プレス発表資料

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カレーのスパイス「クルクミン」が熱変性した
GO-Y022 が胃癌を抑制することを解明

秋田大学大学院医学系研究科臨床腫瘍学講座・腫瘍内科の柴田浩行教授と吉田泰一医員の研究グループは東北大学、金沢大学、癌研究会癌研究所との共同研究により、スパイスのクルクミンが熱変性によってできる化合物"GO-Y022"がヒトの胃癌細胞株の増殖を抑制し、アポトーシスによる細胞死を誘導することを発見しました。また、胃癌を起こすモデルマウスにGO-Y022を含む餌を経口摂取させると、GO-Y022を含まない餌を投与した胃癌モデルマウスと比較して胃癌のサイズが約1/3に縮小されました。GO-Y022は市販のカレーにも含まれていました。今後、「GO-Y022リッチなカレー」のメニューを考案することなどで医食同源を目指すことが期待されます。

本研究成果は10月17日にJournal of Functional Foodsのオンラインで（https://authors.elsevier.com/a/1Xv9U6FNx9edP9）公表されました。

胃癌による死亡は世界第2位を占める悪性腫瘍です。クルクミンは癌化に関連する分子を抑制することが知られています。しかし、クルクミンの活性は低く、改良の余地があります。そこで、我々は様々なクルクミンの誘導体を合成し、抗腫瘍活性が強い誘導体の合成に成功しました。しかし、これらの誘導体はペンタノイドであり、ヘプタノイドであるクルクミンとは化学構造が大きく異なっていました。近年、これらの誘導体の中でGO-Y022と名付けたペンタノイドがクルクミンの熱変性で生じることが判明しました。このGO-Y022は4種類の胃癌細胞株に対してクルクミンよりも約5倍強い抗腫瘍活性を示し、また、GO-Y022はクルクミンよりも強いアポトーシス誘導能を有しています。GO-Y022は胃癌のマウスモデルの腫瘍のサイズを約1/3に抑制し、経口摂取されたGO-Y022は胃の粘膜や胃癌で検出されましたが、血液中には見出されませんでした。GO-Y022は胃粘膜で作用し、全身性の有害事象も認められませんでした。GO-Y022は市販のカレーにも含まれ、GO-Y022には胃癌の抑制効果があり、実はこれまでにもカレーの成分として食べられていました。これによりカレーのGO-Y022は機能性食品になりうる可能性が示されました。
Dietary intake of pyrolyzed deketene curcumin inhibits gastric carcinogenesis
Dietary intake of pyrolyzed deketene curcumin inhibits gastric carcinogenesis

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ABSTRACT
Gastric cancer is the second leading cause of cancer-related mortality worldwide. Curcumin, a phytochemical, possesses molecular inhibitory potentials for regulating malignancies. However, the lower potential of curcumin warrants improvement. Thus, we synthesized diarylpentanoid analogs with higher potency; however, these differed structurally from curcumin—a heptanoid. Recently, one diarylpentanoid was formed following pyrolysis of curcumin, which is identical to our GO-Y022. The growth inhibition of gastric cancer cells by GO-Y022 was five-fold higher than by curcumin; GO-Y022 displayed superior apoptosis induction ability. Besides, it suppressed the gastric tumor growth to a third in a mouse model. GO-Y022 was moved to the epithelial and gastric tumors but not detected in the bloodstream. Moreover, oral GO-Y022 was effective topically, exhibited no adverse events in mice, and was detected in the commercially available curry paste. Briefly, GO-Y022 can inhibit gastric carcinogenesis; it is dietary and can be safely used as an oral functional food.

1. Introduction

Gastric cancer is the second leading cause of cancer-related mortality globally, with a mortality rate of 700,000 in 2012 (Ferlay, Soerjomataram, & Dikshit, 2015). Typical risk factors for gastric cancer comprise Helicobacter pylori infection, obesity, smoking, consumption of red meat and alcohol, and low socioeconomic status (Hundahl, Phillips, & Menck, 2000). However, the precise mechanisms underlying gastric cancer remain unclear, necessitating the determination of effective treatment for gastric cancer. Surgical resection, the only approach for a cure, is not applicable in patients with stage IV gastric cancer, for whom chemotherapy remains the primary treatment (Japanese Gastric Cancer Association, 2017). However, the median survival of stage IV patients is approximately 1 year at best, even with the use of the newer treatment options (Cunningham, Starling, & Rao, 2008; Van Cutsem, Moiseyenko, & Tjulandin, 2006).

Previously, several dietary elements, such as phytochemicals, have been investigated for cancers in these aspects. Curcumin, a dietary pigment in use for > 3000 years in Asia primarily, is being increasingly analyzed for its antitumor potential (Kunnumakkara, Bordoloi, & Harsha, 2017). Reportedly, curcumin can suppress the proliferation of various cancer cell types and induce apoptosis (Kunnumakkara et al., 2017). In addition, it comprises antiangiogenic properties and is known to enhance cancer immunity and inhibit cancer immunotolerance (Mohamed, Jantan, & Haque, 2017). Furthermore, curcumin inhibits the growth of gastric cancer cell lines (Zhang, Lin, & Zhou, 2015). However, curcumin is not used in a clinical setting because of its limited bioavailability and relatively low potential, and the ongoing research is focused on overcoming these limitations.

Thus, we successfully synthesized a series of new diarylpentanoid analogs to attain a new curcumin analog with higher antitumor potential (Ohori, Yamakoshi, & Tomizawa, 2006) and demonstrated that the new analogs inhibited the proliferation of various cancer cell types, including colon cancer stem cells (Lin, Liu, & Li, 2011). In addition, we...
determined that these analogs exhibited the safety profile of the original compound curcumin in mouse models (Lin et al., 2011; Shibata, Yamakoshi, & Sato, 2009) and that one diarylpentanoid, 1,5-bis(3,5-dimethoxy-4-methoxymethoxyphenyl)pentadien-3-one (GO-Y031) inhibited gastric carcinogenesis in vivo with a good safety profile (Uehara, Inoue, & Fukuda, 2014). However, a big gap remains between the mouse models and the first-in-human study. Our diarylpentanoid analogs (C5) are structurally different from curcumin, which is a diarylheptanoid (C7). Recently, a breakthrough demonstrating that a deketene form of curcumin, which is a C5 analog, formed because of curcumin pyrolysis during cooking of curry (Dahmke, Boettcher, & Groh, 2014), this C5 analog is identical to an analog we synthesized, 1,5-bis(4-hydroxy-3-methoxyphenyl)-1,4-pentadiene-3-one (GO-Y022). Thus, this study aims to investigate the efficacy and safety of GO-Y022 in human gastric cancer cell lines and a mouse gastric cancer model and determines the pharmacokinetic profile of GO-Y022.

2. Material and methods

2.1. Compounds

Originally, GO-Y022 was synthesized by the Department of Organic Chemistry, Graduate School of Pharmaceutical Science at Tohoku University (Sendai, Japan) and was subsequently purchased from Nippon Carbide Industries Co., Inc. (Tokyo, Japan; Fig. 1). We dissolved GO-Y022 in dimethyl sulfoxide (DMSO) at 10–50 mmol/L as a stock solution. Curcumin was purchased from Wako Pure Chemical Industries (Osaka, Japan), and the high-fat diet (HFD) 32 was purchased from CLEA Japan (Tokyo, Japan).

2.2. Cell lines

We obtained the gastric cancer cell lines KATO III and GCIY from the American Type Culture Collection through Summit Pharmaceutical International (Tokyo, Japan) and RIKEN BioResource Research Center (Tsukuba, Japan), respectively. In addition, both H-111-TC and SH-10-TC were obtained from Cell Resource Center for Biomedical Research (Osaka, Japan) and RIKEN BioResource Research Center (Tokyo, Japan). We obtained the gastric cancer cell lines KATO III and GCIY from the American Type Culture Collection through Summit Pharmaceutical International (Tokyo, Japan) and RIKEN BioResource Research Center (Tsukuba, Japan), respectively. In addition, both H-111-TC and SH-10-TC were obtained from Cell Resource Center for Biomedical Research (Osaka, Japan) and RIKEN BioResource Research Center (Tokyo, Japan), respectively. We performed immunohistochemistry as previously described (Uehara et al., 2014). As shown in the previous study, gastric cancer could be detected after 12-weeks of age in the HFD-fed Gan mice (Uehara et al., 2014). HFD was used, because GO-Y022 is hydrophobic, and it can be mixed well with oily materials, such as HFD. All animal experiments were performed humanely and complied with the guidelines set by Akita University and were approved by the related ethics committee (certification #a-1-2641). The tumor volumes were evaluated as follows (Euhus, Hudd, & LaRegina, 1986): tumor volume \( (\text{mm}^3) = (\text{shortest tumor diameter})^2 \times (\text{longest tumor diameter})/2 \).

2.3. In vitro growth assay

We assayed the cell viability by quantification of the uptake and metabolism of WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt), per the manufacturer’s instructions (Dojindo Laboratories, Kumamoto, Japan). We determined the half-maximal inhibitory concentration (IC\(_{50}\)) by the MTT assay as described previously (Kudo, Yamakoshi, & Sato, 2011).

2.4. Effect on a normal gastric epithelial cells

A human gastric epithelial cells from 58 year Caucasian, HGaEpC, was purchased from CELL APPLICATIONS, INC (San Diego, CA). HGaEpC cells (lot 3288) were seeded in each well of a 96 well-plate at 5 × 10\(^4\) cells. Cells were cultured with the manufacturers’ supplied medium added by GO-Y022, and the IC\(_{50}\) values were calculated after 72 h incubation.

2.5. Apoptosis analysis

Cells were cultured in a 10 cm-dish to semiconfluence, then they were treated with GO-Y022 at the indicated concentration. After 24 h treatment, cells were lysed and the lysate was applied to the ELISA plate. Apoptosis was determined by the M30-Apoptosense enzyme-linked immunosorbent assay (ELISA; VLVbio) from Funakoshi (Tokyo, Japan), per the manufacturer’s protocol. We treated the control with 1% DMSO alone. The relative values were calculated as the ratio to the control (the control value = 1.0). All cellular experiments were conducted in triplicate, unless otherwise specified.

2.6. ELISA (β-catenin and STAT3)

Cells were cultured in a 10 cm-dish to semiconfluence, then they were treated with GO-Y022 at the indicated concentration. After 24 h treatment, cells were lysed and the lysate was applied to the ELISA plate. We determined the activation of β-catenin and phosphorylation of signal transducers and activators of transcription 3 (STAT3) using the Active β-Catenin MKA ELISA (Symansis, New Zealand) and phospho-STAT3 (pSTAT3) Tyr705 sandwich ELISA (Cell Signaling Technology Japan, Tokyo, Japan), respectively. The obtained values were corrected by the cell numbers and then represented as the relative values against the control (the control value = 1.0).

2.7. Fluorescence-activated cell sorting (FACS) analysis

Cells were seeded in a 6 well plate to grow semi-confluent. Then, they were treated with compounds for 48 h. Cells were scraped and stained with Annexin V apoptosis detection kit APC (Invitrogen, Thermo Fisher Scientific K.K. Tokyo, Japan) and propidium iodide (eBioscience, Thermo Fisher Scientific K.K.) to detect apoptosis/necrosis, according to the manufacturer’s protocol. The stained cells were subjected to flow cytometric analysis with BD FACS AriaTM III (BD bioscience, Tokyo, Japan). Data were analyzed with FlowJo software (Tree Star, OR).

2.8. Mice experiments

We obtained K19-Wnt1/C2 mE (Gan) mice by crossing K19-Wnt1 and K19-C2 mE mice (Oshima, Matsunaga, & Fujimura, 2006). Gan mice are genetically engineered animals where activated oncogene, Wnt-1 signaling and inflammation related genes such as COX-2 and prostanandin E synthase, are up-regulated simultaneously. Resulting gastric cancers arise spontaneously. Every day, Gan mice were fed 5 g High Fat Diet 32 alone (n = 6) or HFD added with 0.5% (weight/weight) GO-Y022 (n = 7), starting since 9 weeks of age and were sacrificed and examined at 16 weeks of age with minor modifications, as described previously (Uehara et al., 2014). As shown in the previous study, gastric cancer could be detected after 12-weeks of age in the HFD-fed Gan mice (Uehara et al., 2014). HFD was used, because GO-Y022 is hydrophobic, and it can be mixed well with oily materials, such as HFD. All animal experiments were performed humanely and complied with the guidelines set by Akita University and were approved by the related ethics committee (certification #a-1-2641). The tumor volumes were evaluated as follows (Euhus, Hudd, & LaRegina, 1986): tumor volume \( (\text{mm}^3) = (\text{shortest tumor diameter})^2 \times (\text{longest tumor diameter})/2 \).

2.9. Immunohistochemistry

We performed immunohistochemistry as previously described (Uehara et al., 2014) using previously described protocols with the following antibodies: anti-mouse β-catenin (1:1500; C2206, rabbit;
To quantify the positivity of pStat3 expression, we randomly selected the specimen and over 80 epithelial cells were counted for the positivity of pStat3 in each mouse group (HFD diet mice; n = 7, GO-022 diet mice; n = 6), and the percentage of positivity is indicated.

2.10. Evaluation of the safety profile of GO-Y022

As a measure of the overall health status, body weight of mice was determined before sacrifice. In addition, the tissue-specific toxicity profile of GO-Y022 was assessed by a blood test. Briefly, we collected blood from the infraorbital venous plexus before sacrifice and analyzed the serum by Oriental Yeast (Tokyo, Japan) to evaluate the total bilirubin (T-Bil), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) for liver toxicity, creatinine (Cre) for renal toxicity, and lactate dehydrogenase (LDH).

2.11. Localization of GO-Y022

We determined tissues localization and blood concentrations of GO-Y022. Briefly, at the time of sacrifice, we collected specimens from the heart, kidney, liver, spleen, and digestive tract, including the esophagus, stomach, and small and large intestines, as well as the gastric tumor. Resected alimentary tracts were incised along their long axis, and washed in the distilled water with vigorous shaking to remove the feed of the surface several times. Then, the tracts were expanded with pins on the dork board. The mucosal surface was swabbed by cotton wetted with the distilled water until the all oily content of High Fat Diet was disappeared. After measuring the weight of the tissue sample, then they were homogenized in the distilled water, and analyzed by HPLC. The parenchymal organs were directly homogenized in the distilled water.

The sample of each tissue was homogenized in 200 μL of the distilled water, and it was applied to an Oasis HLB extraction cartridge (Nihon Waters K.K., Tokyo, Japan) that was preactivated with methanol and water (1.0 mL each). Next, we washed the cartridge with 1.0 mL water and 1.0 mL 60% methanol in water and eluted with 1.0 mL 100% methanol. Then, the eluate was dried by vortex-vacuum evaporation at 70 °C using a rotary evaporator (AS-ONE CVE-2AS, Osaka, Japan). After that, we dissolved the resulting residue in 20 μL methanol and vortexed for 30 s. In addition, 20 μL of the mobile phase was added to the sample, which was vortexed for another 30 s. Next, we processed a 20 μL aliquot of the sample by high-performance liquid chromatography (HPLC), which was conducted using a PU-2080 plus chromatography pump (JASCO, Tokyo, Japan) equipped with a CAPCELL PAK C18 MG II (250 mm × 4.6 mm I.D.; Shiseido, Tokyo, Japan) HPLC column, a UV-2075 light source, and an ultraviolet detector (JASCO). The mobile phase was water–acetonitrile–methanol (55:25:20, v/v/v), which was degassed in an ultrasonic bath before using. In addition, the flow rate was 0.5 mL/min at ambient temperature, and we performed the sample detection at 250 nm. The amount of GO-Y022 was corrected by the tissue wet weight, and indicated as ng/g tissue. Of note, we also measured blood concentrations of GO-Y022 as bellow. Five milligram of GO-Y022 (mixed with 200 μL of 1% methyl cellulose (Wako)) was orally administered by using a flexible sonde (5202, FUCHIGAMI, Kyoto, Japan) to the twelve week-aged B6 male mice, which were anesthetized with intraperitoneal injection of 50 μL of the mixture of 10 mg of midazolam (SANDOZ, Tokyo, Japan), 0.75 mg of medetomidine (ZENOAQ, Koriyama, Japan), and 12.5 mg of butorphanol (Wako) dissolved in 25 mL of distilled water after one-day fasting. Blood samples were collected at 1, 2, 4, 8 and 24 h after administration (n = 3). The same amount of 200 μL of 1% methyl cellulose alone was orally administrated to the control group mice under same anesthesia, and the blood samples were obtained at the same intervals (n = 3). The blood concentration of GO-Y022 and their metabolites were determined by following HPLC method. After addition of 200 μL acetonitrile a 50 μL blood sample, the solution was vortexed for 30 s. This mixture was spun for 5 min at 13,000g. The clear supernatant was filtered through a Millipore filter (0.45 μm; Millex-LH®, Tokyo, Japan) and was then injected into the HPLC apparatus.

Fig. 2. The growth inhibition of gastric cancer cell lines treated with GO-Y022. The growth inhibition was calculated as the percentage of the cell numbers against the mock (vehicle alone, 1% DMSO). Open circle indicates curcumin, and the closed circle indicates GO-Y022. *P < 0.01; **P < 0.05.
2.12. Mass spectrometry

Quantification of GO-Y022 in curry was outsourced to Japan Food Research Laboratories (Tokyo, Japan). The procedures were described briefly. GO-Y022 was dissolved in methanol, and diluted with water/methanol (1:1) solution to make standard solution ranging 0.0001–0.01 mg/L. Five gram of curry sample was mixed with 20 mL of hexane, then homogenized with 40 mL of hexane saturated acetonitrile. After centrifugation (2000 g, 5 min), the under (acetonitrile) layer was collected. Then, remaining hexane layer was again mixed with 40 mL of acetonitrile, and centrifuged. This step was repeated two times. The collected acetonitrile layer was mixed. The volume was measured, and one of 200 mL was applied to Sep-Pak Plus C18 column (Waters, Tokyo, Japan) conditioned by acetonitrile. Then, the sample was eluted with 1 mL of acetonitrile. The eluent was dried, then solved in 2 mL of water/methanol (1:1) solution, and finally it was diluted with water/methanol (1:1) to 5-fold dilution. Two microliter of sample solution was analyzed by liquid chromatography-tandem mass spectrometry (LC unit: Agilent 1100 series (Agilent Technologies, Tokyo, Japan), MS unit: API-4000 (Applied Biosystems, Waltham, MA)). The operation condition was described bellow: column (CAPCELL PAK C18 MG II (SHISEIDO, Tokyo, Japan)), column temperature (40 °C), mobile phase (A; 0.1 vol% formic acid-acetonitrile, B; 0.1 vol% formic acid solution)

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Flow rate: 0.2 mL/min, Injection volume: 2 μL, Ionization mode: ESI (+), Curtain gas: 40 psi, Nebulizer gas: 60 psi, Drying gas: 70 psi, Drying gas temperature: 650 °C, Collision gas: nitrogen, Ionization: 5000 V, Declustering potential: 86 V, Multiple reaction monitoring transition (m/z), collision energy (eV): 327.2 > 89.2 (65), 327.2 > 145.1 (39), 327.2 > 177.2 (29).

Pyrolysis was conducted by heating 100 g of curry roux by microwave (250 °C for 20 min).

2.13. Statistical analysis

Data are presented as mean ± standard deviation. The between-group differences were analyzed using the Fisher's exact probability test or unpaired Student's t-test (two-sided) with StatMate III version 3.14 (ATMS, Tokyo, Japan) or BellCurve for Excel version 2.00 (Tokyo, Japan).

3. Results

3.1. GO-Y022 suppressed the growth of gastric cancer cell lines

We examined the growth inhibitory potential of GO-Y022 in four gastric cancer cell lines, including GCIY, KATO III, SH-10-TC, and H-111-TC. The IC50 value for GO-Y022 after 96 h treatment for GCIY was 7.32 μM, whereas that for curcumin were 30.4 μM. That of GO-Y022 for KATO III was 5.98 μM, whereas that of curcumin for KATO III was 24.8 μM. Those of GO-Y022 were 3.28 μM for SH-10-TC, and 3.67 μM for H-111-TC, respectively, whereas those of curcumin were 31.5 μM for SH-10-TC, and 25.8 μM for H-111-TC, respectively. The inhibition of growth was significantly higher with GO-Y022 than curcumin in all four gastric cancer cell lines (Fig. 2). The IC50 value was 4.2-fold higher in GCIY, and 4.1-fold higher in KATO III. Those were 9.6-, and 7.0-fold higher in SH-10-TC, and H-111-TC, respectively. In addition, the average IC50 of GO-Y022 was 5.06 ± 1.88 μM, which was significantly lower than that of curcumin (27.4 ± 3.55 μM; P < 0.001). These in vitro findings suggested that the growth inhibitory potential of GO-Y022 was 5.4-fold higher than that of curcumin.

Next, we assessed the growth property of each gastric cancer cell line in the presence of 5 μM GO-Y022. The same concentration of curcumin could not inhibit the growth at 24 h following the treatment for all four cell lines (Fig. 3). Except for GCIY, 5 μM GO-Y022 decreased the growth < 50% of the control for three cell lines at 24 h (Fig. 3). For GCIY, 10 μM GO-Y022 decreased the growth < 50% of the control (Fig. 3). In addition, three of four gastric cancer cell lines could not grow in the presence of 5 μM GO-Y022, whereas those could grow in the
presence of 5 μM curcumin. Overall, these findings suggested that GO-Y022 exhibited a stronger growth inhibitory potential than curcumin.

3.2. Apoptosis induction with GO-Y022

We assessed the amount of an apoptosis indicator, soluble caspase-cleaved keratin 18 by ELISA at 24 h after treatment. And those amounts were compared with the mock-treated cells (control) was indicated as 1.0. These relative apoptosis levels to the mock were indicated in each treated cells with 5 μM of curcumin or GO-Y022. The statistical significance is indicated by asterisks.

After 24 h treatment, the mean relative level of apoptosis in GCIY cells treated with 5 μM GO-Y022, evaluated per the rate of apoptosis in cultures treated with the vehicle DMSO that was set to 1.0, was 3.48 ± 0.64, whereas that with 5 μM curcumin was 1.25 ± 0.42 (Fig. 4). For KATO III, the relative apoptosis level was 10.85 ± 0.21, whereas that with 5 μM curcumin was 0.70 ± 0.58. For H-111-TC, that was 3.24 ± 0.44, whereas that with 5 μM curcumin was 0.65 ± 0.28. GO-Y022 exhibited 2.78-fold higher potential of the apoptosis

Fig. 4. The apoptosis induction with GO-Y022 in gastric cell lines. The amount of an apoptosis indicator, soluble caspase-cleaved keratin 18 was determined by ELISA. The amount of soluble caspase-cleaved keratin 18 in the mock-treated cells (control) is indicated as 1.0. The relative values to the mock were indicated in each treated cells with 5 μM of curcumin or GO-Y022. The statistical significance is indicated by asterisks.

Fig. 5. Effects on the phosphorylation of STAT3 and β-catenin by GO-Y022. A. The levels of phosphorylated STAT3 (pSTAT3) were determined by ELISA methods. The amounts of pSTAT3 in the mock-treated cells (1% DMSO) treatment as a control are indicated as 1.0 (open rectangle). The relative values of pSTAT3 to the mock in each treated cells with 5 μM of curcumin (rectangle with diagonal line) and GO-Y022 (rectangle with vertical line) were indicated. The statistical significance is indicated by asterisks. B. The levels of β-catenin were determined by ELISA methods. The amounts of β-catenin in the mock-treated cells (1% DMSO) treatment as a control are indicated as 1.0 (open rectangle). The relative values of β-catenin to the mock in each treated cells with 5 μM of curcumin (rectangle with diagonal line) and GO-Y022 (rectangle with vertical line) were indicated. The statistical significance is indicated by asterisks.
induction than curcumin in GCIY. GO-Y022 also exhibited 15.6-fold and 4.98-fold higher apoptosis induction in KATO III and H-111-TC cell lines, respectively. However, for SH-10-TC, that was 9.40 ± 0.15, whereas that with 5 μM curcumin was 8.99 ± 0.23. That was merely 1.05-fold higher than curcumin. So we confirmed the data by FACS analysis (Fig. S1). Apoptosis/necrosis fraction was 25.5% in 5 μM of curcumin treated SH-10-TC cells, whereas that was 28.8% in the mock-treated cells. On the other hand, that was 34.9% in 5 μM GO-Y022 treated SH-10-TC cells. Further, that was 45.3% in 10 μM GO-Y022 treated SH-10-TC cells, whereas that was 37.9% in 10 μM curcumin treated SH-10-TC cells. Similar result was obtained and indicated that SH-10-TC cells was sensitive to GO-Y022 to induce apoptosis as well as curcumin.

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jff.2018.09.033.

The apoptosis induction might be one of the causes of growth inhibition of GO-Y022.

3.3. In vitro effects on the STAT3 phosphorylation and β-catenin activation with GO-Y022

Several lines of evidence suggest that the suppression of the β-catenin pathway and phosphorylation of STAT3 at Tyr705 contribute to the inhibition of gastric carcinogenesis (Clements, Wang, & Sarnaik, 2002; Tye, Kennedy, & Najdovska, 2012). As our newly synthesized curcumin analogs have similar diarylpentanoid structures, they are supposed to have same targets molecules. For examples, GO-Y030 and GO-Y031 have suppressive effect on the STAT3 phosphorylation and β-catenin activation and STAT3 phosphorylation (Uehara et al., 2014). Therefore, we also examined the degradation of β-catenin with GO-Y022. GO-Y030 and GO-Y031 can induce the degradation of β-catenin. Therefore, we also examined the degradation of β-catenin with GO-Y022.

3.3.1. Inhibition of pSTAT3

The relative pSTAT3 level per cell, compared with mock (1% DMSO) was 0.111 ± 0.011 at 5 μM of GO-Y022, in KATO III, whereas that was 0.621 ± 0.046 in KATO III at 5 μM curcumin. The relative pSTAT3 level was 0.850 ± 0.016 in SH-10-TC with 5 μM GO-Y022, whereas that was 0.661 ± 0.046 in SH-10-TC at 5 μM curcumin. The relative pSTAT3 level was 0.196 ± 0.097 in H-111-TC, whereas that was 0.958 ± 0.060 in H-111-TC at 5 μM curcumin. The relative pSTAT3 level was 1.063 ± 0.007 in GCIY with 5 μM GO-Y022, whereas that was 1.002 ± 0.040 in GCIY at 5 μM curcumin (Fig. 5A).

Inhibition of pSTAT3 with 5 μM of GO-Y022 was apparent in KATO III and H-111-TC, however that was not observed in SH-10-TC and GCIY.

The relationships among the IC₅₀ values, the relative apoptosis induction (at 5 μM) and the relative inhibition of pSTAT3 (at 5 μM) varied among cell lines. To examine the effect of pSTAT3 inhibition on the gastric cancer cell lines, we used cryptotanshinone (CTS), a known STAT3 phosphorylation inhibitor (Donmez, Demirezen, & Beksac, 2016). The IC₅₀ value of CTS for KATO III was 4.29 μM. That for GCIY was 14.02 μM. That for SH-10-TC was 6.91 μM. However, that for H-111-TC was > 100.0 μM (Fig. S2).

3.3.2. Effect on β-catenin

GO-Y030 and GO-Y031 can induce the degradation of β-catenin. At 5 μM, GO-Y022 did not decrease the relative levels of activated β-catenin per cell (Fig. 5B); the relative levels to the control (1% DMSO) was 1.566 ± 0.163 in GCIY. That was 2.988 ± 0.593 in KATO III. Those were 1.136 ± 0.099 in SH-10-TC, and 3.057 ± 0.252 in H-111-TC cells, respectively. In contrast, that with 5 μM curcumin in GCIY was 1.198 ± 0.060, that in KATO III was 0.638 ± 0.112, that in SH-10-TC was 1.117 ± 0.056, and that in H-111-TC was 1.009 ± 0.065

![Image](https://example.com/image1.png)

**Fig. 6.** The in vivo inhibition of gastric cancer in a mouse model with the oral administration of GO-Y022. A, Representing appearance of GO-Y022-treated gastric tumors. Twenty-five milligram of GO-Y022 was mixed in 5 g of HFD were given daily and freely to each mouse. Asterisks indicate the tumors. The average tumor incidence was two in a mouse. B, HFD-alone-treated gastric tumor. Red asterisk indicates tumor. C and D, pathological views of A and B, respectively. Bars indicate 500 μm. E. The volume of each tumor in the GO-Y022-treated (n = 7) and the HFD-mice (n = 6). The average volume with standard deviation of each group is indicated (p = 0.0585). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
(Fig. 5B). In the cases of the 5 μM curcumin treatment, the levels of activated β-catenin per cell were not decreased and comparable with the control. Different from pSTAT3, β-catenin was activated in the surviving cells treated with 5 μM GO-Y022. This phenomenon was similar to the observation where β-catenin showed intense positivity in the non-apoptotic epithelial cells around the apoptotic cells (Ge, Yang, & Chen, 2015).

Degradation of β-catenin by GO-Y022 was not observed. The action mechanisms of GO-Y022 was different from another curcumin analog such as GO-Y030 and GO-Y031 in this aspect.

3.4. In vivo inhibitory potential of GO-Y022 in a mouse model of gastric cancer

We assessed the in vivo ability of GO-Y022 to inhibit gastric carcinogenesis in Gan mice that were freely administered 0.5% (weight/weight) GO-Y022 orally for 8 weeks. Histopathologically, all gastric tumors were suggested to be adenocarcinomas. As the tumors grew in a block, it became difficult to count the multiplicity of tumors; thus, we evaluated the volume of tumors. The average volume of gastric tumors in mice treated with GO-Y022 for 8 weeks was 39.9 ± 41.8 (range: 0.5–150.0) mm³, whereas that in mock-treated mice was 106.7 ± 115.4 (range: 4.0–322.5) mm³ (p = 0.059; Fig. 6), which suggested that GO-Y022 could apparently inhibit the gastric cancer growth in vivo. In addition, we determined the expression of GO-Y022-target molecules β-catenin and pSTAT3 by immunohistochemistry (Fig. S3). The disappearance of the β-catenin signal in the cytosol, which revealed the accumulation of β-catenin, an indicator of the β-catenin activation, was not apparent. However, the inhibition of the nuclear staining for pSTAT3 was apparently detected by immunohistochemistry (IHC). By IHC of Gan mice treated with HFD, it was shown that pStat3 in the nucleus was observed in many cells, however that was not so frequently observed in the epithelial cells in mice treated with GO-Y022 (Fig. S3A and B). Quantitation of the positivity of pStat3 expression indicated that the percentage of pStat3-positive cells was 64.4 ± 10.1% (range: 54.0–82.0) in Gan mice fed with mock, whereas that was 20.3 ± 15.2% (range: 2.0–38.0) in Gan mice fed with GO-Y022 (p = 0.001, Fig. S3C). This data showed that GO-Y022 could inhibit phosphorylation of Stat3 in vivo, and that is a candidate target molecule of GO-Y022.

3.5. Localization of GO-Y022 in mice

We assessed localization of GO-Y022 in Gan mice by HPLC. The tissue GO-Y022 concentrations in the heart, kidney, liver, spleen, as well as the peripheral blood, were negligible in the treated mice (Fig. 7). Conversely, GO-Y022 was detected in the digestive tract, including the esophagus, stomach, and small and large intestines of the treated mice (Fig. 7). In addition, the concentrations of GO-Y022 ranged from 647 ng/g (tissue in the esophagus), 570–2590 ng/g (tissue in the stomach), to 40–446 ng/g (tissue in the small intestine; Fig. 7). Detection of GO-Y022 in the tissues varied among samples. We guess this variation was influenced by free eating, not gavage. Actually, the left over of feed was difficult to count the multiplicity of tumors; thus, we evaluated the volume of tumors. The average volume of gastric tumors in mice treated with GO-Y022 for 8 weeks was 39.9 ± 41.8 (range: 0.5–150.0) mm³, whereas that in mock-treated mice was 106.7 ± 115.4 (range: 4.0–322.5) mm³ (p = 0.059; Fig. 6), which suggested that GO-Y022 could apparently inhibit the gastric cancer growth in vivo. In addition, we determined the expression of GO-Y022-target molecules β-catenin and pSTAT3 by immunohistochemistry (Fig. S3). The disappearance of the β-catenin signal in the cytosol, which revealed the accumulation of β-catenin, an indicator of the β-catenin activation, was not apparent. However, the inhibition of the nuclear staining for pSTAT3 was apparently detected by immunohistochemistry (IHC). By IHC of Gan mice treated with HFD, it was shown that pStat3 in the nucleus was observed in many cells, however that was not so frequently observed in the epithelial cells in mice treated with GO-Y022 (Fig. S3A and B). Quantitation of the positivity of pStat3 expression indicated that the percentage of pStat3-positive cells was 64.4 ± 10.1% (range: 54.0–82.0) in Gan mice fed with mock, whereas that was 20.3 ± 15.2% (range: 2.0–38.0) in Gan mice fed with GO-Y022 (p = 0.001, Fig. S3C). This data showed that GO-Y022 could inhibit phosphorylation of Stat3 in vivo, and that is a candidate target molecule of GO-Y022.

3.6. Safety profile of GO-Y022

First, we examined the effect of GO-Y022 on the normal gastric epithelium using a primary culture of HGaEpC. GO-Y022 has a growth inhibitory effect on HGaEpC, and the IC50 value was 3.08 μM (Fig. S5). Spheroids of HGaEpC were observed at 10 μM, but they were absent at 50 μM. The cells were entirely killed at 50 μM.

We assessed the hepatic and renal toxicity profiles of GO-Y022 by measuring Cre, AST, ALT, LDH, and T-Bil levels (Fig. 8). While Cre levels were 0.12–0.20 mg/dL in GO-Y022-treated mice, they were 0.14–0.20 mg/dL in mock-treated mice. The AST levels were 52–108 IU/L in GO-Y022-treated mice and they were 60–186 IU/L in mock-treated mice. In addition, the ALT levels were 18–70 IU/L in GO-Y022-treated mice and 18–126 IU/L in mock-treated mice. Those of LDH were 322–758 IU/L in GO-Y022-treated mice and they were 334–674 IU/L in mock-treated mice. Finally, the T-Bil levels were 0.04–0.14 mg/dL in GO-Y022-treated mice and 0.06–0.10 mg/dL in mock-treated mice.

The levels of serum AST and ALT are parameters of liver damage. In both mice, the levels of AST and ALT were higher than the normal limits. That was because HFD diet induced a fatty change of liver in both mice. However, there were no differences between the mock-treated mice and in GO-Y022-treated mice. We concluded that additional liver damage was not induced in GO-Y022-treated mice.

The serum Cre level is a parameter of renal damage. The serum creatinine levels were within the normal limit, and there were no differences between both groups. We considered that no renal damage was induced in GO-Y022-treated mice.

These data suggested that additional damages to the liver or kidney were not induced in GO-Y022-treated mice. Furthermore, body weights of GO-Y022-treated mice ranged 23.7–37.6 (average: 32.3 ± 5.88) g, whereas those of the mock mice ranged 21.7–46.1 (average: 33.9 ± 13.8) g; we observed no significant difference in the body weight between the two groups. Overall, these findings demonstrated that 0.5% GO-Y022 administrated had a safe profile in mice.
3.7. GO-Y022, as an ingredient of cooked curry

We determined the GO-Y022 content in cooked curry, which is a regularly consumed food prepared from commercially available retort-pouch preparations, by mass spectrometry. Specifically, we tested nine different curries, revealing that the GO-Y022 concentrations varied from zero to 98 μg/100 g curry (Table S2). In addition, heating the curry at 250 °C for 20 min led to an increase in the GO-Y022 content to 110 μg/100 g curry. Although the amount of GO-Y022 in the commercially available curries was minimal, we already experienced eating GO-Y022, a C5 analog of curcumin.

4. Discussion

This study demonstrates that dietary GO-Y022, a C5 curcumin analog, inhibited the gastric cancer growth both in vitro and in vivo. The growth suppression potential of GO-Y022 was higher than that of curcumin in four gastric cancer cell lines. Except for GCIY, 5 μM GO-Y022 decreased the growth < 50% of the control. In addition, it was a more potent inducer of apoptosis compared with curcumin in all cell lines. Histopathologically, GCIY is a poorly differentiated adenocarcinoma and belongs to a diffuse type based on the Lauren classification (Maki, Nobutake, & Ken-ichi, 2013). Kato III is a signet-ring cell carcinoma and belongs to a diffuse type. SH-10-TC is a mucinous adenocarcinoma and belongs to a diffuse type. H-111-TC is a well-differentiated adenocarcinoma and belongs to an intestinal type. We anticipated that GO-Y022 could be more effective in some gastric cancer cell lines, depending on the pathological subtypes. In this study, we elucidated that GO-Y022 suppressed pSTAT3 in vitro except GCIY, and in vivo.

Inhibition of pSTAT3 with 5 μM of GO-Y022 was strongest in KATO III among four cell lines, and KATO III was also most sensitive to CTS. Apoptosis induction with 5 μM of GO-Y022 in KATO III was also highest. KATO III is most sensitive to pSTAT1 inhibition including GO-Y022 (Table S1). Inhibition of pSTAT3 with 5 μM of GO-Y022 was not observed in GCIY, and GCIY was also rather resistant to CTS. GCIY seemed to be most resistant to pSTAT3 inhibition, including GO-Y022. Apoptosis induction with 5 μM of GO-Y022 in GCIY was lower.

Inhibition of pSTAT1 with 5 μM of GO-Y022 was weaker in H-111-TC, however SH-10-TC was relatively sensitive to CTS. Apoptosis induction with 5 μM of GO-Y022 in SH-10-TC was stronger. SH-10-TC is also sensitive to pSTAT3 inhibition (Table S1). Finally, inhibition of pSTAT3 with 5 μM of GO-Y022 was stronger in H-111-TC, however H-111-TC was most resistant to CTS growth inhibition. H-111-TC seemed to be most resistant to pSTAT3 inhibition. Although GO-Y022 suppressed pSTAT3 expression, apoptosis induction with 5 μM of GO-Y022 in H-111-TC was lowest. There might be some resistant mechanisms to apoptosis occurred in the downstream signaling of pSTAT3. It is speculated that growth suppression was due to different mechanism from apoptosis in H-111-TC (Table S1).

Inhibition of pSTAT3 with GO-Y022 might depend on cell lines. The resistance to a STAT3 inhibitor, CTS varied among cell lines. These differences might be reflected by the molecular backgrounds of the cells. The underlying mechanisms of sensitivity should be elucidated.

In mice, together with the prostaglandin E synthase (PTGES) overexpression, Wnt signaling and cyclooxygenase-2 (COX2) expression contributed to gastric carcinogenesis. Reportedly, a positive feedback loop between STAT3 and COX2 contributes to gastric tumorigenesis (Xiong, Du, & Sun, 2014). Furthermore, the Wnt/β-catenin pathway was reported to cross-talk with STAT3 signaling in the retina (Fragoso, Patel, & Nakamura, 2012). Moreover, PTGES-derived prostaglandin E2 was illustrated to stimulate STAT3 to promote apoptosis of podocytes (Yu, Wu, & Wang, 2017). Notably, STAT3 plays a vital role in the intersection of the Wnt, COX2, and PTGES signaling pathways and could affect them. This study suggests pSTAT3 as one of the key molecules controlling gastric carcinogenesis and that the inhibition of pSTAT3 could suppress the growth of gastric cancer. In GCIY, the inhibition of pSTAT3 with GO-Y022 was insufficient, and GCIY might be relatively resistant to GO-Y022.

This study confirmed that the dietary GO-Y022 is present in commercially available curry pastes. Evidently, GO-Y022 can be ingested by humans. We observed no adverse effects with the administration of 5 mg GO-Y022 to a 20 g mouse per day. A phase I clinical trial reported the tolerability of a daily curcumin dose of 8 g (Cheng, Hsu, & Lin, 2001). However, whether an equivalent GO-Y022 dose is safe in humans remains unclear.

GO-Y022 is insoluble in water and, thus, cannot be administrated intravenously. This study demonstrates that the blood GO-Y022 concentration was insignificant. Nonetheless, orally administrated GO-Y022 can be directly transferred into the tumors, as well as to the luminal surface of the digestive tract. However, we did not detect apparent toxicity in the epithelial cells of the digestive tract. Moreover, GO-Y022 had somewhat a growth suppressive effect on the primary gastric epithelial cells less than 10 μM, but a killing effect was apparent at as high as 50 μM. These aspects of GO-Y022 highlight its benefit in controlling malignancies of the digestive tract by topical application. Furthermore, contingent upon the absence of long-term toxic effects, dietary GO-Y022 should be considered for chemoprevention of gastrointestinal cancers.

In this study, we also assessed whether GO-Y022 could be ingested as a supplement by determining the amount of GO-Y022 in...
commercially available curry pastes and determined that its concentration ranged from zero to 100 μg in 100 g curry paste, which varied among the samples. Notably, there remains a room for the development of better recipes for curry paste with higher GO-Y022 concentrations.

Whether C5 analogs other than dietary GO-Y022 can be ingested safely remains unexplored. Recently, we developed the most potent C5 analog (1E, 4E)-1,5-bis-(3,5-bismethoxymethoxyphenyl) penta-1,4-dien-3-one (GO-Y030) with 10 and 2 times the potency of curcumin and GO-Y022, respectively, to inhibit the growth of various cancer cells (Ohori et al., 2006). The oral administration of GO-Y030 at the same dose as GO-Y022 did not correlate with any apparent toxicities in mouse models. Of note, GO-Y030 is insoluble in water and is not absorbed systemically. If the safety profile of the orally administered GO-Y030 is found to be similar to that of GO-Y022, GO-Y030 could be a better alternative in the prevention of malignancies of the digestive tract by the topical use through oral administration.

5. Conclusions

A diarylpentanoid analog of curcumin, GO-Y022 is included in curry, and human beings have been eating it for many thousands years. This compound formed from pyrolysis of curcumin. It has a growth inhibitory and an apoptosis inducing effects on gastric cancer cells mainly via pSTAT3 inhibition. GO-Y022 could inhibit the growth of gastric tumors in mouse model with safe.

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Ethical issues

All animal experiments were performed humanely and complied with the guidelines set by Akita University and were approved by the associated ethics committee.

Disclosure of potential conflicts of interest

There are no conflicts of interest in this study.

References


