Anti-obesity activities of the yoshinone A and the related marine γ-pyrene compounds

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Marine cyanobacteria are known as important creators of novel natural products. From this valuable source, various bioactive compounds have been found and characterized in terms of their pharmacological and toxicological activities.1 In the previous work, we have reported on the isolation and structure determination of potent cytotoxic compounds, lyngbyacyclamide A and B,2 an inhibitor of osteoclastogenesis, biselyngbyasidic,3 and a protein kinase inhibitor, biselbromamide.4 In the recent work, we have reported the new marine γ-pyrones yoshinone A, B1 and B2 from Leptolyngbya sp., and determined their planar structures using NMR spectral analysis.5 Yoshinone A, as the major compound among them, showed inhibitory activity against the adipogenic differentiation of 3T3-L1 cells with an half maximal inhibitory concentration (IC50) value of 420 nM without cytotoxicity (IC50 > 50 μM). On the other hand, the yoshinone B1 and B2 showed only limited activity against 3T3-L1 cells, with higher concentrations compared with yoshinone A. Further studies of the structure–activity relationship lead us to conclude that the position of a pyrene ring and an olefin in the side chain will be important for the inhibition of adipogenic differentiation. These γ-pyrones have olefins in their side chain at positions 7 and 6 in the cases of yoshinones A and B1/B2, respectively. To express the effects on adipocyte, the olefin should not be conjugated with γ-pyrene moiety, such as yoshinone A (Figure 1). In the previous studies, kalkipyrone6 isolated from cyanobacteria, aureothin,7 and actinopyrones A and B8 isolated from streptomycyes fell into the same γ-pyrones. Then, we confirmed that kalkipyrone and aureothin showed this activity, with IC50 values of 67.5 and 54.2 nM, respectively. On the basis of these data, we are focusing on the 7-en γ-pyrene (unconjugated type) compounds. These pyrones are expected to be candidates for novel lead compounds for the treatment of obesity and related diseases.9 Studies on useful tools that regulate adipocytes will contribute to the prevention and treatment of these diseases. At the present stage of our research, we have evaluated the anti-obesity activities of the 7-en γ-pyrones using in vitro and in vivo experiments. In this study, we report on the interesting properties of these pyrones.

Marine cyanobacteria as sources of γ-pyrene compounds have been collected from Ishigaki and Okinawa islands, Japan, and extracted with aqueous methanol. The isolating procedures of the γ-pyrones were performed according to the original reports with minor modifications. Their purity and structures were confirmed by NMR analysis. From the collected cyanobacteria, we noted that got only a trace amount of yoshinone A (<1.0 mg) and 35.7 mg of kalkipyrone, as purified γ-pyrones for the present experiments.

As the in vitro experiments, the reducing effects on accumulated triglyceride (TG) in the mature 3T3-L1 adipocyte were investigated with yoshinone A. In Figure 2a, typical images of mature 3T3-L1 adipocytes stained TG with oil red O were shown. As shown in Figure 2b, TG amount in mature 3T3-L1 adipocyte-treated yoshinone A significantly decreased with the dose-dependent manner. On the other hand, a significant increase of lactate (LA) in the culture fluid was observed by yoshinone A treatment of the adipocyte, as shown in Figure 2c. These changes in TG and LA were induced with 0.1–0.01 μM of yoshinone A with no effect on cell viability. These results revealed that the 7-en γ-pyrones showed TG reduction activities in mature 3T3-L1 adipocyte, in addition to its inhibitory activities on adipose differentiation. As the differentiation ratio in preadipocyte was evaluated with TG amount in the cells, the findings are acceptable for the previous experimental perceptions in 3T3-L1 cells.

In the following experiment, to verify whether or not the LA production is limited in 3T3-L1 cells, we have repeated the experiment with the same conditions using HeLa cells, the most widely used human cultured cells. After treatment with yoshinone A (0, 0.01 and 0.1 μM) for 48 h, the culture fluids were supplied for HPLC analysis to...
determined LA and glucose (Glc) concentrations. As shown in Supplementary Figure 1, the enhancement of LA production and Glc consumption were observed clearly with dose-dependent manner of yoshinone A even in HeLa cells without cytotoxicities. These results suggested that yoshinone A induced energy metabolic changes relating to Glc via relatively common pathway in cells. On the basis of these in vitro experiments, the induced changes in metabolism will affect the utilization of accumulated TG in mature 3T3-L1 adipocyte with direct or indirect pathway(s). Due to a deficiency of these marine γ-pyrones, further experiments to analyze the mechanism in detail are still needed at this stage.

As with the in vivo experiments, kalkipyrone as a 7-en γ-pyrone was provided to the experiment with a small preliminary sample size. To confirm the anti-obesity activity in vivo, we performed two experiments as described below.

The IC_{50} value of kalkipyrone was reported as 120 nM in HeLa cells, but there are no information about the toxicity in vivo. In the first experiment using mice, the evaluation for acute toxicity of kalkipyrone with oral administration was determined. Male ddY mice (5 weeks old) were divided into three groups (n = 3 each); they received 16.5 and 5.5 mg kg^{-1} per day of kalkipyrone, and vehicle (3% dimethyl sulfoxide solution) orally for 3 days. The physical
measurements, autopsy findings and behavior observations did not indicate a difference among these groups during 7 days from first ingestion. Then, we planned a long-term period treatment test in mice with a dosage of 5 mg kg\(^{-1}\) per day of kalkipyrone.

For the second experiment in vivo, the anti-obesity effects of kalkipyrone in vivo were examined by feeding mice a high-fat diet (HFD) for 5 weeks. Mice were fed a normal diet (ND, \(n = 6\)), a HFD (\(n = 6\)) and HFD with oral ingestion of kalkipyrone at a dosage of 5 mg kg\(^{-1}\) per day (HFD+KAL, \(n = 3\)) during the experiment. The transitional changes of body weight gain, food intake and water intake of the groups are shown in Supplementary Figure 2. After the experimental period, measured parameters were summarized in Table 1. The body weights of mice in the ND and HFD groups showed significant differences, with the values of 39.5 ± 0.2 and 43.4 ± 0.7 g, respectively. The HFD+KAL group (40.6 ± 2.8 g) exhibited pronounced suppressed body weight gain, but no significant differences, owing to the limited sample size for the experiment. Meanwhile, the weight of adipose tissue was significantly suppressed (\(P < 0.05\)) with the kalkipyrone treatment: 0.93 ± 0.23 g in the HFD+KAL group vs 1.62 ± 0.15 g in the HFD group. The other tissues’ weight did not show significant changes with kalkipyrone treatment (Supplementary Figure 3). These results suggest that oral ingestion of kalkipyrone is effective for suppressing adipose tissue weight gain in mice.

As food intake during the experimental period in the HFD (227.9 ± 19.3 g per head) and HFD+KAL (212.8 ± 31.3 g per head) groups were similar, the suppression of adipose tissue gain was not affected by food consumption or appetite in mice. Another main possibility mechanism for anti-obesity is the inhibition of TG absorption in the small intestine. For example, orlistat, a lipase inhibitor in affected by food consumption or appetite in mice. Another main groups were similar, the suppression of adipose tissue gain was not

\[\begin{align*}
\text{Table 1. The body weights of mice in the ND and HFD groups} \\
\text{Measured parameter} & \quad \text{ND} & \quad \text{HFD} & \quad \text{HFD+KAL} \\
\text{Body weight (g)} & 39.5 ± 0.2 & 43.4 ± 0.7 & 40.6 ± 2.8 \\
\text{Food intake (g)}^a & 168.2 ± 4.1 & 227.9 ± 19.3 & 212.8 ± 31.3 \\
\text{Water intake (g)}^a & 205.1 ± 7.1 & 192.0 ± 9.5 & 194.3 ± 14.7 \\
\text{Adipose tissue (g)} & 1.01 ± 0.12 & 1.62 ± 0.15 & 0.93 ± 0.23^* \\
\text{Liver (g)} & 1.31 ± 0.03 & 1.41 ± 0.07 & 1.35 ± 0.11 \\
\text{Hepatic TG (mg g}^{-1}\text{ liver)} & 38.2 ± 6.0 & 41.3 ± 5.7 & 34.7 ± 5.8 \\
\text{Feces (g)}^b & 1.4 ± 0.2 & 2.5 ± 0.6 & 1.8 ± 0.2 \\
\text{Fecal TG (mg g}^{-1}\text{ feces)} & 9.8 ± 0.2 & 41.3 ± 7.4 & 43.6 ± 6.0 \\
\text{Plasma Glc (mg dl}^{-1}) & 133.4 ± 13.7 & 135.7 ± 16.6 & 151.1 ± 28.2 \\
\text{Plasma LA (mg dl}^{-1}) & 65.6 ± 6.1 & 68.8 ± 6.1 & 95.4 ± 2.4^* \\
\text{Plasma TG (mg dl}^{-1}) & 134.5 ± 34.5 & 132.1 ± 25.5 & 88.0 ± 16.1 \\
\text{Plasma NEFA (mEq l}^{-1}) & 0.58 ± 0.17 & 0.74 ± 0.06 & 0.70 ± 0.10 \\
\end{align*}\]

Abbreviations: ANOVA, analysis of variance; Glc, glucose; HFD, high-fat diet; HFD+KAL, high-fat diet with kalkipyrone (5 mg kg\(^{-1}\) per day per os); LA, lactate; ND, normal diet; NEFA, non-esterified fatty acid; TC, total cholesterol; TG, triglyceride.

\(^a\)Accumulated values during experiments.

\(^b\)Total values for 3 days.

\(^*P < 0.05\) vs HFD. Data are presented as the mean ± s.e. and analyzed by ANOVA followed by Dunnett’s test.

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EXPERIMENTAL PROCEDURE

In vitro experiments in 3T3-L1 adipocyte
The reducing effect of yoshinone A on accumulated TG in adipocytes was evaluated using 3T3-L1 cells from different culture media. The preadipocyte 3T3-L1 cells (Riken BRC, Tsukuba, Japan) were cultured in Dulbecco's modified Eagle's medium (Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco) in two 96-well plates at 37 °C, 5% CO2. Two days after confluence, the differentiation was induced by Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 0.5 μM of 3-isobutyl-1-methylxanthine, 0.25 μM of dexamethasone each from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and 10 μg/ml-1 insulin (Gibco) for 7 days.

The differentiated 3T3-L1 adipocytes in a 96-well plate were treated with 2% Triton-X 100 for 30 min at room temperature followed by sonication. Accumulated TG amounts and cell viability were evaluated according to the method with by Inuzuka et al.3

Anti-obesity test in mice
Male ddY mice were obtained from Japan SLC, Inc. (Shizuoka, Japan). They were housed in individual cages under a 12-h/12-h light/dark cycle (lights from 0800 to 2000 hours) in a room with controlled temperature and humidity (25 ± 1 °C and 60 ± 5%, respectively). For the experiment, we formulated experimental diets based on the AIN-93M diet.4 To mimic a westernized diet rich in animal fat, we used HFD-60 (Oriental Yeast Co., Ltd, Tokyo, Japan) including lard as fat (62.2 kcal%). Male mice (5 weeks old) were fed a ND for 1 week and then divided into the following three groups: ND (n = 6); HFD (n = 6); and HFD+KAL (n = 3). Kalkipryrone (5 mg kg-1 per day) was administered orally to the mice fed a HFD (HFD+KAL group). Other mice received vehicle (10 ml kg-1 per day) orally. Body weight, food intake and drinking water were measured every day. After the mice were fed these diets for 5 weeks. The feces were collected for the last 3 days and dried to weigh. The mice were killed by anesthetic overdose with a mixture of 3% isoflurane. And then, blood was collected from the abdominal vein to prepare plasma, and the epididymal adipose tissue and liver were dissected and weighed. The TG in the liver and feces were extracted with methanol-chloroform solution following homogenization. The plasma TG, total cholesterol, non-esterified fatty acid, Glc and LA levels were measured using the commercially clinical assay kit (Wako Pure Chemical Industries, Ltd.) for each. Data were presented as mean ± s.e. and analyzed by one-way analysis of variance and the Dunnett’s test. Differences between groups were considered to be statistically significant at P<0.05.

Animal studies were performed in accordance with notification number 88 of the Ministry of the Environment, Japan, (2006) and the Guidelines for Animal Experimentation of the Tokyo University of Marine Science and Technology, with the approval of the Animal Care and Use Committee of the Tokyo University of Marine Science and Technology.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)