Isoliquiritigenin Inhibits Tumor Growth and Protects the Kidney and Liver Against Chemotherapy-Induced Toxicity in a Mouse Xenograft Model of Colon Carcinoma

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Abstract. A growing amount of attention has been focused on the investigation of the effects of chemopreventive agents on the inhibition of cancer cell growth and toxicity in combination with chemotherapeutics. The objective of this study was to determine whether isoliquiritigenin (ISL) has the potential to serve as a beneficial supplement during cisplatin chemotherapy. We found that the administration of ISL alone significantly reduced the size of the solid tumors in CT-26 cell–inoculated BALB/c mice, without any detectable induction of nephrotoxicity, hepatotoxicity, and oxidative stress, and ISL reduced the viability and DNA synthesis of CT-26 murine colon cancer cells in a dose-dependent manner. ISL did not affect the therapeutic efficacy of cisplatin. Furthermore, ISL suppressed cisplatin-induced kidney damage characterized by increases in serum creatinine and blood urea nitrogen, as well as cisplatin-induced liver damage characterized by increases in serum alanine aminotransferase and aspartate aminotransferase. The repeated oral administration of ISL prior to cisplatin treatment exerted a preventive effect on cisplatin-mediated increases in serum nitric oxide and tissue lipid peroxidation levels, and it recovered depleted GSH levels in the tissues. Therefore, supplementation with ISL may be an effective approach to counteracting the side effects of cisplatin therapy in cancer patients.

Keywords: isoliquiritigenin, anticancer activity, cisplatin, nephrotoxicity, hepatotoxicity

Introduction

Cancer chemotherapy has been shown to play an important role in the treatment of most solid tumors, but has also been associated with substantial short- and long-term side effects. These side effects affect the quality of life in cancer patients. A growing body of evidence suggests that a combination treatment of chemotherapy and chemopreventive agents with anticarcinogenic activity may enhance the efficacy of chemotherapeutics and/or reduce the systemic toxicity induced by chemotherapy (1).

Cisplatin (cis-diaminedichloroplatinum II) is one of the most active anti-tumor drugs, and it is used extensively for the treatment of a variety of solid tumors, including cancers of the ovary, testis, lung, bladder, head and neck, cervix, and endometrium (2). Despite its profound chemotherapeutic properties, the clinical use of cisplatin is frequently limited by severe nephrotoxicity (3, 4). Cisplatin-induced nephrotoxicity is associated with increased renal vascular resistance and morphological damage to the intracellular organelles, including cellular necrosis, loss of microvilli, changes in the number and size of lysosomes, and mitochondrial vacuolization (5). Other less frequently observed toxic effects, including hepatotoxicity, occur and adversely
affect patients to whom high doses of cisplatin were administered (6). The cisplatin-induced alterations in kidney and liver functions are characterized by signs of injury, including glutathione status and lipid peroxidation (7, 8). Although the precise mechanism underlying this cisplatin-induced toxicity remains poorly understood, cisplatin is preferentially taken up and accumulates within human kidney and liver cells (9), resulting in the enhanced production of reactive oxygen species (ROS) and a reduction in the levels of antioxidant enzymes (8, 10). Therefore, antioxidant enzymes and compounds including superoxide dismutase, glutathione peroxidase, glutathione, and flavonoids have been assessed for their potential protective effects against cisplatin-induced nephrotoxicity (11). The results of several studies have indicated that supplementation with antioxidant micronutrients, such as vitamin C, vitamin E, and selenium, may provide protection against cisplatin-induced nephrotoxicity (12, 13).

Isoliquiritigenin (ISL), a flavonoid with a chalcone structure that has been detected in licorice, originated from the dried roots of several Glycyrrhiza species (Leguminosae), has been reported to evidence a variety of biological properties, including vasorelaxant (14), anti-platelet aggregation (15), and antioxidative and superoxide-scavenging activities (16). ISL has been demonstrated to inhibit cell proliferation and/or induce apoptosis in breast, lung, prostate, colon, gastric, and melanoma cancer cell lines (17–23) and also to suppress 7,12-dimethylbenz[a]anthracene (DMBA)-induced mouse skin carcinogenesis (24). With regard to its effects on colon cancer, ISL inhibited the induction of aberrant crypt foci and colon carcinoma development in azoxymethane-treated ddY mice (25) and reduced the viability of mouse, rat, and human colon carcinoma cells (26).

The principal objective of this study was to determine whether ISL with chemopreventive and antioxidative activities harbors the potential to function as a beneficial supplement, which could be used to augment therapeutic efficacy and/or to maintain patients’ health during cisplatin chemotherapy. We evaluated the inhibitory effect of ISL alone and in combination with cisplatin on the growth of xenografted mouse colon carcinoma cells, as well as its protective effects against cisplatin-induced nephrotoxicity and hepatotoxicity in mice. This study is the first report on the protective effect of ISL against cisplatin-induced toxicity in an in vivo model.

**Materials and Methods**

**Chemicals**

Isoliquiritigenin (Lot 034K4111), cisplatin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 1,1,3,3-tetramethoxypropane, sulfanilamide, α-naphthylamine, sodium nitrite, thiobarbituric acid (TBA), potassium chloride, trichloroacetic acid (TCA), ammonium sulfamate, N-(1-naphthyl)ethylenediamine and reduced glutathione (GSH), N-acetyl-L-cysteine (NAC), and Hanks’ balanced salt solution (HBSS) were obtained from Sigma-Aldrich Chemical (St. Louis, MO, USA). Dulbecco’s Modified Eagle’s Medium (DMEM), fetal bovine serum (FBS), trypsin-EDTA, and phosphate-buffered saline (PBS) were acquired from Gibco BRL (Gaithersburg, MD, USA). 5-(and-6)-Chloromethyl 2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H<sub>2</sub>DCFDA) was purchased from Invitrogen (Eugene, OR, USA). All reagents used in this study were of analytical grade.

**Animals**

BALB/c male mice (30 ± 5 g, 5 weeks of age) were purchased from the Central Lab Animal, Inc. (Seoul, Korea), and they were permitted free access to a normal standard chow diet (Daejong Inc., Seoul, Korea) and tap water. All mice were housed for acclimatization for 1 week prior to the experiments and maintained at 25 ± 2°C, with a relative humidity of 55 ± 5% and a 12-h light-dark cycle. Animal studies were conducted after the experimental protocols were approved by the animal ethics committee of the Yonsei University College of Dentistry.

**Mouse xenograft model**

To assess the inhibitory effect of ISL on tumor growth, as well as its protective effects against cisplatin-induced nephrotoxicity and hepatotoxicity, the mice were divided into five groups, each group consisting of 8 mice: PBS-treated group, CT-26 cell-inoculated group, CT-26 cell-inoculated group with cisplatin, CT-26 cell–inoculated group with ISL, CT-26 cell–inoculated group with ISL and cisplatin. The CT-26 mouse colon cancer cells (2 × 10<sup>6</sup> cells in 0.1 ml PBS) cultured in DMEM with 10% FBS were subcutaneously injected into the right flanks of the mice. Twenty-four hours later, BALB/c mice were dosed with ISL [1 mg/kg body weight (BW)] in PBS via oral gavage. At 2 h after treatment with ISL, cisplatin (5 mg/kg BW) in PBS was intraperitoneally injected. ISL and cisplatin were administered once per day for 15 days. The control group received PBS rather than ISL and cisplatin. On day 15, tumor size was measured with calipers and the tumor volumes were calculated in accordance with the following formula: (length × width<sup>2</sup>) / 2. At 16 h after the final cisplatin injection, the mice were sacrificed under anesthesia. The livers and kidneys of the mice
were excised immediately after the blood was collected from each mouse and then homogenized for the following experiments.

Cell viability and DNA synthesis in colon cancer cell lines

The viability of CT-26 mouse colon cancer cells was determined via a MTT assay. In brief, colon cancer cells (2.5 \times 10^4 cells/ml) were seeded into each well of a 96-well plate with DMEM containing 10% FBS and cultured to adhere overnight. The cells were then treated with various concentrations of ISL in serum-free medium for 24 or 72 h. A 20-\mu l aliquot of MTT solution (5 mg/ml) was added to each well, and the cells were incubated for 4 h at 37°C. The medium was then removed, and 200 \mu l of DMSO was added to each well. The absorbance was determined at 570 nm using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

DNA synthesis was evaluated by measuring the quantity of 5-bromo-2‘-deoxyuridine (BrdU) incorporated into the colon cancer cells. Colon cancer cells (5 \times 10^3 cells/ml) were plated onto each well of a 96-well plate and grown to 70% confluence. The cells were treated with serum-free medium in the absence or presence of ISL at the indicated concentrations for 24 and 72 h, respectively. The cells were labeled with 10 \mu M of 5-bromo-2‘-deoxyuridine for an additional 4 h. The cells were fixed for 30 min with 70% acidic ethanol at −20°C. Cell proliferation was then evaluated using a Roche BrdU labeling and detecting kit III (Roche Diagnostics, Germany) in accordance with the manufacturer’s protocols. The absorbance was measured at 450 nm by using a microplate reader.

Determination of serum biochemical parameters

Blood samples were maintained at room temperature for 1 h and then centrifuged for 10 min at 3000 rpm to obtain serum. As indicators of kidney function, serum blood urea nitrogen (BUN) and creatinine levels were assessed. In order to evaluate liver function, serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were evaluated. All biochemical assays were conducted spectrophotometrically, using commercially available kits (Asan Pharmaceutical, Seoul, Korea).

Nitric oxide (NO) determination

Serum nitrite was estimated as an index of NO production using Griess reagent. A 100-\mu l aliquot of Griess reagent (1:1 solution of 0.2% \alpha-naphthylamine in distilled water and 2% sulphanilamide in 5% phosphoric acid) was added to 100 \mu l of appropriately diluted serum.

Total nitrite was spectrophotometrically measured at 540 nm after the conversion of nitrate to nitrite for 5 min at room temperature (27). Nitrite concentration was calculated using a standard curve for sodium nitrite. Nitrite levels were expressed as \mu M of serum.

Preparation of the homogenates from kidney and liver tissues

Kidney and liver tissues were immediately perfused with ice-cold saline (0.9% KCl) to remove the blood. The tissues were sliced into small pieces with scissors on ice and then homogenized in 10 volumes of ice-cold 1.15% KCl. The homogenates were centrifuged at 2000 rpm for 10 min at 4°C. Aliquots of the homogenate were collected and stored at −80°C in order to determine the levels of malondialdehyde (MDA) and glutathione (GSH). Total protein concentrations were determined using a BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA).

Determination of lipid peroxidation

The content of MDA, as an index of the extent of lipid peroxidation, was assayed in the form of thiobarbituric acid-reactive substances (TBARS) as previously described (28). The reaction mixture (4 ml) consisted of 0.2 ml of 8.1% sodium dodecyl sulfate, 1.5 ml of 20% acetic acid (pH 3.5), 1.5 ml of 0.8% TBA, 0.2 ml of the homogenate, and distilled water. The mixture was incubated for 1 h at 95°C, cooled for 5 min with tap water, vigorously mixed with 5 ml of a n-butanol-pyridine (15-1, v/v) mixture and centrifuged for 10 min at 3000 rpm. The absorbance of the organic layer (upper n-butanol phase) was determined at 532 nm. 1,1,3,3-Tetramethoxypropane was utilized to establish the standard curve, and the final MDA concentration was expressed as nmol MDA per mg protein.

Determination of GSH level

To determine GSH content in accordance with the method described by Higach (29), 0.1 ml of the tissue homogenate was added to an equal volume of 10% TCA solution, and then centrifuged for 20 min at 3000 rpm. A 0.1-ml aliquot of the supernatant was added to 0.5 ml of a 0.01 M NaNO₂ - 0.2 N H₂SO₄ (1 – 9, v/v) mixture and incubated for 5 min at room temperature, followed by the addition of 0.2 ml of a 0.5% sulfamic acid ammonium solution, 1 ml of a 1% HgCl₂ - 3.4% sulfanilamide / 0.4 N HCl mixed solution (1 – 9, v/v), and 1 ml of a 0.4 N HCl solution containing 0.1% N-(1-naphthyl)ethylenediamine. Five minutes later, the absorbance was determined at 540 nm. The GSH content was expressed as nmol GSH per mg protein.
**ROS measurement**

LLC-RK1 cells, a cultured renal epithelial cell line derived from the rabbit kidney, were obtained from the Korea Cell Line Bank (Seoul, Korea). LLC-RK1 cells were maintained as monolayer cultures in RPMI 1640 medium supplemented with 10% FBS. For estimation of intracellular ROS levels, a cell membrane permeable and oxidant-sensitive fluorescent dye CM-H$_2$DCFDA was employed. This dye passively diffuses into cells, where its acetate groups are cleaved by intracellular esterases and its chloromethyl group reacts with intracellular glutathione and other thiols. Subsequent oxidation yields a fluorescent product, 2',7'-dichlorofluorescein (DCF) that is retained inside the cell. Briefly, LLC-RK1 cells ($1 \times 10^4$ cells) were seeded in 96-well plates for 24-h incubation. Cells were incubated with ISL at the indicated concentrations or 20 µM of NAC, a GSH precursor as a positive control, 2 h prior to treatment with 50 µM of cisplatin. Six hours later, the medium was aspirated. A 100-µl aliquot of warm HBSS buffer (pH 7.4) and 5 µM of CM-H$_2$DCFDA was added to each well and incubated further at 37°C for 30 min. The cells were then washed with warm HBSS twice and the generation of ROS was measured as the fluorescence intensity of DCF on a Perkin-Elmer LS 50B Fluorescence Spectrometer (Waltham, MA, USA) with an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

**Statistical analyses**

The results are expressed as means ± S.E.M. In order to determine the effects of treatment, data were analyzed via repeated measures of one-way ANOVA followed by Dunnett’s t-test. P values of less than 0.05 were considered to be statistically significant. All analyses were conducted using SPSS statistical software. (Version 10.0 software; SPSS Inc., Chicago, IL, USA).

**Results**

**ISL alone and in combination with cisplatin inhibits the growth of CT-26 colon carcinoma cells inoculated into BALB/c mice**

In an effort to investigate the antitumor activity of ISL and whether the oral administration of ISL reduces cisplatin-induced toxicity without any loss of the anti-neoplastic capacity of cisplatin, we initially evaluated the effects of ISL (1 mg/kg BW), cisplatin (5 mg/kg BW), and ISL together with cisplatin on tumorigenicity in a xenograft model, in which murine colon carcinoma CT-26 cells were inoculated into BALB/c mice. Orally administered ISL and intraperitoneally administered cisplatin treatment applied daily for 15 days significantly inhibited the growth of tumors, by 52% and 76%, respectively, as shown in Fig. 1. The mice receiving ISL together with cisplatin reduced tumor growth by 79%. To confirm the anticancer activity of ISL, we performed in vitro experiments to determine if ISL reduced the viability and DNA synthesis of CT-26 colon cancer cells in a dose-dependent manner (Fig. 2).

**ISL alleviates cisplatin-induced nephrotoxicity and hepatotoxicity**

The nephroprotective and hepatoprotective activities of ISL were determined in a xenograft model of colon carcinoma. As shown in Table 1, cisplatin administration induced a marked reduction in renal functions, as characterized by significant increases in serum BUN and creatinine levels, in all mice receiving cisplatin. Pretreatment with ISL 2 h prior to cisplatin administration exerted a remarkable preventive effect against cisplatin-induced increases in serum BUN and creatinine levels, as well as in serum AST and ALT levels. However, ISL itself did not affect kidney and liver function.

**ISL blocks cisplatin-induced oxidative stress**

Serum nitrite level as an indicator of NO production and tissue MDA level as a measure of lipid peroxidation were found to be elevated significantly, whereas tissue GSH levels were markedly reduced, in mice treated for 15 days with cisplatin in a xenograft model. The
cisplatin-mediated increases in serum nitrite (Fig. 3) and tissue MDA levels (Fig. 4) were prevented by ISL pretreatment. The reduction of tissue GSH levels by cisplatin treatment were also suppressed in mice treated with a combination of ISL and cisplatin (Fig. 5). Treatment with ISL alone did not influence the levels of serum nitrite, nor did it affect tissue MDA and GSH levels.

ISL reduces cisplatin-induced intracellular ROS

The amount of ROS inside LLC-RK1 renal epithelial cells stimulated by cisplatin or and ISL was assessed by measuring changes in DCF fluorescence as shown in Fig. 6. While cisplatin caused a significant increase in intracellular ROS level, pretreatment with ISL or NAC for 2 h remarkably inhibited the production of cisplatin-induced intracellular ROS in a dose-related manner.

Discussion

Chemotherapy improves the disease-free and overall survival in cancer patients, but its clinical applications are limited by profound side effects. Thus, strategies for cancer treatment using combined therapies or combined agents with distinct molecular mechanisms are considered to be more promising for higher efficacy and/or lower toxicity, thereby resulting in superior survival rates. There has been a growing amount of interest in investigating the effects of chemopreventive agents on the inhibition of cancer cell growth and toxicity in combination with chemotherapeutic agents.

Cisplatin is one of the most active cytotoxic agents used in the treatment of cancer, and induces mitochondrial dysfunctions, particularly the inhibition of the

Table 1. Effect of ISL on the cisplatin-induced nephrotoxicity and hepatotoxicity

<table>
<thead>
<tr>
<th>Groups</th>
<th>BUN (mg/dl)</th>
<th>Creatinine (mg/dl)</th>
<th>AST (U/L)</th>
<th>ALT (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.1 ± 0.1</td>
<td>4.2 ± 0.3</td>
<td>17.8 ± 0.8</td>
<td>3.6 ± 0.4</td>
</tr>
<tr>
<td>CT-26 cells</td>
<td>5.4 ± 0.8</td>
<td>5.3 ± 0.3</td>
<td>33.4 ± 0.8</td>
<td>7.1 ± 1.2</td>
</tr>
<tr>
<td>CT-26 cells + ISL (1 mg/kg BW, p.o.)</td>
<td>4.4 ± 0.1</td>
<td>4.7 ± 0.2</td>
<td>21.5 ± 2.1</td>
<td>5.8 ± 0.6</td>
</tr>
<tr>
<td>CT-26 cells + cisplatin (5 mg/kg BW, i.p.)</td>
<td>50.1 ± 2.0*</td>
<td>14.8 ± 0.7*</td>
<td>91.7 ± 7.2*</td>
<td>29.5 ± 1.2*</td>
</tr>
<tr>
<td>CT-26 cells + ISL + cisplatin</td>
<td>34.2 ± 1.5*</td>
<td>9.2 ± 0.6**</td>
<td>51.1 ± 1.4**</td>
<td>16.3 ± 2.6*</td>
</tr>
</tbody>
</table>

Values are expressed as the mean ± S.E.M. (n = 8). #P<0.05 vs CT-26 cell–inoculated group. *P<0.01, **P<0.01 vs the CT-26 cell–inoculated group with only cisplatin treatment.
electron transfer system, thereby resulting in enhanced ROS production (30, 31) and subsequent tissue damages. Therefore, the administration of antioxidants prior to cisplatin treatment has been used to protect against nephrotoxicity and hepatotoxicity (32). Recent studies have shown that dietary chemopreventive agents with antioxidative activity, including selenium (33), vitamin C, vitamin E (34), capsaicin from hot red peppers (35), and caffeic acid phenethyl ester from honeybee propolis (36), can attenuate cisplatin-induced nephrotoxicity and hepatotoxicity. However, the impact of chemopreventive agents with antioxidative activity on chemotherapeutic efficacy remains controversial. Some have argued that antioxidants scavenge the ROS integral to the activity of certain chemotherapy drugs, thereby diminishing therapeutic efficacy. A recent study showed that dietary curcumin with antioxidative and chemopreventive activities reduced camptothecin-, methotrexate-, and doxorubicin-induced apoptosis in human breast cancer cells via the inhibition of ROS generation and significantly inhibited cyclophosphamide-induced tumor regression in an in vivo model of human breast cancer (37). By way of contrast, several trials have suggested that chemopreventive antioxidants mitigate toxicity and increase survival times and tumor responses (1).

The results of both in vitro and in vivo studies have indicated that ISL inhibited cell viability and induced apoptotic cell death by reducing cyclooxygenase-2 expression in colon cancer cell lines and also exerted a preventative effect on colon carcinogenesis caused by carcinogens in mice (25, 26). In addition, ISL has been shown to have antioxidative and superoxide scavenging...
properties. First, we found that the administration of ISL alone significantly reduced the size of the solid tumors in CT-26 cell-inoculated BALB/c mice without inducing nephrotoxicity, hepatotoxicity, and oxidative stress, and ISL reduced the proliferation of CT-26 murine colon cancer cells in a dose-dependent manner. Therefore, its anti-tumor activity may be attributed to its anti-proliferative activity as well as apoptosis-inducing capacity. Several studies have demonstrated that cisplatin mediates its antineoplastic effects via the generation of ROS following the formation of DNA adducts (38, 39). Thus, ISL with antioxidative and superoxide scavenging activities has the potential to inhibit the effectiveness of cisplatin therapy. However, the combination of ISL and cisplatin in this study was not shown to affect the therapeutic efficacy of cisplatin.

Furthermore, in our xenograft model, ISL suppressed cisplatin-induced kidney damage characterized by increases in serum creatinine and BUN, as well as cisplatin-induced liver damage characterized by increases in the levels of serum ALT and AST. Cisplatin therapy induces oxidative stress, principally involving ROS, in renal tubular cells. The interaction of ROS with cellular components may result in damage to biomolecules, including DNA, proteins, and lipids. Cisplatin treatment also induces a significant increase in the activity of calcium-independent nitric oxide synthase in rat kidney and liver tissues, resulting in an increase in serum NO levels as well as in tissue NO formation (40, 41). Peroxynitrite, which is generated by the reaction between nitric oxide and superoxide anion, also oxidizes biomolecules. In addition, cisplatin-induced oxidative stress is a consequence of GSH depletion, and cellular GSH concentration is negatively correlated with the levels of oxidatively damaged biomolecules. The balance between oxidants and antioxidants is crucial for the maintenance of the biological integrity of the tissues. Therefore, the depletion of GSH is an early and necessary event occurring during cisplatin-induced lipid peroxidation and subsequent toxicity. It has been suggested that the levels of GSH in the liver are significantly reduced by cisplatin treatment, although very little information is currently available regarding cisplatin-induced liver injury and the mechanisms underlying its hepatotoxicity (42, 43), and this suggestion was corroborated in our repeated preliminary experiments (data not shown). In the present study, our results showed that the repeated oral administration of ISL in combination with cisplatin treatment prevented cisplatin-mediated increases in the levels of serum nitric oxide and tissue lipid peroxidation, and also recovered the depleted GSH levels in tissue to a significant degree. Moreover, we explored scavenging activity of ISL on ROS inside cisplatin-treated renal epithelial cells. ISL inhibited considerably cisplatin-induced intracellular ROS level. These results suggest that ROS scavenging activity of ISL is directly associated with its protection against tissue damages by cisplatin-induced oxidative stress.

In conclusion, the administration of ISL inhibits the growth of tumors without the induction of toxicity and attenuates nephrotoxicity and hepatotoxicity by reducing cisplatin-induced oxidative stress with no attendant loss of therapeutic efficacy in a xenograft model of colon carcinoma. Therefore, supplementation with ISL may be one approach that could be utilized to counteract the side effects of cisplatin therapy in cancer patients.

Acknowledgments

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