Purification, antitumor activity in vitro of steroidal glycoalkaloids from black nightshade (Solanum nigrum L.)

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ABSTRACT

Six steroidal glycoalkaloids (1–6) were isolated and purified from Solanum nigrum L. (SNL) by acid extraction and alkaline precipitation, various chromatographic techniques, and their structures were elucidated by spectroscopic data. Antitumor activity, structure–activity and its molecular mechanism were investigated by methyl thiazolyl tetrazolium (MTT) method, flow cytometry, colorimetric assay and an immunocytochemical method. Experimental results showed that compounds 1 (solasonine), 2 (b1-solasonine), 3 (solamargine) and 6 (solanigroside P) have cytotoxicity to MGC-803 cells. Compounds with three sugar units and a-L-rhamnopyranose at C-2 or a hydroxyl group on the steroidal backbone may be potential candidates for the treatment of gastric cancer. The mechanism of action may be related to the decrease of mutation p53, the increase of the ratio of Bax to Bcl-2 and the activation of caspase-3 to induce apoptosis.

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1. Introduction

Gastric cancer is the second leading cause of cancer-related death (Scartozzi et al., 2009). In the metastatic setting, chemotherapy is the gold standard of palliative therapy. Traditional Chinese medicine has served as an important source of potent anti-cancer drugs for decades (Bradbury, 2005; Efferth, Li, Konkimalla, & Kaina, 2007). Steroidal compounds are an important class of secondary metabolites, which have been reported to exhibit wide range of pharmacological properties including anticancer activity (Bhutani, Paul, Fayad, & Linder, 2010).

Solanum nigrum L. (SNL), belonging to the nightshade of the Solanaceae family, is an herbal plant and wild vegetable that commonly grows in temperate climate zones. It has been used as a traditional folk medicine because of its diuretic and antipyretic effects in China (Chinese Pharmacopoeia Commission., 1978). Recently, this plant has attracted much attention because it has been proved to have remarkable antitumor activity (Li, Li, Feng, & Li, 2008; Son et al., 2003). Pharmacological experiments indicated that total steroidal alkaloids from SNL selectively destroys sarcoma 180 in vivo (Cham & Daunter, 1990) and two steroidal glycoalkaloids (SGAs) from SNL, solamargine (SM) and solasonine (SS), possess antiproliferative activity on many human tumor cells, e.g. colon, breast, lung and hepatoma cells, except human gastric cancer cells (Ding, Zhu, Li, & Gao, 2012; Liu et al., 2004; Luo et al., 2007; Shiu et al., 2007). Phytochemical studies revealed that SNL contains multiple SGAs (Hu et al., 1999). In our previous study, it was found that water extract, ethanol extract and n-butanol extract of Solanum nigrum L. could inhibit the growth of human gastric cancer MGC-803 cells (Ding, Gao, & Li, 2011). However there has not been any report available on whether SGAs from SNL are the active components in the whole process. Moreover, little is known about the anticancer activity of steroidal glycoalkaloids on human gastric cancer, their mechanism of action and the structure–activity relationship. Thus, in order to address the problem, diverse SGAs were isolated and purified from SNL, the in vitro anticancer activity of SGAs on MGC-803 cells was observed in the present study.

2. Materials and methods

2.1. Materials and methods

Dried SNL (the overground parts of SNL, herbarium number: 20080802) was purchased from Anhui Jingquan Chinese Herbal Medicine Co., Ltd. (Anhui, China) and identified by Dr. Xiao-Bin Jia, Jiangsu Province Academy of Traditional Chinese Medicine. The dried herb was ground in a mechanical grinder and sieved then stored at 20 °C in an air tight container until further use.
2.2. Extraction and isolation of SGAs

Air dried whole herb of SNL (10 kg) was refluxed twice with 70% ethanol for 3 h. The pooled extract was evaporated to dryness under vacuum at 45 °C. From the alcoholic extract of SNL, total SGAs were extracted with 2% aqueous HCl solution as water-soluble hydrochloride salts. And the acidic extract was basified under cooling with 10% sodium hydroxide solution up to pH 11 and extracted with n-butanol saturated by water. The pooled butanol extract was washed with water and evaporated under vacuum to afford a dark brown residue (24.5 g). For the isolation of SGAs, the brown residue was isolated and purified according to literature procedures (Zhou, 2006). Briefly, the n-butanol extract was subjected to chromatographic purification over a silica gel chromatographic column (CC) with a step gradient solvent system of CHCl3–MeOH mixtures (17:3, 4:1, 7:3, v/v) to give five fractions (Fr. I–V). Fr. I (5.12 g) was purified further by Sephadex LH-20 CC eluted with MeOH–H2O (1:1), ODS CC with MeOH–H2O (3:2), and Rp-18 HPLC with MeOH–H2O (4:1) to give compounds 5 (180 mg) and 4 (86 mg). Fr. III (6.58 mg) were isolated and purified using a similar procedure for isolation of Fr. I to afford compounds 2 (β1-SS, 2.72 mg), 3 (161 mg) and 6 (38 mg). Fr. V (2.56 g) was purified further by a similar procedure to obtain compound 1 (55 mg). The six compounds were identified as SS (compound 1), β1-solasonine (β1-SS/compound 2), SM (compound 3), β2-solamargine (β2-SM/compound 4), γ-solamargine (γ-SM/compound 5) and solanigroside P (SGP/compound 6), respectively, on the basis of their physical and spectroscopic data and by comparison with literature data (Mahato, Sahu, Ganguly, Kasai, & Tanaka, 1980; Novruzov, Aslanov, Ismailov, & Imanova, 1975; Yoshida et al., 1987). And all spectroscopic data were in complete agreement with the reported data. The structures of compounds 1–6 were shown in Fig. 1. As shown in Fig. 1, the six compounds belong to SGAs.

2.3. Cell culture

Human gastric cancer cell line MGC-803 was obtained from Life Sciences College of Nanjing University. All cells were maintained in DMEM medium supplemented with 10% (v/v) heat-inactivated foetal bovine serum, 100 μg/ml streptomycin and 100 U/ml penicillin. The cells were grown at 37 °C in a humidified atmosphere of 5% CO2.

2.4. Detection of cell viability and analysis of structure–activity relationship

The in vitro assay for the six SGAs was performed according to a MTT method. Briefly, MGC-803 cells were seeded at a density of 2 × 104 cells/well onto a 96-well plate in 100 μl volumes and treated with serial concentrations of SGAs (0, 2.5, 5, 10, 20, 40 μg/ml) for 24, 48 and 72 h, respectively (Fig. 2). 5-Fluorouracil (5-FU) was used as positive control. Each of the treated or control group was contained of six parallel wells. The absorbance was measured at 490 nm with an ELISA reader (Bio-Rad 680, USA). Growth inhibition rate (%) = (1 - A/B) × 100, where A and B are the average absorbance of the treated group and the negative control group, respectively. IC50 value was taken as the concentration that caused 50% inhibition of cell proliferation (Ding et al., 2012).

The activity of SGAs was investigated comparatively based on IC50 values. The structure–activity relationship was studied by comparing the structure of the compounds and IC50 values.

2.5. Quantitative detection of apoptosis and cell cycle distribution

Cell cycle distribution and apoptotic rate were detected by flow cytometry. Briefly, MGC-803 cells (1 × 105 cells/ml) were seeded onto a 6-well plate in 3 ml volumes and treated with different concentrations (0, 5, 10, 20 μg/ml) of SM (compound 3) for 48 h. The treated cells were harvested, washed twice with ice-cold PBS, fixed overnight at 4 °C with 70% EtOH and stained at 37 °C with 500 μl PI solution consisting of 50 μg/ml PI and 100 μg/ml RNase A by shaking in the dark for 30 min. The stained cells were analysed using a flow cytometer (BD FACSCalibur, USA). 1 × 104 cells were collected for analysis, implemented by ModFit LT 3.0 software. The percentage

Fig. 1. The molecular structures of six SGAs.
of apoptotic cells was assessed by calculating the percentage of cells in the sub-G1 phase (Gülçin et al., 2012).

2.6. Detection of caspase-3 activity

The activity of caspase-3 was determined using a caspase-3 colorimetric assay kit according to the manufacturer’s protocol. Briefly, MGC-803 cells (1 × 10⁶ cells/ml) were treated with SM according to the methods in Section 2.5. The treated cells were harvested and lysed by addition of lysis buffer. The protein content in the cell lysates was determined by Bio-Rad protein assay. The samples of the cell lysates were mixed with colorimetric substrate (Ac-DEVD-pNA) and incubated at 37 °C in the dark for 4 h. The absorbance was measured at 405 nm in an ELISA reader (Bio-Rad, USA) and the caspase-3 activity was expressed as percentage of enzyme activity compared with control (Hu et al., 2009).

2.7. Detection of the expression of p53, Bcl-2 and Bax

The expression of Bcl-2, Bax and mutation p53 was examined by immunocytochemistry according to the manufacturer’s instructions. Briefly, MGC-803 cells (1 × 10⁶ cells/ml) were seeded in a 6-well plate containing a glass slide and treated with SM (20 µg/ml) for 48 h. The treated cells were washed twice with PBS and fixed at 4 °C with ice-cold acetone for 10 min. After PBS washing and goats serum closing for 10 min, the cells were incubated at 37 °C with the primary antibody against p53/Bcl-2/Bax for 1 h and then incubated with the secondary antibody at room temperature for 15 min. The cells were then stained brown by diaminobenzidine (DAB) reaction and counter-stained with hematoxylin (Grace, Shalini, Sree lekhac, Devaraj, & Devaraj, 2003; Khalfaoui et al., 2011). The expression of the proteins was assayed by automatic microscope (LEICA DM-1000, Leica Inc., Germany) and computer-image analysis system (software Image Pro Plus version 6.0, Media Cybernetics Inc., USA). Optical density value represented the protein level (Fig. 5). A minimum of five fields were examined in each case (Baba, Koji, Itoh, & Mizuno, 1999; Charpin et al., 1995).

2.8. Statistical analysis

All data was expressed as the mean ± standard deviation (S.D.). Data analysis was carried out using SPSS statistical software package. The p-value of <0.05 was adopted as statistically significant.

3. Results

3.1. Four SGAs had potent growth inhibitory activity in vitro

The cytotoxic effect of six SGAs on MGC-803 cells was evaluated by MTT assay. The results indicated that compounds 1 (SS), 2
(β-SS), 3 (SM) and 6 (SGP) dramatically inhibited the proliferation of MGC-803 cells in a dose- and time-dependent manner, and compounds 4 (β2-SM) and 5 (γ-SM) had no significant cytotoxicity (the maximum inhibition rate < 30%). IC50 values treated with compounds 1, 2, 3, 6 and 5-FU for 72 h were 25.2, 26.5, 8.77, 20.1 and 10.4 µg/ml, respectively. The maximum inhibition rate reached 74.8%, 66.1%, 95.1%, 75.1% and 75.2%, respectively. Based on IC50 values, the activity order of the four active compounds was as follows: compound 3 > compound 6 > compound 1 > compound 2. The activity of compound 3 surpassed that of positive control drug (5-FU).

As shown in Fig. 1, compounds 1–5 have the same steroidal alkaloid backbone and differ on the number, the nature and the connective position of sugar units. Compound 6 was a derivative of compound 4 with the substitution of a 12-hydroxyl for the hydrogen atom on the steroidal alkaloid backbone. The results suggest that the number, the type, the location of sugar or the substitution of hydroxyl group on steroidal alkaloid backbone had an effect on the activity.

3.2. SM induced S phase arrest and rising apoptosis rate

To investigate the effect of SGAs on the apoptosis and the cell cycle distribution of MGC-803 cells, flow cytometry was used. Results showed that a typical sub-G1 peak, which represented the apoptosis cell population, began to appear after the treatment with SM. The percentage of apoptosis increased in a dose-dependent manner and the changes in the cell cycle progression were notable (Fig. 3). Fig. 3 displayed that the cell population of the G2/M phase reduced concurrently with the increase of the S phase population after the treatment with SM for 48 h. These data suggested that SM induced the cell cycle arrest in S phase, which might be contribute to the apoptosis.

3.3. SM up-regulated the expression of caspase-3

The caspase-3 activity was assessed by a colorimetric method. As shown in Fig. 4, SM increased caspase-3 activity in a dose-dependent manner, suggesting a possible relation between the SM-induced apoptosis and the activation of caspase-3.

3.4. SM up-regulated the ratio of Bax to Bcl-2 and down-regulated the expression of mutant p53

To ascertain if p53 and Bcl-2 family are critical factors for the anticarcinogenic effect of SGAs, the expression of p53, Bcl-2 and Bax in MGC-803 cells was assayed by immunocytochemistry analysis. As shown in Fig. 5, a remarkable increase in the expression of Bax and a significant decrease in the expression of Bcl-2 or p53 were observed after treatment with SM (20 µg/ml) for 48 h. The results indicated the suppression of mutant p53 and the augment of
the ratio of Bax to Bcl-2 might be the primary contributor to the apoptosis mediated by SM.

4. Discussion

This study is the first to examine the antiproliferative effect of six SGAs from SNL on MGC-803 cells. MTT experimental results showed that compounds 1, 2, 3 and 6 have good cytotoxicity to MGC-803 cells, based on their low IC50 values (Fig. 2). Fig. 1 showed that compounds 1–5 have the same steroid alkaloids aglucon and different sugars units, and that compound 6 was a derivative of compound 4 with the 12-hydroxyl replaced. Compounds 1, 2 and 3 contain α-1-rhamnopyranose located at the C-2 of β-D-glucose or galactose, which have good cytotoxicity on MGC-803 cells. Compound 4 contains α-1-rhamnopyranose at the C-4 and compound 5 does not contain α-1-rhamnopyranose. The two compounds have hardly exhibited inhibition activity, which suggested that α-1-rhamnopyranose at the C-2 plays an important role in the antiproliferative activity. Compound 6, without α-1-rhamnopyranose at the C-2, has a good activity (IC50 = 20.10 μg/ml) compared to compound 4, which could be related to substitution of a hydroxyl group for the hydrogen at steroidal alkaloid backbone.

Moreover, it was found that the steroidal glycoalkaloids with trisaccharides (compounds 1 and 3) were higher activity than those with disaccharides (compound 2 and 4) or monosaccharides (compound 5). Compound 3, the glycoalkaloids with trisaccharides and two α-1-rhamnose, had the best in vitro inhibitory activity among the active compounds, suggesting the number of rhamnose groups may be one of the key factors in the activity. The activity of compound 2 was far greater than that compound 4, which showed that the location of rhamnose or the type of sugar influenced the activity.

During the past decades, the killing of tumors through the induction of apoptosis has been recognised as a novel strategy for the identification of anticancer drugs (Smets, 1994). During apoptosis, DNA fragmentation and apoptotic body formation can be observed. In the flow cytometry study, a sub-G1 peak, which is considered an indicator of cell apoptosis, was clearly observed and the peak height rose in a dose-dependent manner (Fig. 3). These results suggested that MGC-803 cells treated with SM undergo typical apoptosis, which might partially contribute to the anti-proliferative effect of SM. Since cell proliferation was finely regulated by promoting or blocking cell cycle progression, the effect of SM on cell cycle progression was examined. Fig. 3 showed that SM induced an accumulation of cells in S phase with an increasing apoptotic rate, which indicated that SM induced apoptosis perhaps through S phase arrest.

Apart from the cell cycle regulation, caspase family proteases play an important role in the mechanism of apoptosis. Hence, we investigated whether caspase-3, an executioner caspase (Lu et al., 2004), was involved in SM-induced apoptosis in MGC-803 cells, as it would contribute to the understanding of the mechanism responsible for apoptosis execution. Experimental results demonstrated that the higher the concentrations of SM during treatment, the greater expression of caspase-3 in MGC-803 cells (Fig. 4), which indicated that SM could activate caspase-3 and caspase-3 might be involved in SM-induced apoptosis.

It was reported that anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax are also important members involved in apoptosis. If the pro-apoptotic function wins, caspase-3 is activated, which finally results in apoptosis (Ahn et al., 2009; Hou et al., 2008). Furthermore, the p53 gene is the most commonly mutated gene in human cancers (Hsieh et al., 1996). Some previous studies have demonstrated an inverse correlation between mutant p53 and Bax expression (Hussein, 2009; Leiser et al., 2006; Shabnam et al., 2004). The altered p53 protein is detectable by immunohistochemistry because of its prolonged half-life (Chan et al., 2000; Logullo et al., 2000). Based on the hypothesis, the expression of p53, Bcl-2 and Bax proteins in MGC-803 cells after the treatment with SM was examined by immunocytochemical methods. In this study, immunocytochemical staining analysis showed that SM enhanced the expression of Bax and down-regulated the expression of Bcl-2 and mutant p53 (Fig. 5), which promoted the expression of caspase-3 to induce apoptosis. The results suggested that the mechanism of apoptosis may be associated with apoptosis-associated proteins taking part in intrinsic apoptosis pathway.

In conclusion, SGAs exhibited antitumor activity on MGC-803 cells in vitro and induced apoptosis in MGC-803 cells by activating caspase-3 and leading to S phase arrest, which might be related to up-regulation of the ratio of Bax to Bcl-2 and down-regulation mutant p53. The number and the location of α-1-rhamnose, the type of sugar and the substitution of a hydroxyl on steroidal alkaloid backbone play an important role in the anti-proliferative activity.
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