Two Glutathione S-transferase Inhibitors from Radix Angelicae sinensis

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Chemotherapy for malignant tumors fails because of multidrug resistance (MDR). The overexpression of glutathione S-transferase (GST) with MDR is considered one of the major obstacles to successful cancer chemotherapy. High-throughput screening was performed to search for inhibitors of GST from natural products. Two compounds isolated from Radix Angelicae sinensis had an inhibitory effect on GST, with 50% inhibitory concentration (IC50) values of 16.80 and 7.30 \text{mM}. Kinetic analyses showed that the two compounds acted as reversible noncompetitive inhibitors of GST. They also increased apoptosis of B-MD-C1 (ADR+/-) cells induced by adriamycin with low cytotoxic activity. Radix Angelicae sinensis may be a potential source of GST inhibition for pharmaceutical use. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: cancer; multidrug resistance; glutathione S-transferase; 11-angeloylsenkyunolide F; Tokinolide B; Radix Angelicae sinensis.

INTRODUCTION

Chemotherapy is one of the most important methods used to treat cancer. However, drug resistance during chemotherapy is the leading cause of treatment failure and decreased survival in cancer patients. Multidrug resistance (MDR) represents a major obstacle to the success of cancer chemotherapy (Ullah, 2008). MDR is defined as resistance of tumor cells to the cytotoxic action of multiple structurally dissimilar and functionally divergent drugs commonly used in chemotherapy. The resistant phenotype is characterized by alterations in multiple pathways (Tsuruo, 1988; Mellor and Callaghan, 2008; O’Connor et al., 2007; Ramachandran et al., 1995). Two important mechanisms involved in MDR are increased activity of efflux pumps, such as those of the MDR proteins, and the detoxification by phase II conjugating enzymes, such as glutathione S-transferases (GSTs).

Glutathione S-transferases are a superfamily of xenobiotic metabolizing enzymes that catalyse the conjugation of various electrophilic compounds with glutathione (GSH). Of all GSTs, GSTP1-1 is the most important isozyme in MDR as derived from overexpression of GSTP1-1 in a large number of solid tumors and is involved in the development of resistance to several anticancer drugs (O’Brien and Tew, 1996). Most of the MDR reversal agents, such as verapamil, phenothiazine, cyclosporin A and ethacrynic acid, are often limited by side effects in normal tissues and cells in clinical trials (Lage, 2008). Finding MDR reversing agents with better effect and less toxicity in cancer therapy is important. In in vitro trials, some natural products, including curcumin, quercetin, plant sterols, schizandrin, panaxaxiphen and sophorcarpin have been shown to antagonize MDR (Um et al., 2008; Shen et al., 2008; Nabekura et al., 2008; Huang et al., 2008).

The natural resources of Chinese medicine materials are abundant, with long-term clinical screening and high safety. They should possess few side effects and may represent a new generation of MDR modulators. Angelica sinensis (Oliv.) Diels is an umbelliferae angelica perennial herb. Radix Angelicae sinensis (RAS), known as Danggui in China, is used frequently in traditional Chinese medicine for tonifying blood and for treating anemia, female irregular menstruation and amenorrhoea. Recent research indicated that RAS could have multiple pharmacological activities, such as beneficial hematopoietic effects (Bradley et al., 1999) and antitumor activity by immunomodulation or cytotoxic effects (Chen et al., 2007; Hsieh et al., 2003).

High-throughput screening of GST inhibitors was performed and two potential compounds isolated from RAS were found. The study also investigated the structure-activity relationship of the compounds to...
better understand the pharmacologic action of the compounds and to develop a new use for RAS.

**MATERIALS AND METHODS**

**Chemicals.** GST (mainly GSTπ, from human placenta), GSH, 1-chloro-2,4-dinitrobenzene (CDNB), RPMI-1640, 3′-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), RNase A, propidium iodide, adriamycin and ethacrynic acid were from Sigma (St Louis, MO, USA). All other chemicals were from Beijing Chemical Co. All the chemicals used were of analytical grade.

**Plant materials.** RAS was collected in Gansu province, China, in 2002, and was identified by Professor Zongwan Xie of the Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences. A voucher specimen (2002031101) has been deposited in the Herbarium of the Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences.

**Extraction and isolation.** The isolation procedure for the active compounds was as follows. Briefly, dried RAS (10 kg) was ground into farina and extracted with 95% EtOH (100 L). After the insoluble farina was removed, the EtOH solvent was evaporated under reduced pressure to give a viscous residue. The viscous residue was suspended in water and extracted with petroleum ether until oily (400 g), and then the petroleum ether extract was chromatographed on a silica gel column (160–200 mesh). The column was eluted with a gradient of n-hexane/acetone from 90:10 to 10:90 to obtain nine fractions. The fraction eluted with n-hexane/acetone (80:20) was further separated by silica gel-CC (260–300 mesh, petroleum ether/chloroform from 100:0 to 0:100) and high-performance liquid chromatography (mobile phase: 80% methanol) to give compound P14 (40.0 mg). The fraction eluted with n-hexane/acetone (70:30) was further separated by silica gel-CC (260–300 mesh, petroleum ether/chloroform from 100:0 to 0:100), and thin-layer chromatography (developing solvent: methylene dichloride) to give compound P7 (46.2 mg). The structures of these compounds was as follows. Briefly, dried RAS was collected in Gansu province, China, in 2002, and was identified by Professor Zongwan Xie of the Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences. A voucher specimen (2002031101) has been deposited in the Herbarium of the Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences.

**GST activity assay.** The inhibitory effects of the compounds on GST were measured with GSH and CDNB used as substrates as described (Habig et al., 1974) with modification: 10 μL of GST (0.0018 unit) was dissolved in 50 mM potassium phosphate buffer (pH 7.4) and 1 μL of purified compounds (final concentration from 0.1 μM to 100 μM) dissolved in DMSO was added to each well of a 384-well plate. After incubation with GST at 37°C for 5 min, 10 μL of GSH and CDNB dissolved in 50 mM potassium phosphate buffer was added to a final concentration of 5 mM GSH and 0.1 mM CDNB to each well. After incubation at 37°C for 30 min, the rate of product formation was monitored by measuring the change in absorbance at 340 nm wavelength by use of a single-beam UV/visible spectrophotometer (Spectra Max M5, Molecular Devices, USA).

**Inhibitory kinetics.** To understand how the compounds inhibit GST, the initial rate of the enzyme was analysed by measuring the formation of catalysate at 340 nm wavelength. The reaction was carried out at 37°C for 5 min after pre-incubating GST with various concentrations of the compounds for 5 min. A plot of ν (μmol/mL/min) versus [E] was obtained with different compound concentrations (P7, P14 of 0%, 50%, 100% inhibition rate to GST) and different GST concentrations of 0.0075–0.18 U/mL to distinguish between reversible and irreversible inhibition. A Hanes plot of [S]/ν versus [S] was performed at GSH concentrations of 0.07–2.33 mM, or CDNB concentrations of 0.5–43.3 μM, with the compound concentrations of 0.8 and 16 μM (P7) and 0.5 and 10 μM (P14).

**Computational docking methods.** Computational docking was carried out with FlexX run in SBVS (Rational drug design v7.0, Tripos Inc.). The protein data bank (PDB) file (PDB code 2GSS) of the co-crystal of the inhibitor ethacrynic acid in the active site of GST (human GSTP1-1) were optimized and used (Zhao et al., 2005). Ethacrynic acid was used as a reference ligand structure, which was a fixed conformation docked into the active site of the enzyme. The docking conformations of ethacrynic acid were created with the use of FlexX, with 30 docking conformation options. Each conformation was energy-minimized with the use of a molecular mechanics program. All ligands were predicted on the basis of active sites being within a 6.5 Å radius from the bound ligand. Water and metals not involved in binding were removed from the protein.

**Cytotoxic activity by MTT assay.** The MTT assay was performed as described (Carmichael et al., 1987), with slight modification. A549 or MDA-MB-231 cells were maintained in RPMI-1640 medium with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin in a CO2 incubator in humidified atmosphere with 5% CO2 at 37°C. Cells in the exponential growth phase were seeded at 6000 cells/well in a 96-well plate. After 48 h of treatment with the compounds at various concentrations, MTT was added into each well (0.5 mg/mL) and further incubated for 4 h. The cells from each well were solubilized with 150 μL DMSO for optical density reading at 540 nm.

**Flow cytometry analysis.** Flow cytometry was used to evaluate the effect of 11-angeloylsenkyunolide F and tokinolide B on apoptosis of multidrug-resistant B-MD-C1 (ADR+/−) cells induced by adriamycin. B-MD-C1 (ADR+/−) cells were treated with the compounds at various concentrations for 48 h, then the medium was replaced with fresh medium containing 5 μg/mL adriamycin. After treatment for 24 h, the cells were collected and fixed in 70% cold ethanol (−20°C) overnight. After two washes with PBS, the cells were resuspended in phosphate-buffered saline containing 1% fetal calf serum (vertex). RNA in the fixed cells was digested with RNase A (0.5 mg/mL) at 37°C for 1 h. Finally, the cells were stained with propidium iodide (2.5 μg/mL). The DNA content of cells was then analysed with use of an EPICS-XL FACScan instrument (Beckman Coulter, USA).
RESULTS

Structure of P7 and P14

UV, MS and NMR spectra showed that P7, P14 were identical to two known compounds, 11-angeloylsenkyunolide F and tokinolide B (purity 95%) (Li et al., 2003; Lu et al., 2003).

Inhibition of GST

The two compounds showed similar inhibition of GST (mainly GSTπ, from human placenta) in a dose-dependent manner, with IC_{50} values of 16.80 and 7.30 μM, respectively. However, they did not react with GSH even at 100 mM (data not shown). In our assay system, ethacrynic acid as a positive control inhibited GST, with an IC_{50} value of 4.89 μM (see Fig. 1).

Kinetic studies

The mechanism of inhibition to GST by the two compounds was investigated by kinetic analysis. The inhibition of GST of the two compounds was dose dependent and appeared to be reversible (Fig. 2). Figures 3 and 4 show the Hanes plots with various concentrations of GSH or CDNB. The Hanes plot results indicated that the compounds inhibited GST noncompetitively (Michaelis constant [K_m] remained unchanged, whereas the maximum rate of clearance [V_{max}] decreased) with respect to GSH and CDNB. Therefore, the compounds were likely to act as a reversible noncompetitive inhibitor of GST.

Computational docking

To gain insight into the structural basis by which 11-angeloylsenkyunolide F and tokinolide B exert their inhibitory activity on GST, the active site of GST was analysed, the complex of GST with ethacrynic acid, and molecular docking studies were performed on the compounds. A computational docking method was used with FlexX run in SBVS software to dock the compounds into the active site of GST and it was found that the two compounds might insert into the enzyme molecule active cavity. The compound with high scoring conformation would display high inhibitory activity on GST, and the computational docking scoring conformations were according to the results of high-throughput screening. The computational docking with the use of the PDB file 2GSS was a reference structure: the 11-angeloylsenkyunolide F oxygen atom in a five-membered ring formed a hydrogen bond with active site ASN, and the conformation scoring was −3.6; the tokinolide B carbonyl oxygen atom in a five-membered ring formed a hydrogen bond with active site LYS42, and the oxygen atom in another five-membered ring formed a hydrogen bond with active site GLN1. The conformation scoring was −4.0 (Fig. 5).

Figure 1. The inhibitory activity of the compounds to glutathione S-transferase (GST). After incubation of GST and the compounds at 37°C for 30 min with a final concentration of 5 mM glutathione (GSH) and 0.1 mM 1-chloro-2,4-dinitrobenzene (CDNB), the rate of product formation was monitored by measuring the change in absorbance at 340 nm wavelength. 50% inhibitory concentrations (IC_{50}) for (a) 11-angeloylsenkyunolide F, 16.80 μM; (b) tokinolide B, 7.30 μM; and (c) ethacrynic acid, 4.89 μM, as a positive control. Each point shows mean ± SD of triplicate determinations.

Figure 2. Kinetics plot of 11-angeloylsenkyunolide F and tokinolide B to GST. The reaction was carried out at 37°C for 5 min after pre-incubating GST with the compounds for 5 min. A plot of v (μmol/mL/min) versus [E] was obtained for GST concentrations from 0.0075–0.18 U/mL. Kinetics plots of inhibition to GST at 0%, 20%, 50% IC for (a) 11-angeloylsenkyunolide F and (b) tokinolide B. Each point shows mean of triplicate determinations.
Cytotoxic activity

The in vitro cytotoxicity of the two compounds against cancer cell lines was evaluated by MTT assay. Both compounds inhibited the proliferation of cells in a concentration-dependent manner. The IC_{50} values were 65.28 and 81.26 μM for 11-angeloylsenkyunolide F and tokinolide B, respectively, for the A549 cell line, and 74.17 and 52.58 μM, respectively, for the MDA-MB-231 cell line. The IC_{50} values for cis-platinum were 13.88 μM for A549 cells and 14.78 μM for MDA-MB-231 cells. Therefore, the compounds showed low cytotoxic activity.

Flow cytometry of effect of 11-angeloylsenkyunolide F and tokinolide B on apoptosis of B-MD-C1 (ADR+/+)

To further investigate the reversal effect of the two compounds on cancer MDR cell lines, their effects were determined on apoptosis of B-MD-C1 (ADR+/+) cells induced by Adriamycin by flow cytometry. After the cells were exposed to the two compounds for 48 h, a distinct sub-G1 peak, the apoptotic fraction, was observed in the B-MD-C1 (ADR+/+) cells compared with the controls (Table 1, Fig. 6).
Figure 6. Flow cytometry results for the effect of the compounds on apoptosis of B-MD-C1 (ADR+/+) cells induced by adriamycin. (a) control; 11-angeloylsenkyunolide F concentrations of (b) 20 μg/mL and (c) 40 μg/mL; Tokinolide B concentrations of (d) 9 μg/mL, (e) 15 μg/mL, and (f) 30 μg/mL. This figure is available in colour online at http://wileyonlinelibrary.com/journal/ptr

Table 1. Flow cytometry data for effect of 11-angeloylsenkyunolide F and tokinolide B on apoptosis of B-MD-C1 (ADR+/+) cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Apoptosis (%)</th>
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<tbody>
<tr>
<td>Control</td>
<td>4.8 ± 1.4</td>
</tr>
<tr>
<td>11-angeloylsenkyunolide F, 20 μg/mL</td>
<td>7.1 ± 2.9</td>
</tr>
<tr>
<td>11-angeloylsenkyunolide F, 40 μg/mL</td>
<td>15.5 ± 2.1*</td>
</tr>
<tr>
<td>Tokinolide B, 9 μg/mL</td>
<td>13.5 ± 1.9*</td>
</tr>
<tr>
<td>Tokinolide B, 15 μg/mL</td>
<td>17.8 ± 3.3*</td>
</tr>
<tr>
<td>Tokinolide B, 30 μg/mL</td>
<td>31.9 ± 4.2*</td>
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Values are presented as mean ± SD (n = 3). *p < 0.01 vs control.

DISCUSSION

The mechanism of MDR in cancer is associated with multiple biochemical changes, such as changes in the enzymatic system, expression of the ATP-binding cassette membrane transport protein family, and cell cycle and apoptosis regulator control system. Although GST as a resistance factor may not be as effective as p-glycoprotein, GST may be primarily involved in intrinsic drug resistance and in the early stage of acquired resistance (Tew et al., 1997). With regard to the mechanism of drug resistance with GST, the following six mechanisms can be hypothesized on the basis of the physiological properties of GST (Johansson et al., 2007; Townsend and Tew, 2003; Townsend et al., 2005): (1) detoxifying anticancer drugs by GSH conjugation, (2) sequestering anticancer drugs by binding with GST, (3) chemically reducing lipid peroxides, (4) reducing the amount of DNA peroxides and repairing DNA, (5) certain GST isozymes regulating mitogen-activated protein kinases or facilitating the addition of GSH to cysteine residues in target proteins, and (6) antiapoptotic activity by interacting with c-Jun N-terminal kinase. These multiple functionalities have contributed to the recent efforts to target GST with novel therapeutics.

Targeting GST by small-molecular compounds is an effective strategy to overcome MDR in cancer. Several studies have investigated overcoming anticancer drug resistance with GST inhibitors (Tew et al., 1997, 1988). Ethacrynic acid, an inhibitor of GST, represented a first attempt in this direction. However, the deleterious side effects of the inhibitor have discouraged its use in clinical practice. Natural products are expected to possess fewer side effects and may represent a new generation of GST inhibitors. A high-throughput screening assay was developed to screen a library of 10000 natural products for their ability to inhibit GST. Two compounds isolated from RAS were able to inhibit GST activity in a concentration-dependent manner. To gain insight into the structural basis by which 11-angeloylsenkyunolide F and tokinolide B exert their
inhibitory activity on GST, a computational docking method was used with a FlexX run in SBVS software to dock the two compounds into the active site of the GST and it was found that they might insert into the gorge of GST. As well, the two compounds showed low cytotoxic activity and increased apoptosis of B-MD-C1 (ADR+/+) cells induced by adriamycin, thereby showing significant reverse MDR in cancer. Therefore, RAS is a potential source of inhibitors of GST that may be used as an MDR reversal agent for cancer. On the basis of their high GST-inhibitor activity and low cytotoxic activity, the two compounds could be used as a lead compound for drug discovery and warrant further investigation.

Conflict of Interest

The authors have declared that there is no conflict of interest.

REFERENCES


