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Malabaricone C as Natural Sphingomyelin Synthase Inhibitor against Diet-Induced Obesity and Its Lipid Metabolism in Mice

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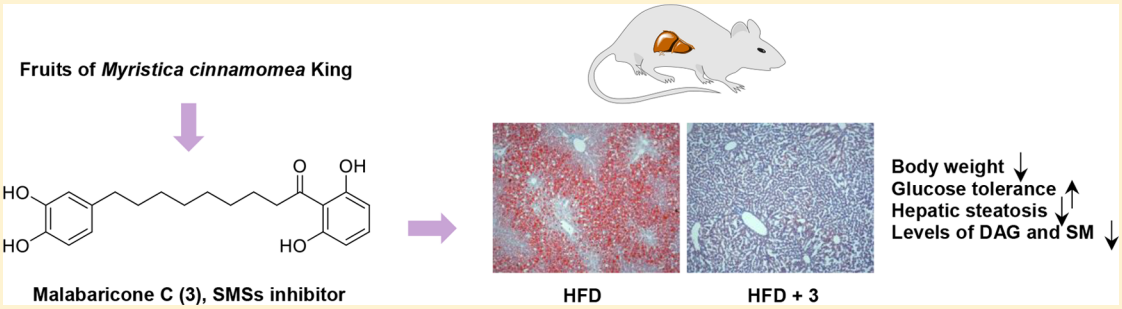
Supporting Information



ABSTRACT: The interaction between natural occurring inhibitors and targeted membrane proteins could be an alternative medicinal strategy for the treatment of metabolic syndrome, notably, obesity. In this study, we identified malabaricones A−C and E (**1**−**4**) isolated from the fruits of *Myristica cinnamomea* King as natural inhibitors for sphingomyelin synthase (SMS), a membrane protein responsible for sphingolipid biosynthesis. Having the most promising inhibition, oral administration of compound **3** exhibited multiple efficacies in reducing weight gain, improving glucose tolerance, and reducing hepatic steatosis in high fat diet-induced obesity mice models. Liver lipid analysis revealed a crucial link between the SMS activities of compound **3** and its lipid metabolism *in vitro* and *in vivo*. The nontoxic nature of compound **3** makes it a suitable candidate in search of drugs which can be employed in the treatment and prevention of obesity.

KEYWORDS: *Membrane protein, sphingomyelin synthase, malabaricone C, myristica cinnamomea, obesity*

Worldwide prevalence of obesity has increased substantially over the past 40 years and continues to cause metabolic syndrome, which is associated with dyslipidaemia, insulin resistance, cardiovascular diseases, and type 2 diabetes mellitus (T2DM).[1−3] These intersecting risks are controlled by a critical and complex metabolic pathway which involves the membrane protein. Having said that, the membrane protein could be the initial key in enhancing the understanding of pharmacology for common metabolic related diseases, notably, obesity. The membrane protein regulates cell communication with its surroundings which is activated by a wide variety of physiological and environmental stimuli including peptides, proteins, small organic molecules, and even ions.[4−6] About more than 50% of all known low molecular drugs bind to the membrane protein.[7,8] Thus, discovering an enzyme inhibitor will be a direct approach in developing low molecular drugs.

This study of ours focuses on the sphingomyelin synthase (SMS) membrane protein family which consists of two isozymes, SMS1 and SMS2.[9,10] Both SMS 1 and 2 catalyze ceramide and phosphatidylcholine (PC) as substrates to produce sphingomyelin (SM) and diacylglycerol (DAG).[11,12] The SMSs modulate SM and other sphingolipids levels, thereby regulating membrane fluidity, ceramide-dependent apoptosis, lipid metabolism, and signal transduction.[13−16] The increasing levels of SM and DAG produced by the SMSs will lead to obesity and insulin resistance.[17,18] SMS knockout mice are resistant to Alzheimer's disease, tumorigenesis, diet-induced obesity, and T2DM and are also know to exhibit decreased levels of plasma inflammatory cytokines.[19,20,15,21] Therefore, the inhibition of the SMSs enzymes by natural occurring substrates would be an ideal therapeutic approach for metabolic syndrome.

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Very recently, the inhibitory activity of gingkolic acid from the leaves of *Gingko biloba* was reported by our group.[22] Though, gingkolic acid has been proven to be an effective inhibitor with equal inhibiting potentials (IC_{50} = 1.5 μM) against both enzymes, studies have revealed that gingkolic acid is toxic, thus making it an unsuitable candidate for the further development of it as a drug.[23,24] With regard to this, in the present work, we report the isolation of malabaricones A−C and E (**1**−**4**) as the first naturally occurring SMS inhibitor from edible plants in an effort to display a safe alternative with lesser side effects.[25] Additionally, we performed a diet-induced obesity test with malabaricone C (**3**) that showed significant prevention of high fat diet-induced fatty liver.

Preliminary screening of the ethyl acetate extract from the fruits of *M. cinnamomea* showed potential inhibitory activity against SMS1 (13 μg/mL) and SMS2 (10 μg/mL), respectively. Therefore, the bioassay-guided fractionation of the extract resulted in the isolation of malabaricones A−C and E (**1**−**4**) as the active compounds (Figure 1).[26,27]

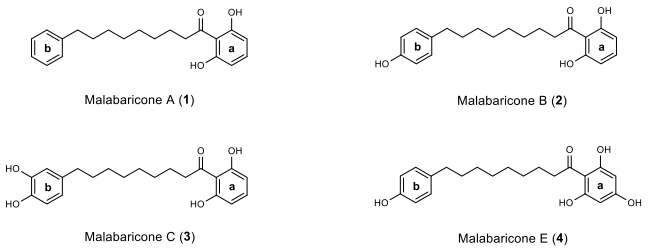

Figure 1. Bioassay-guided extraction of the fruits of *M. cinnamomea* afforded four naturally occurring inhibitors for SMS inhibitory activity.

Subsequently, compounds **1**−**4** were subjected to SMS inhibition assay by lysate-based assay of SMS1- or SMS2-expressed SMS1/2 double knockout mouse fibroblasts. Each of the compounds showed relatively moderate inhibition activities compared with that of previously synthesized inhibitors (Table 1).[22] A closer look at the structures of compounds **1**−**4** provided further insight as to how the activities of these compounds might have been influenced by the chemical groups in their respective structures (Figure 1). The SMS inhibiting potentials of compounds **1**−**3** could have enhanced with the increase in the number of hydroxyl groups in their ring b. The lower SMS inhibiting potentials of compound **4** upon comparison to compound **2** may have resulted from the additional hydroxyl group in its ring a.

Table 1. Inhibitory Activity of Sphingomyelin Synthase (Lysate Based Assay)[a]

		NBD − Ceramide (IC_{50}, μM)	
No.	Compounds	SMS 1	SMS 2
1	Malabaricone A	4	4
2	Malabaricone B	3.5	2.5
3	Malabaricone C	3	1.5
4	Malabaricone E	6	4.5

[a]IC_{50} values are the means of three separate determinations on SMS1 or SMS2 expressed SMS1/2 double knockout mouse fibroblast cell lysate and were determined by more than four concentrations of each inhibitor.

To determine the mode of action for major compounds **1**−**3**, cell lysate assay of the SMS inhibitory activity was carried out by using different substrate concentrations. The IC_{50} values of 2 to 3 μM for SMS1 and 1 to 3 μM for SMS2 were obtained in the presence of 5 and 10 μM of NDB-Ceramide. As a result, changes in substrate concentration did not significantly affect the IC_{50} values of compounds **1**−**3**, thus suggesting that compounds **1**−**3** were noncompetitive inhibitors of both SMS 1 and 2 (Table S1). Cell counting kit-8 assay was used to evaluate the cytotoxic activity of compound **3** against wild-type mouse embryonic fibroblasts cells, MEF. 56−97% of the cells were viable after 3 h of treatment with compound **3** at concentration levels of 1−0.01 mM (Figure S1). Acute toxicity studies of compound **3** at the concentration of 500 mg/kg were previously conducted on mice liver and kidneys. The absent of inflammation, necrosis, and hemorrhaging in the respective organs further supported our findings.[28]

Furthermore, in the current investigation, the SMS inhibition assay of compound **3** was carried out with live cells (cell-based assay) and the IC_{50} values were 13 μM and 11 μM for SMS1 and SMS2 enzymes, respectively (Table S2). These results suggested that compound **3** could be a suitable candidate for further *in vitro* and *in vivo* studies based on its previously reported world drug index, Lipinski's rules, nonmutagenicity, and noncarcinogenicity.[29]

It has been reported that a high fat diet (HFD) activates the nuclear receptor PPAR-γ, which is responsible for the hyper-expression of CD36/FAT.[15] The SMS2 enzyme facilitates CD36/FAT to take up the PPAR-γ ligands, which leads to the accumulation of triglycerides and lipid droplets, thus resulting in fatty liver formation. Since compound **3** exhibited SMS1 and SMS2 inhibitory activities, an oleic acid uptake analysis assay with hepatocytoma HepG2 cells was further conducted to examine the levels of intracellular triglycerides and free fatty acids. Compound **3** decreased the levels of intracellular triglycerides in a dose-dependent manner while it exhibited no significant changes in the free fatty acids levels as compared to the control (Figures 2A−B). With the promising *in vitro* effects on oleic acid uptake, we performed a Nile red staining assay to examine the effect of compound **3** on lipid droplet formation in the HepG2 cells. Remarkably, compound **3** for the first time was found to significantly decrease lipid accumulation in a dose-dependent manner (Figures 2C−D). These data indicated that compound **3** was able to prevent cellular uptake by CD36/FAT in a dose dependent manner, which is in good agreement with the results of the previous *in vivo* effects of SMS2 knockout mice.[15]

With regard to the *in vitro* results, the selected natural occurring inhibitor was further investigated using (C57BL/6J) mice which were fed with high-fat diet (HFD), normal chow diet (ND), and HFD supplemented with 0.1% of compound **3**. The HFD + **3** mice were healthy and behaved normally over 2 months with the exception of a noticeably leaner phenotype (Figure 3A). Despite not having any statistically difference in the daily food intake between the HFD control and the HFD + **3** group (Figure S2), the body weight of the HFD + **3** group was significantly lower than those of the controls starting from 20 days of treatment with an inclusive weight loss of 42.7% (Figures 3B−C). In addition, an oral glucose tolerance test was performed after 8 weeks of the daily oral administration of the vehicle controls and HFD + **3**. Treatment of mice with compound **3** also displayed a more significant improvement in

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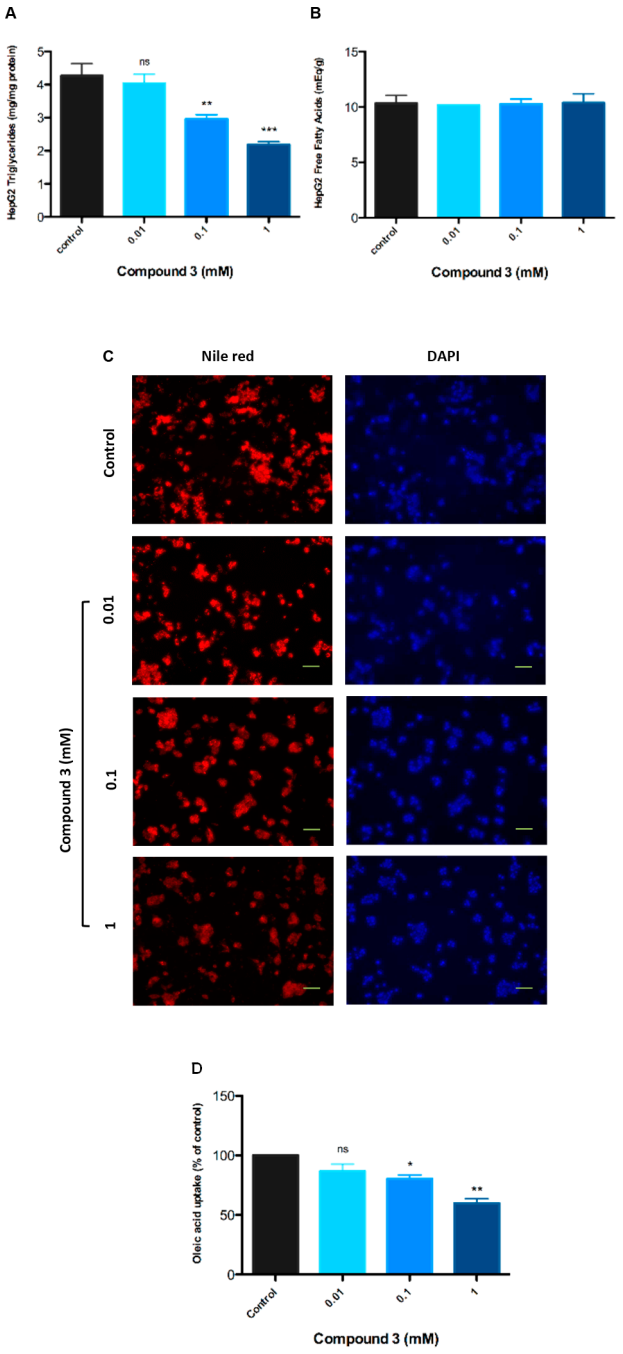

Figure 2. *In vitro* results of HepG2 cell analysis. Intracellular levels of (A) triglycerides and (B) free fatty acid when treated with different concentrations of compound **3** with oleic acid uptake. (C) Representative images of Nile Red staining and DAPI staining. (D) Oleic acid uptake analysis. Lipid droplets were stained with Nile Red and the numbers of lipid droplets were counted using fluorescent microscopy. Scale bar, 100 μm. Data are presented as the mean ± standard error of the mean (SEM). Statistical analysis was done by using *t* test: (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$, (****) $P < 0.0005$, ns = no significant difference versus the control.

the glucose tolerance than that of the vehicle-treated mice (Figure 3D).

The liver plays a key role in lipid metabolism.[30] Liver weight reduction was observed for the HFD + **3** as compared to the HFD group (Figure 4A), but the liver of the HFD + **3** group was noticeably redder, possibly implying a decreased fat

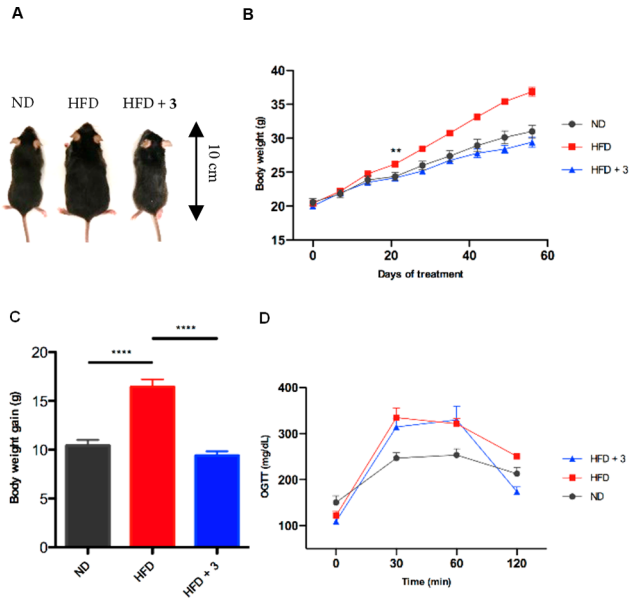

Figure 3. *In vivo* results of compound **3** on body weight gain and blood glucose levels. (A) Representatives images of the whole mice body. (B–C) Body weight gain. (D) Oral glucose tolerance test. Control: ND, normal chow diet and HFD, High fat diet. Test group: HFD + **3**, High fat diet with 0.1% of Malabaricone C. Data are presented as mean ± standard error of the mean (SEM); $N = 7–8$ mice per group. *t* test: (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$, (****) $P < 0.0005$, ns = no significant difference versus the control.

content in the organ (Figure 4B). Previous study has shown that up-regulation of the hepatic lipid metabolism may contribute to the suppression of the liver fat and visceral fat accumulation.[31] Examination of the histological analysis of the oil red O-stained sectioned of the liver showed the presence of numerous steatosis in the HFD control group as indicated by microscopy observation (Figure 4C). The HFD + **3** group on the other hand exhibited resistance in the development of liver steatosis and improved lipid metabolism. Steatosis controls the development of obesity along with metabolic syndrome related disorder.[32] Consistent with the histochemical results, we found that HFD + **3** effectively reduced the hepatic TG levels (Figure 4D). In addition, feeding the mice with HFD + **3** significantly reduced the levels of triglycerides (TG) and free fatty acids (FFAs) in the blood plasma (Figures 4E–F). In comparison with previous plasma free fatty acids in the SMS2 knockout mice *in vivo*, there is a possibility that the uptake of fatty acids into the liver tissues may not fully be prevented, which further explains the decrease of plasma free fatty acids upon feeding with HFD + **3**. Finally, we assessed the synthesis of DAG and SM via liver tissue lysate assays to further confirm the *in vivo* SMS inhibitory activities by compound **3**. Indeed, we have proved that, for the first time, compound **3** as a natural SMS inhibitor, has significantly reduced the synthesis of the DAG and SM in the liver (Figures 4G–H). Herein, we underlined the *in vitro* and *in vivo* efficacies of compound **3** in its inhibition of the SMS2 enzyme and its putative mechanism involving the prevention of obesity. Interestingly, we demonstrated that compound **3** results in body weight reduction, improves glucose tolerance, and lowers hepatic steatosis *in vivo*. Further studies on gene expression related to lipogenesis and gluconeogenesis are required to better understand the exact metabolism which is involved.

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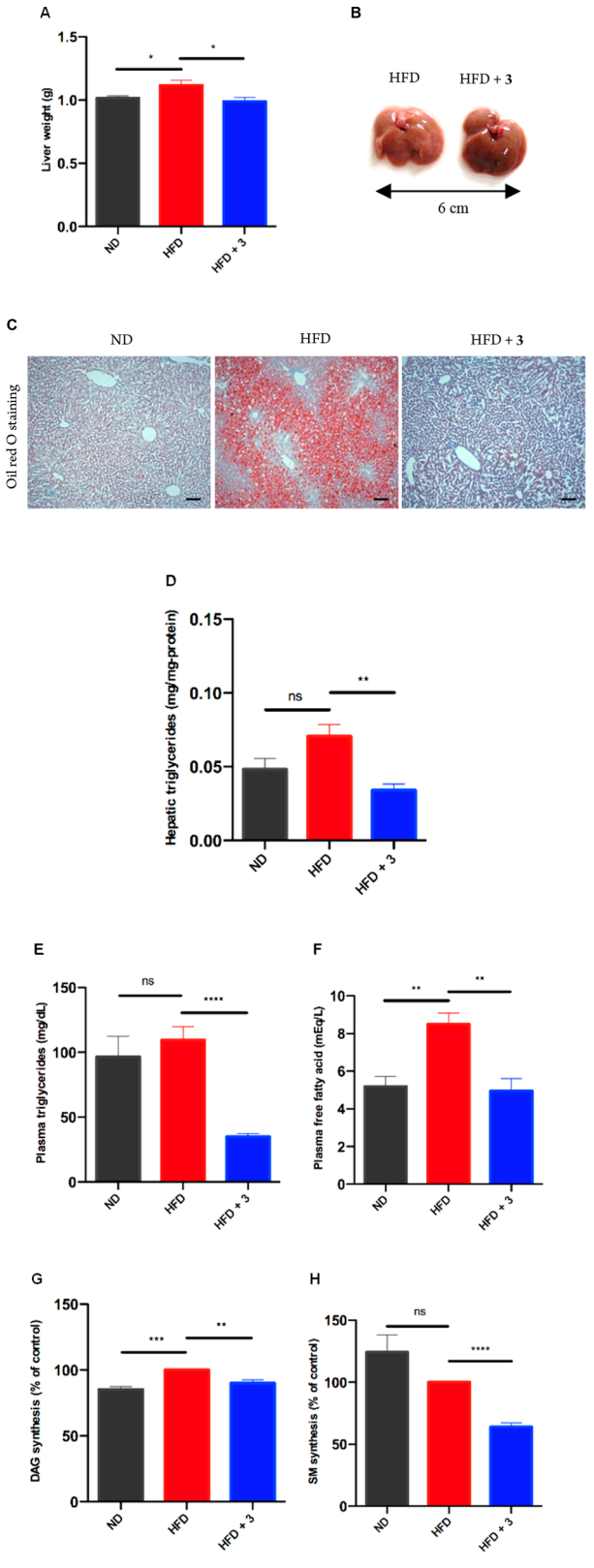

Figure 4. *In vivo* results of mice liver, lipid metabolism, and SMS inhibitory activity. (A) Liver weight of the mice. (B) Representatives images of liver gross appearance. (C) Representatives images of Oil Red O staining (N = 3 mice per group). (D) Hepatic triglycerides. (E) Plasma triglycerides. (F) Plasma free fatty acids. (G) Conversion of NBD-Phosphocholine. (H) Conversion of NBD-Ceramide. Measurements were taken from distinct samples. Scale bar, 100 μm. Control: ND, normal chow diet, and HFD, high fat diet; Test group: HFD + **3**, high fat diet with 0.1% of compound **3**. Data are presented as mean ± standard error of the mean (SEM); N = 7−8 mice per group. t test: (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$, (****) $P < 0.0005$, ns = no significant difference versus the control.

In summary, malabaricone C (**3**), an acylphenol isolated from the fruits of *M. cinnamomea*, has been identified as a lead natural sphingomyelin synthase inhibitor. Having the same mechanisms of action as the previously reported SMS knockout studies, malabaricone C was highly efficacious in preventing oleic acid uptake across the membrane, which in turn reduced lipid droplet formation *in vitro*.[15] Malabaricone C was also found to be able to reduce body weight gain, improve glucose tolerance, and decrease lipid accumulation in the liver *in vivo*, thus making this the first report involving a plant derived SMS inhibitor against high fat diet-induced obesity. Its nontoxic nature makes malabaricone C a suitable candidate for its further development as a new drug or medicinal supplement to treat and prevent obesity.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.9b00171.

All experimental procedures, tables on mode of inhibition and cell-based assay inhibition, figures on cytotoxicity of compound **3**, and *in vivo* result for daily food intake (PDF)

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Author Contributions

M.A.O., K.Y., Y.M., and D.M. contributed to the design, execution, and analysis of the *in vitro* and *in vivo* experiments. Y.S. and K.A. contributed to plant samples. M.A.O., K.Y., Y.M., Y.S., and K.M. wrote the manuscript. Y.I and K.M. designed and supervised the study. All of the authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

SMS, sphingomyelin synthase; PC, phosphatidylcholine; SM, sphingomyelin; DAG, diacylglycerol; ND, normal chow diet; HFD, high fat diet; PPAR-γ, peroxisome proliferator-activated receptor gamma; CD36, cluster of differentiation; FAT, fatty acid translocase; TG, triglycerides; FFA, free fatty acid; NBD, N-[6-[(7-nitro-2−1,3-benzoxadiazol-4-yl)amino]hexanoyl]; T2DM, type 2 diabetes mellitus; OGTT, oral glucose tolerance test; DAPI, 4′,6-diamidino-2-phenylindole.

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