the blot. Values are the means ± S.E.M. of at least three independent experiments. *P<0.05, **P<0.01 relative to the control.

FIGURE 4. R-LA normalized the phase II antioxidant enzyme system. HEK293T cells were treated with 50 μM C75 for 24 h and then exposed to 100 μM R-LA for another 24 h. A-C. The mRNA levels of Nrf2, NQO1 and HO-1 were examined by real-time PCR. D. The protein expressions of NQO1 and HO-1 were examined by western blotting (upper panel) with quantitative analysis (lower panel). E. The protein expressions of cytoplasmic and nuclear Nrf2 were examined by western blotting (upper panel) with quantitative analysis (lower panel). The predicted protein size has been marked in the blot. Values are the means ± S.E.M. of at least three independent experiments. *P<0.05, **P<0.01 relative to the control.

FIGURE 5. HsmtKAS knockdown impairs mitochondrial function. HEK293T cells were transfected with FASN siRNA, the efficiency of FASN and HsmtKAS knockdown was evaluated
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by real-time PCR and western-blot analysis (A). Cells were transfected with HsmtKAS siRNA, the efficiency of FASN and HsmtKAS knockdown was examined (B). Reactive oxygen species (ROS) content was measured using H2DCFDA staining (C). Cell viability was analyzed by MTT assay (D). The MMP was determined using JC-1 staining (E). After transfected with HsmtKAS siRNA for 24 h, cells were incubated with 10 mM NAC for another 24 h and then were examined for ROS content (F), cell viability (G), and MMP level (H). The predicted protein size has been marked in the blot. Values are the means ± S.E.M. of at least three independent experiments. *P<0.05, **P<0.01 relative to the control.

FIGURE 6. Effects of HsmtKAS knockdown and C75 treatment on protein lipoylation. Western blot analysis was used to detect lipoic acid that was bound to the E2 subunit of PDC (upper panel) or KDH (lower panel) using a polyclonal anti-lipoic acid rabbit antibody. A. The levels of protein lipoylation were detected in cells transfected with HsmtKAS siRNA for 48 h. B. HEK293T cells were treated with 0, 10, 50 or 100 μM C75 for 24 h, and the cellular protein lipoylation levels were determined. The quantitation of bands was calculated and the predicted protein size has been marked in the blot. Values are the means ± S.E.M. of at least three independent experiments. *P<0.05, **P<0.01 relative to the control.

FIGURE 7. Effects of R-LA on HsmtKAS knockdown-induced mitochondrial dysfunction. HEK293T cells were treated with 0, 5, 10, 50, 100 or 200 μM R-LA, the mRNA levels (A) and protein expressions (B) of HsmtKAS were determined. HEK293T cells were transfected with HsmtKAS siRNA for 24 h and exposed to 100 μM R-LA for another 24 h. The ROS content was analyzed (C), and the protein expression levels of mitochondrial complex subunits were measured by western blotting (D). Quantitation of the bands is shown and the predicted protein size has been marked in the blot. Values are the means ± S.E.M. from at least three independent experiments. *P<0.05, **P<0.01 relative to the control.

FIGURE 8. HsmtKAS overexpression protected cells against low dose of C75-induced damage. HEK293T cells were transfected with pcDNA3.1-HsmtKAS for 24 h and then exposed to 50 μM C75 for another 2, 6, 12, 24 and 48 h. A. The MMP was analyzed by JC-1 staining. B. ROS generation was analyzed using H2DCFDA staining. C. Protein expression levels of mitochondrial complex I, Nrf2, NQO1 and HO-1 were measured by western blotting (upper panel), and quantification of the bands is shown (lower panel). D. Cell viability of C75 with 50 and 150 μM concentration treated HsmtKAS-overexpressed cells. The predicted protein size has been marked in the blot. Values are the means ± S.E.M. of at least three independent experiments. *P<0.05, **P<0.01 relative to the control.
Figure 1

A

Cell viability (%)

B

Relative MMP (%)

C

Relative ROS level (%)

D

Cell viability (%)

E

Relative MMP (%)

F

Relative ROS level (%)

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Figure 2

A

Cell Viability (%)  

50 μM C75  -  +  +  +
R-LA (μM)  -  -  20  100

B

Relative MMP (%)  

50 μM C75  -  +  +  +
R-LA (μM)  -  -  20  100
Figure 3
Figure 4
Figure 5
Figure 6

A

LA-PDC-E2 (63 kDa)
LA-KDH-E2 (60 kDa)
HmtKAS (46 kDa)
Beta-actin (46 kDa)

C    HmtKAS sRNA

Relative protein expression (%)

LA-PDC-E2  LA-KDH-E2  HmtKAS

B

LA-PDC-E2 (63 kDa)
LA-KDH-E2 (60 kDa)
Beta-actin (46 kDa)

C75  0  10  50  100 (μM)

Relative protein expression (%)

LA-PDC-E2  LA-KDH-E2

Control  10 μM  50 μM  100 μM
Figure 7
C75, An inhibitor of fatty acid synthase, suppresses the mitochondrial fatty acid synthesis pathway and impairs mitochondrial function

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