A stereo-selective growth inhibition profile of ginsenoside Rh2 on human colon cancer cells

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1. Introduction

Ginseng (the root of Panax ginseng Meyer, family Araliaceae) has long been used as a traditional medicine in East Asia (China, Korea, and Japan) (Xu, Choi, & Huang, 2017). Recently, ginseng has attracted attention not only in the Asian market but also in many Western countries as a medicinal herb (Arring, Millstine, Marks, & Nail, 2018). Ginseng contains multiple substances that act as immune, stress and cholesterol modulators, such as acidic polysaccharides, ginsenosides, polyacetylenes, sesquiterpenes and polyphenolic compounds (Cho et al., 2013). Among these, ginsenosides, belonging to the dammarane triterpene saponin group, are regarded as key functional components of the health benefits of ginseng (Ku, You, Park, & Ji, 2015; Lee & Kim, 2014). Ginsenosides have been reported to have significant in vivo hypoglycemic, hepatoprotective, anti-allergic, and anti-carcinogenic effects (Chen et al., 2015; Li et al., 2018; Ning et al., 2018; Yang et al., 2017; Zhou et al., 2018). To date, >100 kinds of ginsenosides have been isolated from ginseng (Kim, Yi, Kim, & Cho, 2017). Since these ginsenoside molecules have different structures, their biofunctional effects in the human body differ (Ku, 2016). Generally, the structure-specific effects of ginsenosides have been investigated in two major ways: (i) level of sugar and (ii) OH side chains attached to the dammarane skeleton (17 carbons in a four-ring structure). First, many researchers have studied how the numbers and positions of sugar moieties attached to ginsenosides can affect the physiological changes of ginsenosides, and it has been reported that ginsenoside aglycones are more potent than glycoside forms. Several groups reported that ginsenosides with fewer sugar moieties, such as Rh2, Compound K, and PPD, had stronger anti-tumor effects than those with more sugar residues such as Rb1, Rb2, and Rc (Dong et al., 2011; Popovich & Kitts, 2002). Recently, various food and cosmetic companies have released ginseng products containing ginsenoside aglycones and used their inclusion as a marketing tool. Second, ginsenosides can be divided into two groups based on the number of hydroxyl groups...
attached to the ginsenoside skeleton: protopanaxadiols (PD), which contain two hydroxyl groups at positions C-3 and C-20 (e.g., ginsenosides Rb1, Rb2, Rc, Rd, Rg3, and Rh2), and protopanaxatriols (PT), which contain three hydroxyl groups at positions C-3, C-6, and C-20 (e.g., ginsenosides Re, Rg1, and Rh1, and notoginsenoside R1). Among these ginsenoside molecules, PD types such as Rh2, Compound K, and Rg3 have been reported to show potent apoptotic effects on various cancer cell lines and in vivo models (Dong et al., 2011; Kim et al., 2009; Li et al., 2018; Liu, Bu, Yan, & Jia, 2007; Zheng, Jeong, Song, & Ji, 2011; Zou, Wang, Gao, Han, & Fang, 2018).

In addition to sugar levels and numbers of hydroxyl groups, recent scholarship is increasingly interested in studying the functional changes of ginsenoside molecules by stereoisomers. Specifically, 20(S) and 20(R) ginsenoside Rh2s are stereoisomers that have the same molecular (C36H32O9) formula and sequence of bonded atoms. However, these two ginsenoside Rh2s have different three-dimensional shapes due to the dissimilar orientation of the C-20 OH group in ginsenoside Rh2. The OH group of 20(S) is three-dimensionally closer to the C-12 OH group of ginsenoside Rh2. However, the OH group in 20(R) is relatively far away from the C-12 OH side chain compared to 20(S). This difference in the three-dimensional orientations of the hydroxyl groups of ginsenoside Rh2s potentially results in dissimilar biofunctionalities (Figure 1).

The colon cancer is a malignant tumor consisting of cancer cells that develop in the large intestine. Though early detection and treatment of colon cancer can improve the therapeutic effect, many patients find colon cancer after progression due to the lack of early-stage symptoms (Alonso-Abreu et al., 2017). When colon cancer is diagnosed, surgery and medicinal therapy with 5-fluorouracil (5-FU), bevacizumab, and cetuximab are generally performed (Kuipers et al., 2015). However, the chemotherapy and targeted therapy showed diverse side effects such as alopecia and hypertension, so supplement with traditional non-toxic medicinal substance could be beneficial for prevention and treatment (Kuipers et al., 2015; Scheithauer et al., 2003).

Recently, ginsenosides have been in the spotlight as anticancer agents in the nutraceutical market. However, the literature based on objective evidence regarding the stereoselective activity and growth pattern of cancer cells in real time is still lacking. Therefore, the present study examined the efficacy of Rh2-dependent cancer cell repression on enantiomer status using two Rh2 stereoisomer forms (i.e. 20(S) and 20(R)). Further, using a non-invasive, real-time cellular analysis (RTCA) method allowed for the understanding of cellular status and growth pattern of the HT-29 colon cancer cells with different concentrations of both types of two Rh2 stereoisomer forms.

1.1. Growth inhibitory effect of 20(S)-Rh2 on a colon cancer cell line

HT-29 colon cancer cells were treated with 50 μM of each form of Rh2 for 72 h, then an MTT assay was performed to assess their growth-inhibitory effects. The growth inhibitory effect on HT-29 colon cancer cells was observed through a cell viability ratio and compared with the vehicle control (0.1% (v/v) DMSO) as 100%. A non-treated group was used to determine whether the 0.1% DMSO used in the vehicle control group had any effect. The results indicated that the DMSO had no impact. As shown in Figure 2, each substance was pretested in 50 μM of concentration, and the viability of HT-29 cells treated with 20(S)-Rh2 was found to be significantly lower than that of the control (p < .05). Also, as shown in Figure 3(a), the growth of HT-29 cells treated with 20(S)-Rh2 was reduced in a dose-dependent manner compared with the vehicle control and the 20(R)-Rh2 ginsenoside-treated group. Cancer cell viability was significantly reduced by 20(S)-Rh2 treatment. However, 20(R)-Rh2 ginsenoside treatment did not show any significant decrease in cancer cell viability (Figure 3(b)). We also performed a viability assay of fetal human cells, FHC cell line (ATCC, Manassas, VA, USA) as normal colon cells at the concentration of 50 μM of 20(S)-Rh2 for 72 h. The viability of normal cell was 90 ± 4.6% (vehicle: 100%) presenting very low growth inhibitory effect compared to the viability of HT-29 cells at the same concentration, 32.2 ± 4.5%, so we could clarify the anticancer activity of 20(S)-Rh2 was cancer cell-specific effect.

1.2. Real-time growth pattern of colon cancer cells treated with Rh2

To monitor simultaneous cellular status and growth pattern, RTCA was performed in the presence of 2% fetal bovine serum (FBS) with 10, 25, 50, and 75 μM of both types of Rh2s. At 10 μM, neither of the Rh2 types caused a cellular profile change, but at higher concentrations, the two types of Rh2 produced different profiles. The treatment with 25 μM of 20(S)-Rh2 showed a cytostatic profile from 18 to 42 h after treatment, followed by a loss of viability (Figure 4(a)), whereas 20(R)-Rh2 showed no change of profile (Figure 4(b)). Cells treated with 50 μM of 20(S)-

Figure 1. Chemical structures of the ginsenosides 20 (S)-Rh2 and 20 (R)-Rh2.

Figure 1. Estructuras químicas de los ginsenosidos 20 (S)-Rh2 y 20 (R)-Rh2.
Rh2 maintained growth for 7 h, but after that, a loss of viability was shown in the profile. When the HT-29 cells were treated with 75 μM 20(S)-Rh2, the cells were detached from the surface of the culture well and died immediately after treatment (Figure 4a).

2. Discussion

According to a number of stereochemistry studies, certain compounds such as ibuprofen and thalidomide can produce different physiological effects depending on their racemic status (Sanganyando, Lu, Fu, Schlenk, & Gan, 2017). The pharmaceutical effects of ibuprofen on pain reduction and anti-inflammation are mainly due to S-form ibuprofen (Evans, 2001; Kaehler, Phleps, & Hesse, 2003). By removing R-form ibuprofen from an existing racemic mixture, the side effects and dosages could be reduced, and medicinal effects could be enhanced (Kaehler et al., 2003). Thalidomide, used as a sedative for pregnant women in the 1960s who subsequently gave birth to numerous-deformed babies, is also a racemic mixture. Although R-type thalidomide has a sedative effect, S-type thalidomide has a teratogenicity effect (Blaschke, Kraft, Fickentscher, & Kohler, 1979; Eriksson, Bjorkman, Roth, & Hoglund, 2000; Heger et al., 1994; Hoglund, Eriksson, & Bjorkman, 1998). As shown in the example above, when an enantiomer of a useful substance exists under development, it should be verified that there is a difference in activity depending on each form.

Although studies on the anti-cancer activity of ginsenoside Rh2 have been performed on lung, breast, liver, colorectal cancers, and leukemia, there has been no study of the cellular pattern of cancer cells when Rh2 was treated in real-time (Ge, Yan, & Cai, 2017; Han et al., 2016; Kim & Choi, 2016; Li, Li, Dong, Wang, & Li, 2017; Ren, Shi, Teng, & Yao, 2018; Wan et al., 2017). Among the existing studies, there is a study on how Rh2 using HCT-116 cells can act as a specific mechanism of anti-cancer activity for a colorectal cell line (Han et al., 2016). This study reported that only 20(S)-Rh2 showed anti-cancer activity when 20(R)-Rh2 or 20(S)-Rh2 were treated with HCT-116 cells. It also demonstrated that 20(S)-Rh2 had the effect of inhibiting the IL-6-mediated tumor invasion process of HCT-116 cells. However, studies on the specific mechanisms that inhibit the growth of colon cancer cells have not yet been conducted. Other groups and researchers have observed that the viability of cancer cells is inhibited by ginsenoside Rh2 through an end-point assay similar to the study method used in this study. However, the end-point assay approach did not use RTCA to determine a timely pattern for how ginsenoside Rh2 inhibited cell growth.

Observation of real-time cellular patterns reveals information that cannot be obtained from an end-point assay (Xing, Zhu, Gabos, & Xie, 2006; Yu et al., 2006). With RTCA, it is possible to evaluate how soon the cell inhibitory effect is activated after treatment (Jeong, You, & Ji, 2012; Lohberger et al., 2013). It can also distinguish whether the treatment is a cytotoxic reagent that acts immediately, an apoptosis-inducing substance or substances that delay the growth of cells. In RTCA, the cell index did not increase or decrease during the 72-h treatment with 20(R)-Rh2. This result seems to be due to the fact that the cells in wells had changed to a confluent form. In addition, even though the cells were confluent, it was confirmed that sufficient nutrient in the medium did not lead to cell death. However, no significant difference in cell growth inhibition was observed when 20(S)-Rh2 or 20(R)-Rh2 at 10μM was treated with cancer cells under the...
same experimental conditions. When the concentration of ginsenoside was increased to 25 µM, a cytostatic pattern from 18 to 42 h later, the cell index had dramatically decreased. This outcome suggests that HT-29 cells exhibit a pattern similar to that observed in the process of apoptosis after G1 phase cell cycle arrest, as reported in a previous study of ginsenoside compound K, an isomer of ginsenoside Rh2 (Jeong et al., 2012). However, further studies that include cell cycle analysis and mechanism-related gene or protein expression are needed in order to clarify the stereo-selective growth inhibitory activity of ginsenoside Rh2 on colon cancer cells.

3. Conclusions

Among the two enantiomers of ginsenoside Rh2 tested in this study, 20(S)-Rh2 proved to be an effective reagent for cancer cell inhibition. The patterns of real-time cellular response are either different dependent on the enantiomeric form. Taken together, these results provide novel evidence that the chiral characteristics of ginsenoside Rh2 enantiomeric pairs exhibit stereo-selective growth inhibition effects on cancer cells.

4. Materials and methods

4.1. Chemicals and reagents

We purchased commercially available 20(S)-ginsenoside Rh2 (LKT Laboratories, Saint Paul, MN, USA) and 20(R)-ginsenoside Rh2 (LKT Laboratories, Saint Paul, MN, USA), and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). The materials used for cell culture including media, fetal bovine serum (FBS), antibiotic solutions, and related reagents were obtained from Gibco® (Invitrogen Life Technologies, Carlsbad, CA, USA).
4.2. Cell culture

The human colorectal adenocarcinoma cell line, HT-29(KCLB, Seoul, Korea), was maintained in Dulbecco’s Modified Eagle Medium (DMEM) and contained 10%(v/v) of fetal bovine serum (FBS) and 1%(v/v) of antibiotic-antimycotic solution (Invitrogen, Calsbad, CA, USA). The normal colon cell line, FHC was purchased from ATCC (Manassas, VA, USA) and cultured in DMEM/F12 media containing 25 mM HEPES, 10 ng/mL hydrocortisone, 10%(v/v) FBS, and 1%(v/v) antibiotic-antimycotic solution. Cells were incubated at 37°C in a humidified incubator containing 5% CO2 and subcultured prior to the experiment in order to be confluent in the T-75 flask. After the incubation, cells were harvested and a hemacytometer count was performed using trypan blue dye exclusion methods.

4.3. Cell viability

To estimate the viability of both Rh2s on the human cancer cells, MTT assay was performed. The cells were seeded in 96-well plates at 6 × 10³ cells per well and attached to the bottom of the plate in a humidified incubator (37°C, 5% CO2) for 24 h. Then, the medium was replaced with an FBS-free medium to adjust the growth phase of each cell equally. After 24 h, the medium was replaced with 2% FBS medium, and test compounds were treated to each well. Then, incubation with test compounds for 72 h, 20μL of the MTT solution (5mg/ml) were added to each well and plates were incubated at 37°C for 2-4 h. The absorbance at 570nm was recorded using a microplate reader (Bio-Rad Laboratories, Philadelphia, PA, USA).

4.4. Real time analysis of cell growth pattern

Monitoring and recording of cellular growth patterns were carried out using real time cell analysis (RTCA) with the xCELLigence RTCA system (ACEA Biosciences, San Diego, CA, USA) and 96-well E-plate™ (ACEA Biosciences, San Diego, CA, USA). The RTCA SP instrument equipped with the E-plate™ and the seeded Rh2 treated cells was placed inside the CO2 incubator and the measured data were transferred to the connected analyzer outside under the control of integrated software (Xing et al., 2006; Yu et al., 2006, pp. 40–43). One hundred μL of HT-29 cell suspension was added to each well of the E-plate™ and monitored every hour. Approximately 48 h after seeding, the cells were treated with both forms of Rh2 in various concentrations and vehicle controls and were monitored for 72 h.

4.5. Statistical analysis

Data are expressed as the mean ± standard deviation (SD). The student’s t-test was performed for the comparison between two groups and one-way ANOVA with Tukey’s post hoc test was implemented for multiple comparisons. All analyses were done with Prism 7 software.

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Author contributions

Y.J. initiated this work in partial fulfillment of her PhD degree at Seoul National University under the supervision of G.E.J. and the mentorship of S.K. and H.J.Y. Y.J. performed cell line experiments under the mentorship of H.J.Y. and G.E.J. Y.J. and S.K. collaboratively wrote the manuscript and performed the literature review. S.K. edited and revised the manuscript based on a non-disclosure research agreement between Middle Tennessee State University and BiFIDO Co., Ltd. All authors discussed the drafts and approved the final manuscript for publication.

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