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RESEARCH ARTICLE

Inhibitory effects of thioethers on fatty acid synthase and 3T3-L1 cells

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Abstract

Thioethers are the main flavor compounds found in Liliaceae *Allium* vegetables and have been shown to have beneficial effects against several diseases correlated with metabolic syndrome. The inhibitory effects of six thioethers on fatty acid synthase (FAS) were investigated. Dose-dependent and time-dependent inhibitions of FAS by one trisulfide and two disulfides were revealed. Diallyl trisulfide (DATS, IC_{50} = 8.37 μM) was the most active of these thioethers. Inhibition kinetics, substrate protection analysis, and stoichiometric assay revealed that DATS interacted with both essential sulfhydryl groups on the acyl-carrier protein and β-ketoacyl synthase domain of FAS to inactivate the enzyme. The inactivation by DATS represented affinity-labeling kinetics. The active thioethers also inhibited the differentiation and lipid accumulation of 3T3-L1 preadipocytes, and the effect was related to their inhibition of FAS. It is suggested that the inhibition on FAS by thioethers and *Allium* vegetables is an important factor for their effects against metabolic syndrome.

Keywords: *Fatty acid synthase; thioether; diallyl trisulfide; 3T3-L1 cells; metabolic syndrome*

Introduction

The animal fatty acid synthase (FAS, EC 2.3.1.85) is an important enzyme in energy metabolism *in vivo*. It catalyzes the *de novo* synthesis of fatty acid from acetyl-coenzyme A (CoA) and malonyl-CoA in the presence of NADPH (reduced nicotinamide adenine dinucleotide phosphate) through the sequential functions of its seven active sites[1]. Recently, it has been shown that FAS function is related to many human diseases such as obesity and cancer. Human cancer cells express high levels of FAS, and inhibitors of FAS such as cerulenin, C75, and EGCG (epigallocatechin gallate) are known to have inhibitory effects on some human cancers[2],[3]. It was reported at the beginning of the 21st century that mice treated with FAS inhibitors exhibited dramatic decreases in food intake and body weight[4]. Therefore, FAS is attracting more and more attention worldwide for its association with human diseases, and it might be a potential therapeutic target for these diseases[5]. Our laboratory has previously reported that some natural plants can inhibit FAS activity, such as galangal[6], tea[7], and parasitic loranthus[8]. However, safer and more potent inhibitors are worth researching.

Thioethers are the main flavor component of Liliaceae *Allium* plants such as garlic and onion[9]. DATS (diallyl trisulfide), commonly known as allitridin, is the most attractive thioether as a potential therapeutic molecule. It has been reported that many *Allium* plants and thioethers are preventive or therapeutic to various cancers and other diseases[10–13]. However, the mechanisms of these effects are not yet entirely clear.

In the present work, we report that diallyl trisulfide and several disulfides exhibited dose-dependent and time-dependent inhibition on FAS. The inhibition kinetics and mechanisms were also studied. Furthermore, the inhibition of 3T3-L1 preadipocytes by DATS and several disulfides was investigated. The results provide valuable information for understanding the health protective mechanisms of DATS and *Allium* plants.

Materials and methods

Materials

Diallyl trisulfide was obtained from the National Institute for the Control of Pharmaceutical and Biological Products,

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China. Other thioethers were obtained from RunLong Flavors and Fragrances Co., Ltd. (TengZhou, ShanDong, China). The sulfides were all dissolved in dimethylsulfoxide (DMSO). DMSO, dithiothreitol (DTT), acetyl-CoA, malonyl-CoA, NADPH, 5-(iodoacetamido)fluorescein (5-IAF), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), 3-isobutyl-1--methylxanthine, insulin, dexamethasone, and oil red O (ORO) were purchased from Sigma. Dulbecco's modified Eagle's medium and fetal bovine serum were pruchased from Gibco BRL. All other reagents were local products of analytical grade.

FAS preparation and activity assays

The preparation, storage, and use of FAS isolated from chicken liver were performed as described previously[14]. The final purified enzyme was determined to be homogeneous by polyacrylamide gel electrophoresis (PAGE) analysis in the presence and absence of SDS (sodium dodecyl sulfate). FAS activity was determined by monitoring the decrease in absorbence at 340 nm resulting from the oxidation of NADPH using an Amersham Pharmacia Ultrospec 4300 pro UV-Vis spectrophotometer at 37°C. The assay mixture contained 100 mM potassium phosphate buffer, pH 7.0, 1 mM EDTA (ethylenediaminetetraacetic acid), 2.5 μM acetyl-CoA, 10 μM malonyl-CoA, 32 μM NADPH, and 10 μg chicken liver FAS in a total volume of 2.0 mL[15].

FAS inhibition assays

Fast inhibition was determined by adding thioether to the reaction system before FAS initiated the reaction. The initial velocity was measured to calculate the remaining activity (RA) of FAS, and each assay was repeated three times. The concentrations of thioether required for 50% inhibition (IC_{50}) were obtained from the dose–response curves of inhibition.

The time course of time-dependent inhibition was determined by taking aliquots to measure the remaining activity at the indicated time points after the enzyme solution was mixed and incubated with the thioether. Usually, this is an irreversible inactivation and is induced by chemical reaction. The observed first order rate constant (k_{obs}) was obtained from the slope of the plot of ln RA versus time.

The inactivation rate of FAS by higher concentrations of DATS is too fast to enable the rate constant to be determined exactly by the above method, so the following measurement was used. DATS was mixed with FAS activity assay solution before initiating the reaction by FAS. A continuous monitoring assay for the FAS catalyzing reaction was conducted. Due to the FAS activity being time-dependently inhibited by DATS, the assay line should be deflexed. The maximum product concentration was named as P_∞. If P_∞ was in the range of the initial velocity of the same activity assay reaction without DATS, the deflexion of the assay line would result absolutely from the inactivation of FAS by DATS. Tsou educed the product–time expression of this reaction:

$$P = P_\infty(1 - e^{-AYt}) \quad (1)$$

in which Y is the inhibitor concentration and A is the apparent second order rate constant for inactivation of the enzyme by the inhibitor[16]. The logarithmic expression of equation (1) is:

$$\ln(P_\infty - P) = \ln P_\infty - AYt \quad (2)$$

The rate constant A can be calculated from the slope of the plot of ln (P_∞ – P) vs. time. For a reaction such as E + I → EI, the constant is a real second order rate constant. If the reaction of the inhibitor with the enzyme is affinity-labeling such as E + I⇔E•I → EI, in which E•I is a non-covalent dissociable complex and E•I → EI is an irreversible chemical reaction, the reaction should follow saturated kinetics. Thus, A would decrease with an increase of inhibitor concentration, and the plot of 1/A versus Y should be a straight line with a positive slope[16].

SDS-PAGE studies

DTT and 5-IAF (a fluorescently labeled thiol-reagent) were added to the enzyme mixtures after 2 h of inactivation by DATS. Following another 2 h of incubation, the samples were subjected to SDS-PAGE. The acrylamide concentrations were 5% for the stacking gel and 6% for the separating gel.

Stoichiometric assay of essential target groups

The number of essential DATS target groups on FAS was determined by the graphical method reported elsewhere[17]. The following equation was used:

$$a^{1/i} = 1 - m/n \quad (3)$$

where a is the remaining FAS activity, n is the total number of sulfhydryl groups, m is the total number of modified groups, and the number of essential target groups is assumed as i. Values of a were measured over a range of DATS concentrations. Curves of $a^{1/i}$ versus m could be obtained when i was endowed with a simple natural number different from 1. The value of i that resulted in a linear relationship between $a^{1/i}$ and m was the number of essential groups on FAS that were modified by DATS.

Cell culture and ORO staining

3T3-L1 preadipocytes were obtained from Servier Laboratories (France) and were used at passage 8–15. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C in 5% CO_2. The medium was changed every 2 days. Two days after reaching confluence, adipogenic agents (0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 1.7 μM insulin) were added to the medium to induce differentiation (day 0). Following 2 days of treatment (day 2), cells were cultured within 1.7 μM insulin for another 2 days. Thereafter (day 4), the medium was changed to normal culture medium.

Thioethers were added at the beginning of the differentiation process (day 0) and whenever a medium change was performed.

Intracellular lipid accumulation was determined by ORO staining at day 8. Cells were washed twice with phosphate-buffered saline and were stained with 0.3% (w/v) ORO solution in 60% (v/v) isopropanol for 1 h. After removing excess stain by washing exhaustively, stained oil droplets in isopropanol were measured by spectrophotometry at 520 nm.

Results

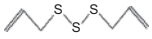
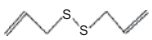
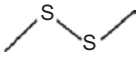
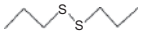
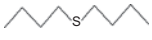
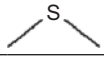
We examined the fast inhibitory effects of six thioethers on the enzymatic activity of animal FAS (structures shown in Table 1). The diallyl trisulfide (DATS), diallyl disulfide, and dimethyl disulfide exhibited a dose-dependent inhibitory effect on FAS. A plot of inhibition by DATS is shown in Figure 1A, and the IC_{50} values of these thioethers are listed in Table 1. The inhibition of dipropyl disulfide, dibutyl sulfide, and dimethyl sulfide was not detected.

In addition, besides DATS, three thioethers also exhibited time-dependent inhibition of FAS. Figure 1B shows the time course of the inhibition, and Table 1 lists the $k_{obs}/[I]$ values of these sulfides. As time-dependent inhibition is generally considered to be irreversible, dialysis was performed to explore the reversibility of the inhibition. The results showed that the activity of FAS treated with 13.28 μM DATS did not recover after 3 h of dialysis, whereas the FAS control retained 90% activity.

In subsequent experiments, DATS was used as a representative thioether to elucidate the mechanism of FAS inhibition by thioethers. Different concentrations of DTT were added to FAS solutions that had been inactivated by moderate levels of DATS, and the activity of the enzyme was continuously assayed for 90 min. It was observed that the activity of FAS gradually recovered over time following the addition of DTT (Figure 2A).

Furthermore, FAS cross-linking was detected in the presence of DATS, and treatment with DTT caused disassembly. In SDS-PAGE analysis, the density of the FAS cross-linking band increased with increasing DATS concentration (Figure 2B). We also observed that fluorescence emitted by the binding of 5-IAF to FAS weakened as the DATS concentration increased (Figure 2C). This confirmed that DATS

Table 1. IC_{50} values and rate constants of sulfides on FAS.

Sulfide	Structure	IC_{50} (μM)	IC_{50} (μg/mL)	$k_{obs}/[I]$ ($min^{-1}\,mM^{-1}$)
Diallyl trisulfide		8.37	1.49	12.5
Diallyl disulfide		145	21.2	1.93
Dimethyl disulfide		2213	208	0.0182
Dipropyl disulfide		—	—	0.00183
Dibutyl sulfide		—	—	—
Dimethyl sulfide		—	—	—

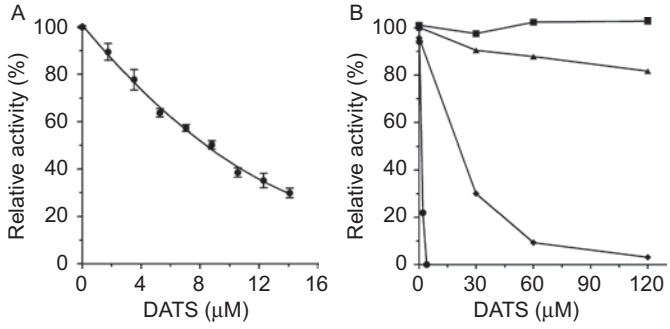


Figure 1. Inhibition of fatty acid synthase (FAS) activity by thioethers. (A) Dose-dependent inhibition of FAS activity by diallyl trisulfide (DATS). Data are represented as mean ± SD ($n = 3$). (B) Time-dependent inhibition of FAS activity by 0.53 mM diallyl disulfide (●), 1.54 mM dimethyl disulfide (◆), 0.87 mM dipropyl disulfide (▲), and 1.85 mM dimethyl sulfide (■). The concentration of FAS in the inactivation system was 0.82 μM.

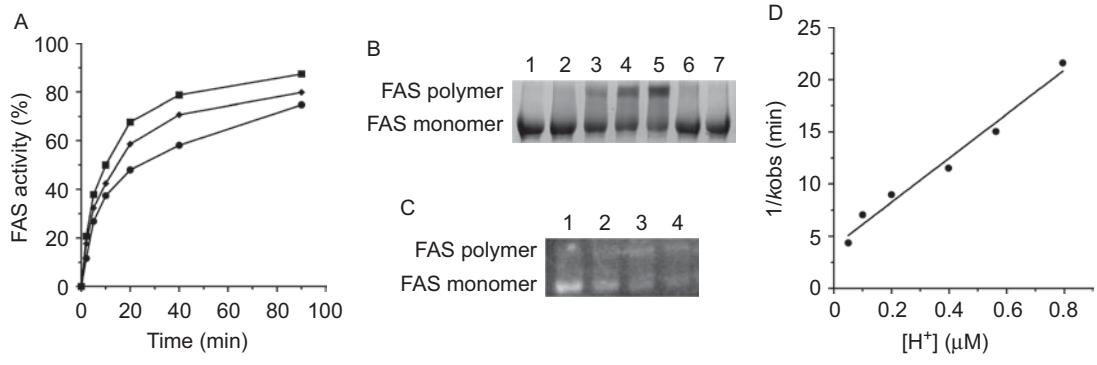


Figure 2. Inactivation of FAS by DATS in external influence on sulfhydryl groups. (A) Dithiothreitol (DTT) reactivation of DATS-inactivated FAS. Reactions contained 1.23 μM FAS and 17.03 μM DATS. The concentrations of DTT in the mixtures were: 10 mM (●), 20 mM (◆), 40 mM (■). (B) Cross-linking of DATS-modified FAS analyzed by SDS-PAGE. The samples contained 1.11 μM FAS. Following 2 h incubation of FAS with DATS, DTT was added and the enzyme mixtures were incubated for 2 h more. (1) 1 mM DTT. (2) FAS control. (3) 17.6 μM DATS. (4) 35.2 μM DATS. (5) 52.8 μM DATS. (6) 52.8 μM DATS and 1 mM DTT. (7) 52.8 μM DATS and 2 mM DTT. (C) Gel image of the fluorescence of 5-(iodoacetamido)fluorescein (5-IAF)-tagged FAS. The reactions contained 1.11 μM FAS and half-saturated concentrations of 5-IAF. Following 2 h incubation of FAS with DATS, 5-IAF was added to the enzyme mixtures and they were incubated for 2 h more. (1) FAS control. (2) 17.6 μM DATS. (3) 35.2 μM DATS. (4) 52.8 μM DATS. (D) Dependence of the apparent first order rate constant of inactivation on pH. The reaction contained 0.62 μM FAS and 20 μM DATS.

reacted with the sulfhydryl groups on FAS, and that thiol–disulfide exchange had occurred.

In order to ascertain whether the DATS-reacted sulfhydryl groups were dissociated or not, the effect of pH on FAS inactivation was assessed by measuring the observed first order rate constant k_{obs}. The inactivations of FAS by DATS at different pH values of 6.0–7.5 were assayed. It was showed that k_{obs} increased with a decrease of the concentration of H^+, and the plot of $1/k_{obs}$ versus $[H^+]$ was linear (Figure 2D). The result demonstrated that DATS reacted with the dissociated sulfhydryl group. The reaction agrees with the equation:

$$1/k_{obs} = 1/k + [H^+]/kK \quad (4)$$

in which k is the real rate constant of reaction of the inhibitor with the enzyme and K is the dissociation constant of the sulfhydryl groups in FAS. K can be calculated from the y-axis intercept versus slope of the plot of $1/k_{obs}$ versus $[H^+]$. The values of K and pK were 1.91×10^{-7} M and 6.72, respectively.

To our knowledge there are two essential sulfhydryl groups on FAS, the sulfhydryl group of cysteine on the β-ketoacyl synthase (KS) domain and the sulfhydryl group on the phosphopantetheine linked to the acyl carrier protein (ACP) domain. In order to identify the number of essential sulfhydryl groups that are targeted by DATS, we used the graphical method of Tsou[17]. First, we examined the activities of FAS remaining after 30 min of inactivation by increasing the concentration of either DATS or DTNB (a classical SH reagent). The graphical results demonstrated that DATS acted on two essential sulfhydryl groups (Figure 3A). In contrast, DTNB only reacted with one essential SH (Figure 3B), which is accordance with previously reported results[15].

The inhibition kinetics of DATS on FAS with each substrate was examined. The Lineweaver–Burk plot shows that DATS inhibited FAS competitively against acetyl-CoA (Figure 4A). DATS exhibited noncompetitive inhibition against NADPH (Figure 4B). For malonyl-CoA, DATS appeared to be a mixed type of inhibitor (Figure 4C).

Furthermore, substrate protection for time-dependent inhibition was examined in the measurement of FAS inactivation by DATS with 10 μM acetyl-CoA, 10 μM malonyl-CoA, or 10 μM NADPH, respectively. Inactivation in the same condition but without any substrate was the control. The apparent first order rate constant k_{obs} was 0.025 min^{-1}, 0.037 min^{-1}, 0.063 min^{-1}, and 0.070 min^{-1}, respectively. Comparison of the constants showed that acetyl-CoA and malonyl-CoA could protect FAS against inactivation by DATS, whereas NADPH had no protection. Further analysis of acetyl-CoA and malonyl-CoA protection was performed by premixing FAS with different concentrations of substrate prior to the addition of DATS. The k_{obs} decreased as the concentration of substrate increased. A linear relationship was obtained from a plot of $1/k_{obs}$ versus acetyl-CoA concentration (Figure 4D), showing that acetyl-CoA can competitively protect FAS against DATS.

The continuous monitoring assay for FAS activity in the presence of a series of DATS concentrations was conducted. Figure 5A displays these assay curves of product versus time, and Figure 5B shows plots according to Equation (2). A series of A values, the apparent second order rate constants for different concentrations of DATS, were calculated from the slopes of these plots. It was found that the constants decreased with an increase of DATS concentration, and the plot of 1/A versus [I] is a straight line with a positive slope

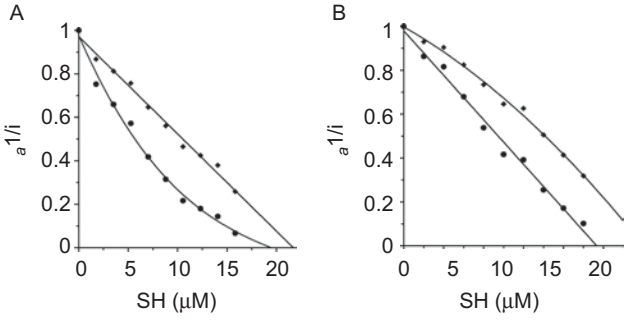


Figure 3. Stoichiometry plots of sulfhydryl modifiers on FAS activity. The concentration of FAS in the inactivation system was 0.82 μM. i = 1 (●), i = 2 (◆). (i values were set to reflect the number of essential groups targeted) (A) DATS concentrations were varied from 0 to 15.82 μM. (B) 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) concentrations were varied from 0 to 9.0 μM.

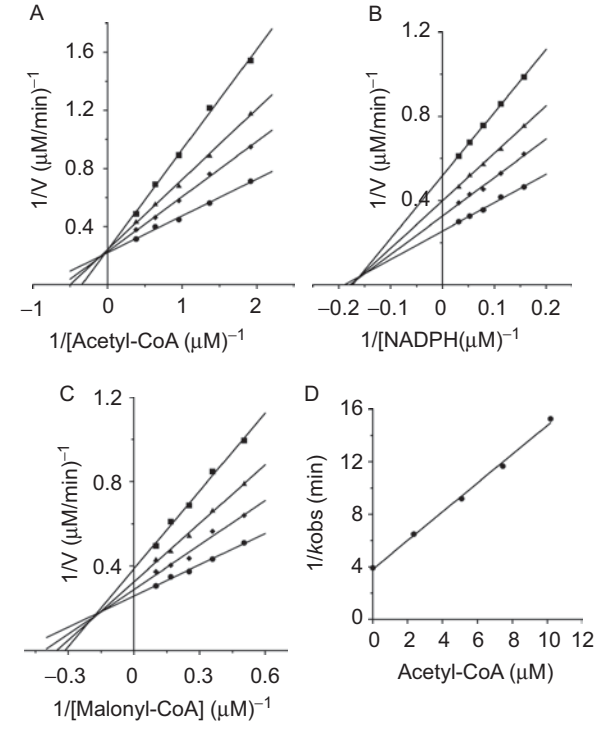


Figure 4. Inhibition kinetics plots of FAS by DATS. (A) Double-reciprocal plots for inhibition of the reaction activity of FAS by DATS when acetyl-CoA concentration was the variable. DATS concentrations were 0 μM (●); 1.76 μM (◆); 3.52 μM (▲); 5.28 μM (■). (B) Double-reciprocal plots for inhibition of the reaction activity of FAS by DATS when NADPH concentration was the variable. DATS concentrations were 0 μM (●); 2.64 μM (◆); 5.28 μM (▲); 7.92 μM (■). (C) Double-reciprocal plots for inhibition of the reaction activity of FAS by DATS when malonyl-CoA concentration was the variable. DATS concentrations were 0 μM (●); 2.64 μM (◆); 5.28 μM (▲); 7.92 μM (■). (D) Substrate protection with increasing concentrations of acetyl-CoA. The reaction contained 0.58 μM FAS and 6.59 μM DATS.

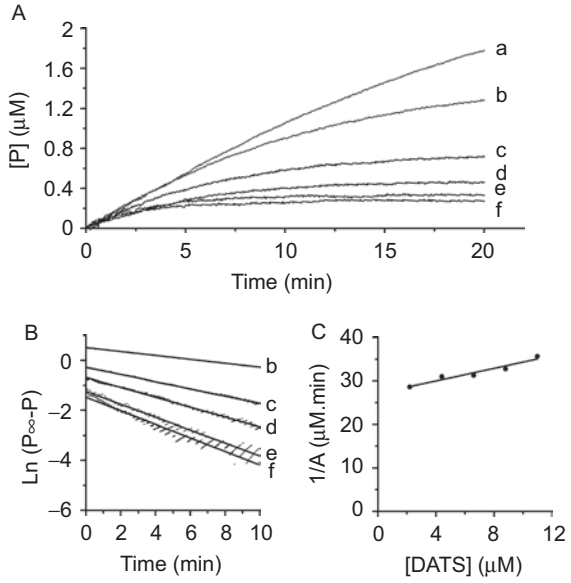


Figure 5. (A) Reaction monitoring curves for FAS activity assays in the presence of different concentrations of DATS: 0 μM (a), 2.2 μM (b), 4.4 μM (c), 6.6 μM (d), 8.8 μM (e), 11.0 μM (f). The concentration of FAS in this system was 5.15 nM, the concentrations of acetyl-CoA, malonyl-CoA, and NADPH were 13.1 μM, 50.0 μM, and 155.3 μM, respectively. (B) Plots of ln (P_∞ – P) vs. time, data from curves of (A). (C) Plot of 1/A vs. concentration of DATS, apparent constants A taken from plots of (B).

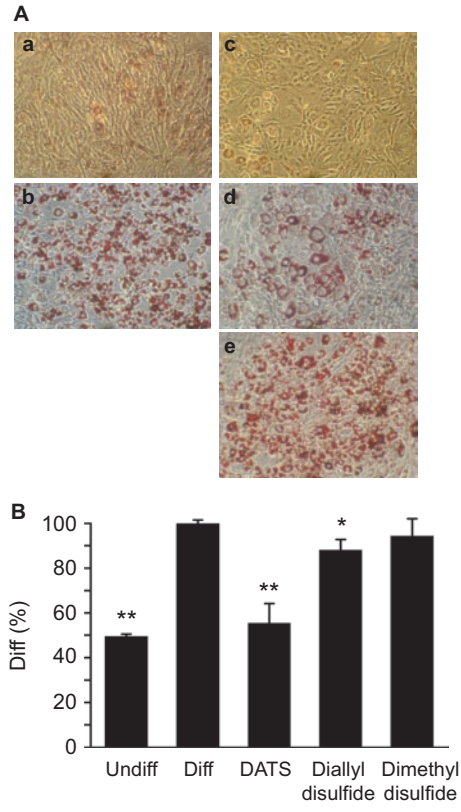


Figure 6. Effect of 100 μM sulfide treatment during differentiation on morphology of 3T3-L1 cells. (A) Photos were taken after oil red O (ORO) staining and the original magnification was ×100. (a) Undifferentiated control cells. (b) Differentiated control cells. (c) DATS-treated. (d) Diallyl disulfide-treated. (e) Dimethyl disulfide-treated. (B) Stained oil droplets in the cells were dissolved in isopropanol and spectrophotometrically measured at 520 nm. Undiff, undifferentiated cells; Diff, differentiated cells. Data are represented as mean ± SD ($n = 3$), p values obtained using a two-tailed t-test. $^{**}p < 0.001$ and $^{*}p < 0.05$ compared to Diff.

(Figure 5C). These results indicate that the reaction of DATS with FAS is a two-step affinity-labeling reaction where a dissociable complex is initially formed prior to the irreversible modification of the enzyme.

Treatment of 3T3-L1 preadipocytes with 100 μM DATS, diallyl disulfide, or dimethyl disulfide resulted in inhibition of both cell differentiation and lipid accumulation (Figure 6A). Figure 6B shows the total lipid content of 3T3-L1 preadipocytes treated with each thioether. DATS, diallyl disulfide, and dimethyl disulfide inhibited cell lipid accumulation to 55.5%, 88.3%, and 94.5% of that of the control differentiated adipocytes. DATS-treated cells exhibited the greatest differentiation defect.

Discussion

In the present work, it was demonstrated that DATS and several disulfides inhibit FAS activity in a both dose-dependent and time-dependent manner. Comparing the inhibitory ability of these thioethers, it can be concluded that the trisulfide is more active than the disulfide and monosulfide for inhibiting FAS, and the number of sulfur atoms of the thioether is very important for their inhibitory activity. Also, the allyl group is the most conducive to FAS inhibition. The IC_{50} value of DATS (8.37 μM or 1.49 μg/mL) is much lower than that of other, known classical FAS inhibitors, such as cerulenin with an IC_{50} values of 89 μM (20 μg/mL)[18] and EGCG with an IC_{50} value of 52 μM (24 μg/mL)[19]. For time-dependent inhibition, DATS completely inhibited FAS within 4 min with $k_{obs}/[I]$ of 12.5 min^{-1} mM^{-1}, which is 10 times higher than that of C75[19]. This indicates that thioethers may have a wider application prospect, and they may be suitable for the development of products related to health care or obesity therapy.

The competitive result between DATS and 5-IAF shown in Figure 2C suggests that DATS chemically reacts with sulfhydryl groups on FAS. The DTT recovery of FAS activity inactivated by DATS supports this suggestion. This interaction mechanism is similar to that of the reaction between DTNB and the sulfhydryl group, which is presented as a thiol–disulfide exchange and is essentially explained by a nucleophilic reaction between the negatively charged dissociated sulfhydryl group and the sulfur atom in the disulfide bond of the reagent. This estimation is consistent with the result of the pH effects on FAS inactivation by DATS.

Furthermore, we found that DATS acted on two essential groups of FAS (Figure 3A). The result is different from the reaction of DTNB with FAS (Figure 3B). During the series of reactions of fatty acid synthesis, the essential cysteine SH on KS only combines with saturated acyl groups, and the phosphopantetheine SH on ACP combines with all acyl groups involved in the reactions. The kinetic competitive results between DATS and acetyl-CoA rather than malonyl-CoA

and NADPH for both fast and time-dependent inhibition would indicate that DATS reacted with both essential SH groups on KS and ACP, which are the same sites combined by acetyl-CoA.

In the cell experiments, the inhibition of differentiation and lipid accumulation of 3T3-L1 preadipocytes by thioethers positively correlated with their inhibitory abilities on FAS. It is suggested that the effects of thioethers on preadipocytes are related to their inhibition on FAS. It is also shown that the lipid accumulation of fat tissue is not only dependent on fat absorption but also related to the synthesis of fatty acid.

In this investigation, it was concluded that the inactivation of DATS is due to the irreversible modification of essential SH groups on FAS. The problem is that the essential sulfhydryl group exists in many enzymes in our body. Could DATS inactivate these enzymes? Could DATS be dangerous? Nevertheless, the fact is that the *Allium* vegetables, such as garlic and onion, have been widely used as common foods for many years, and no toxicity has been found. Our understanding is that the inactivation of FAS by DATS is relatively specific. It was shown in this study that DATS inactivated FAS with affinity-labeling kinetics, and the IC_{50} was only 8.37 μM or 1.49 μg/mL. This means that low concentrations of DATS could inhibit FAS activity. In contrast, DATS inhibited human squalene monooxygenase by reacting with the essential SH group with an IC_{50} of 195 μM[20]. We showed that 1 mM DATS did not inhibit thioredoxin reductase, which also contains the essential SH group. These results indicate that DATS reacts relatively exclusively with the essential SH on FAS. Lower concentrations of DATS can effectively inhibit FAS activity, but cannot affect that of other enzymes. Thus, DATS should be safe for application. Thioethers would be promising candidates for further, thorough, pharmacological and clinical investigations.

In this investigation, we offer an explanation from a novel viewpoint for the mechanism of thioethers and *Allium* vegetables against obesity and cancer. Thioethers are compounds with distinct structure and character such as cerulenin, C75, EGCG, theaflavin, and flavone, which are reported as effective FAS inhibitors. All of them exhibit similar effects against diseases correlated with metabolic syndrome. It is further validated that FAS is an effective target for prevention and treatment of obesity and cancer.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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