Jaceosidin, isolated from dietary mugwort (Artemisia princeps), induces G2/M cell cycle arrest by inactivating cdc25c-cdc2 via ATM-Chk1/2 activation

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ABSTRACT

Jaceosidin, a flavonoid derived from Artemisia princeps [Japanese mugwort], has been shown to inhibit the growth of several human cancer cells. However, the exact mechanism for the cytotoxic effect of jaceosidin is not completely understood. In this study, we investigated the molecular mechanism involved in the antiproliferative effect of jaceosidin in human endometrial cancer cells. We demonstrated that jaceosidin is a more potent inhibitor of cell growth than cisplatin in human endometrial cancer cells. In contrast, jaceosidin-induced cytotoxicity in normal endometrial cells was lower than that observed for cisplatin. Jaceosidin induced G2/M phase cell cycle arrest and modulated the levels of cyclin B and p21-cdc2 in HeLa cells. Knockdown of p21 using specific siRNAs partially abrogated jaceosidin-induced cell growth inhibition. Additional mechanistic studies revealed that jaceosidin treatment resulted in an increase in phosphorylation of Cdc25C and ATM-Chk1/2. Ku55933, an ATM inhibitor, reversed jaceosidin-induced cell growth inhibition, in part. Moreover, jaceosidin treatment resulted in phosphorylation of ERK, and pretreatment with the ERK inhibitor, PD98059, attenuated cell growth inhibition by jaceosidin. These data suggest that jaceosidin, isolated from Japanese mugwort, modulates the ERK/ATM/Chk1/2 pathway, leading to inactivation of the Cdc2-cyclin B1 complex, followed by G2/M cell cycle arrest in endometrial cancer cells.

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1. Introduction

Artemisia princeps (Japanese mugwort) is a familiar plant that is widely used in East Asia as a food substance and medicinal herb. In Japan and Korea, it is called yomogi and ssuk, respectively, and its widely used in East Asia as a food substance and medicinal herb. In Japan and Korea, it is called yomogi and ssuk, respectively, and its widely used in East Asia as a food substance and medicinal herb. In contrast, jaceosidin-induced cytotoxicity in normal endometrial cells was lower than that observed for cisplatin. Jaceosidin induced G2/M phase cell cycle arrest and modulated the levels of cyclin B and p-Cdc2 in HeLa cells. Knockdown of p21 using specific siRNAs partially abrogated jaceosidin-induced cell growth inhibition. Additional mechanistic studies revealed that jaceosidin treatment resulted in an increase in phosphorylation of Cdc25C and ATM-Chk1/2. Ku55933, an ATM inhibitor, reversed jaceosidin-induced cell growth inhibition, in part. Moreover, jaceosidin treatment resulted in phosphorylation of ERK, and pretreatment with the ERK inhibitor, PD98059, attenuated cell growth inhibition by jaceosidin. These data suggest that jaceosidin, isolated from Japanese mugwort, modulates the ERK/ATM/Chk1/2 pathway, leading to inactivation of the Cdc2-cyclin B1 complex, followed by G2/M cell cycle arrest in endometrial cancer cells.

Abbreviations: ATM, ataxia-telangiectasia mutated; Caspase, cystein aspartyl-specific protease; Cdk, cell division cycle 2; CDKi, cyclin-dependent kinase inhibitor; ERK, extracellular signal-regulated kinase; MAPK, mitogen activated protein kinase; MTI, 3(4-dimethylthiazol-2-71]-2,5-diphenyl tetrazolium bromide; PI, propidium iodide; PI3K, phosphatidylinositol 3-kinase; siRNA, small interfering RNA.

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1. Introduction

Artemisia princeps (Japanese mugwort) is a familiar plant that is widely used in East Asia as a food substance and medicinal herb. In Japan and Korea, it is called yomogi and ssuk, respectively, and its leaves are commonly used for teas, spices, and cooking ingredient. The main constituents of A. princeps are eupatilin and jaceosidin (Fig. 1A), which are part of the flavonoid family. A. princeps extract and its flavonoid components have been shown to have anti-hyperglycaemic (Choi et al., 2011), lipid lowering (Jung et al., 2009; Yamamoto et al., 2011), anti-oxidative (Choi et al., 2008; Nugroho et al., 2010), and anti-inflammatory (Chang et al., 2009; Min et al., 2009) properties. Additionally, they have been reported to have anti-cancer effects against several cancers (Cho et al., 2011; Ju et al., 2012; Park et al., 2008; Sarath et al., 2007). Only a few studies on jaceosidin have been reported while there are many reports on the bioactive properties of eupatilin, and the molecular mechanisms underlying jaceosidin’s anti-cancer activity are poorly understood. Therefore, in the present study, we investigated the anti-cancer effect of dietary flavonoid jaceosidin isolated from A. princeps and its molecular mechanism of action in human endometrial cancer cells.

Endometrial cancer is the most prevalent gynaecological malignancy. Although most women with endometrial cancer have a relatively good prognosis (Jemal et al., 2009), therapeutic options for advanced and recurrent endometrial cancer remain limited. The survival rates for patients with terminal stage and recurrent endometrial cancer are only 18% and 7.7%, respectively (Creasman et al., 2001). Thus, there is an urgent need for new preventive and therapeutic agents for late-stage and recurrent endometrial cancer.

Cell cycle de-regulation resulting in uncontrolled cell proliferation is one of the most frequent alterations that occurs during tumor development (Collins et al., 1997). Therefore, cell cycle blockade is regarded as an effective strategy for eliminating cancer
cells (Buolamwini, 2000; Hajduch et al., 1999). Among the major regulated cell cycle checkpoints, the G2/M checkpoint is known to maintain chromosomal integrity by allowing cells to repair DNA damage before entering mitosis. In response to DNA damage, molecular sensors such as ataxia telangiectasia mutated (ATM) can be activated, which initiate signal transduction pathways that lead to cell cycle arrest and allow time to correct the damage (Sherr, 2000). ATM phosphorylates Chk2 on threonine 68 (Thr-68) and Chk1 on serine 317 and 345 (Ser-317 and Ser-345), resulting in their activation (Matsuoka et al., 1998; Melchionna et al., 2000). Phosphorylation of Cdc25C, which is controlled by Chk1 and Chk2 complexes are bound by p21CIP1/WAF1, a member of the cyclin-dependent kinase inhibitor (CDKI) family, rendering the complex inactive (Ando et al., 2001; Harper et al., 1993).

2. Materials and methods

2.1. Materials

Jaceosidin (Fig. 1A) used for this study was isolated from Artemisia princeps Pampanini (AP) as described previously (Min et al., 2009). Briefly, AP cultivated in the field of GangHwa-Do, Korea, was collected and deposited in the Laboratory of Natural Product Chemistry, Kyung Hee University, Korea, with a voucher specimen (KHU05067). AP was extracted with 80% ethanol, evaporated under reduced pressure, suspended in water, and extracted, stepwise, with ethyl acetate (EtOAc). The EtOAc fraction (47 g) was chromatographed on silica gel (4 cm × 20 cm) with a step gradient of n-hexane-EtOAc (7:1, 5:1, 3:1, 1:1, v/v) to give 20 fractions (SSE-1–SSE-20) at the first column. The SSE-16 fraction (1.53 g) was re-separated on silica gel (4 cm × 20 cm) with CHCl3–MeOH solvent pairs (30:1, v/v) to obtain jaceosidin (3.125, 6.25, 12.5, 25, 50, and 100 μM) or cisplatin (12.5, 25, 50, 100, 200, and 250 μM) were added. After 48 h, 25 μl of MTT (5 mg/ml stock solution) was added, and the plates were incubated for an additional 4 h. The medium was discarded, and the formazan blue, which was formed in the cells, was dissolved in 50 μl DMSO. The optical density was measured at 540 nm using a microplate spectrophotometer (SpectraMax; Molecular Devices, Sunnyvale, CA, USA).

2.2. Trypan blue assay

The in vitro growth inhibitory effect of jaceosidin on the Hec1A cells was determined by trypan blue dye exclusion. The reduction in viable cell number was assessed for each day. The cells were seeded at a concentration of 3 × 104 cells/ml and were maintained for logarithmic growth by passaging them every 2–4 days, and incubated for 1–4 days with jaceosidin at various concentrations. Jaceosidin dissolved in DMSO was added to the medium in serial dilution (the final DMSO concentration in all assays did not exceeded 0.1%). Cells were loaded on a hemocytometer, and viable cell number was determined based on exclusion of trypan blue dye.

2.3. Cell culture and MTT assay

The endometrial cancer cell lines Hec1A and KLE are originally from American type culture collection. The normal endometrial cells HES, recently established by Dr. Krikun (Yale University, New Haven, Connecticut, USA), and HESC were kindly provided by Dr. Asgi Fazleabas of University of Illinois at Chicago (USA). Cells were cultured in DMEM supplemented with 5% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin sulfate (100 μg/ml). The cytotoxicity was assayed using a MTT assay. Briefly, the cells (5 × 103) were seeded in each well containing 50 μl of DMEM medium in a 96-well plate. After 24 h, various concentrations of jaceosidin (3.125, 6.25, 12.5, 25, 50, and 100 μM) or cisplatin (12.5, 25, 50, 100, 200, and 250 μM) were added. After 48 h, 25 μl of MTT (5 mg/ml stock solution) was added, and the plates were incubated for an additional 4 h. The medium was discarded, and the formazan blue, which was formed in the cells, was dissolved in 50 μl DMSO. The optical density was measured at 540 nm using a microplate spectrophotometer (SpectraMax; Molecular Devices, Sunnyvale, CA, USA).

2.4. Propidium iodide (PI) staining for cell cycle analysis

On the day of collection, the cells were harvested and washed twice with ice-cold PBS. The cells were fixed and permeabilised with 70% ice-cold ethanol at 4 °C for 1 h. The cells were washed once with PBS and resuspended in a staining solution containing propidium iodide (50 μg/ml) and RNase A (250 μg/ml). The cell suspensions were incubated for 30 min at room temperature followed by fluorescence-activated cell sorting (FACS) gating plus flow cytometry (Becton Dickinson Co., Germany) using 10,000 cells per each group.

2.5. Western blot analysis

Jaceosidin-treated cells were washed with ice-cold PBS and extracted in protein lysis buffer (Intron, South Korea). Protein concentration was determined by a Bradford assay. Protein samples of cell lysate were mixed with an equal volume of 5× SDS sample buffer, boiled for 5 min, and then separated on 10–12% SDS–AGE gels. After electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in 5% non-fat dry milk for 1 h, washed, and incubated with specific antibodies (cycin B1, p21, p27, β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and phospho-Cdc25C (Ser 216), Cdc25C, phospho-Akt, total-Akt, phospho-ERK1/2, total-ERK1/2, phospho-H2AX, H2AX and phospho-Chk2 from Cell Signaling (Beverly, MA, USA). We also pre-treated the cells with 10 μM of PD98059 (Invitrogen, Carlsbad, CA) and 3 μM of Ku55933 (Selleck, USA) for 60 min in inhibitor study.

Fig. 1. Growth inhibitory effects of jaceosidin on human endometrial cancer Hec1A cells. (A) Chemical structure of jaceosidin. (B) Exponentially growing cells were treated with the indicated concentration of jaceosidin for 4 days. Control, •, 25 μM; ▪, 50 μM; ○, 100 μM; ●, 150 μM. Inhibition of cell growth was assessed by the trypan blue exclusion test, as described in method. The data shown represent the mean ± SD of three independent experiments. *p < 0.05 vs. the control group.
(1:1000–2000). Following three washes in TBS-T, immuno-positive bands were visualized by enhanced chemiluminescence and exposed to ImageQuant LAS-4000 (Fujifilm Life Science, Japan).

2.6. Transfection of siRNA and expression vector

P21, p27, and control small interfering RNAs (siRNAs) were synthesized by Bio-neer technology (Daejon, South Korea). Hec1A cells were transfected with siRNA at a final concentration of 20 or 50 nmol/L using Lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer’s suggested protocol.

2.7. Statistical analysis

Data are presented as the mean ± SD of three individual experiments performed in triplicate. Student’s t-test and one-way ANOVA were used to identify statistically significant differences. P-values < 0.05 were considered to be statistically significant.

3. Results

3.1. Jaceosidin inhibits the growth of endometrial cancer cells

To examine the effect of jaceosidin on the viabilities of various cancer cells, we assessed its effects using IC50 by MTT assays (Table 1 and Supplementary Table 1). The IC50 of jaceosidin in Hec1A and KLE cells was 70.54 and 147.14 μM, respectively, which was significantly lower than that of cisplatin (IC50 = 225.63 and 186.3 μM, respectively). In contrast, cisplatin had significant cytotoxicity in the HES and HESC normal endometrial cells with an IC50 range of 21.6–22.9 μM; in contrast, the IC50 for jaceosidin was in the 52.68–55.1 range. These data suggest that the growth inhibitory effects of jaceosidin are greater than that of cisplatin in endometrial cancer cells, but they are much less than that of cisplatin in normal endometrial cells. It is of note that the cytotoxicity of cisplatin was greater than that of jaceosidin in the other cancer cell lines tested (Supplementary Table 1). In this regard, we choose Hec1A cells for further experiments. Exponentially growing Hec1A cells were exposed to various concentrations of jaceosidin for 24, 48, 72, and 96 h, and their growth was monitored using the trypan blue assay. A significant decrease in cell growth was observed in cells treated with jaceosidin (Fig. 1B). Jaceosidin showed cytostatic activity at concentration ranges from 50–100 μM in Hec1A cells.

3.2. Jaceosidin induces G2/M cell cycle arrest in Hec1A cells

Additional experiments were performed to elucidate jaceosidin’s cell growth inhibitory mechanism of action in Hec1A cells. To determine whether the growth inhibitory effect was associated with cell cycle arrest, the distribution of cells in each phase of the cell cycle was analyzed using flow cytometry. As shown in Fig. 2, jaceosidin treatment resulted in an increase in the number of Hec1A cells in the G2/M phase. After treatment with 100 or 150 μM jaceosidin for 16 h, the percentage of cells in the G2/M phase was 35.6% and 40.2%, respectively, compared to 25.1% in the control cells (Fig. 2A). Additionally, the G2/M arrest induced by jaceosidin occurred in a time-dependent manner (Fig. 2B). In contrast, jaceosidin did not significantly increase the number of apoptotic cells (sub-G1 phase). Jaceosidin also induced G2/M cell cycle arrest in KLE cells (Supplementary Fig. 1). Western blot analysis revealed that treatment with jaceosidin did not have a significant effect on caspase-3 activation (Supplementary Fig. 2A), which is an essential enzyme for the induction of apoptosis, or the expression of CDK2 and CDK4, which are G1/S transition-associated proteins (Supplementary Fig. 2B).

Cyclins, cyclin-dependent kinases (CDKs) and their inhibitors regulate cell cycle progression and arrest. Cyclin B1 and Cdc2 form a complex and cooperate to promote the G2/M phase transition. We investigated whether jaceosidin affected the expression of cyclin B1 and Cdc2 in cells treated with 100 μM jaceosidin. Treatment with jaceosidin considerably suppressed the levels of cyclin B1 at 8 h. Additionally, jaceosidin induced a remarkable increase in the phosphorylation of Cdc2 at Tyr 15, while no significant change in Cdc2 expression was observed up to 32 h (Fig. 3). Increased phosphorylation of Cdc2 at Tyr 15 suppresses its kinase activity and decreases the amount of cyclin B1, leading to the inactivation of the Cdc2-cyclinB1 kinase complex. These data suggest that the jaceosidin-induced growth inhibitory effect occurs via G2/M cell cycle arrest, not through the induction of apoptosis or other types of cell cycle arrest. Furthermore, jaceosidin-induced G2/M arrest is associated with the negative regulation of cyclin B1 and Cdc2 in Hec1A cells.

3.3. Jaceosidin increases the levels of p21 expression in Hec1A cells

To determine whether p21 and p27 play a role in the jaceosidin-induced cell cycle arrest, the effect of jaceosidin on the expression of p21 and p27 was investigated using Western blot analysis. As shown in Fig. 4A, treatment with jaceosidin markedly increased both p21 and p27 expression in a time-dependent manner. To determine whether the up-regulation of p21 and p27 expression by jaceosidin was involved in jaceosidin-induced growth inhibition, we investigated the effect of jaceosidin on cell viability in Hec1A cells following down-regulation of p21 and p27 using siRNA (Fig. 4B and C). The gene silencing efficiency of p21 and p27 siRNA was shown in Supplementary Fig. 3. Knockdown of p21 partially abrogated jaceosidin-induced growth inhibition. In contrast, down-regulation of p27 had no effect on jaceosidin-induced effects on cell growth. These data suggest that the jaceosidin-induced G2/M cell cycle arrest is mediated in part by p21, but not p27. This finding is consistent with a previous one suggesting that jaceosidin elevated the expression of p21 in ras-transformed human breast epithelial cells (Kim et al., 2007).

3.4. Jaceosidin facilitates the phosphorylation of Cdc25C, Chk1/2, and ATM

The Cdc25C phosphatase is believed to regulate the phosphorylation state of Cdc2 at Tyr 15. For this reason, the phosphorylation of Cdc25C was examined following treatment with jaceosidin (100 μM). The level of p-Cdc25C (Ser 216) was notably increased by jaceosidin treatment (Fig. 5). Chk1/2 kinases act up-stream of Cdc25C (Matsuoka et al., 1998). Therefore, loss of Cdc25C phosphatase activity can result from the phosphorylation of inhibitory sites by Chk1/2. As shown in Fig. 5, jaceosidin up-regulates the phosphorylation status of Chk1/2 kinases. ATM is a central kinase in triggering cellular responses to DNA damage and can phosphorylate several substrates that are involved in cell cycle checkpoints, including Chk1 (Sanchez et al., 1999) and Chk2 (Chaturvedi et al., 1999). Because we observed strong phosphorylation of both Chk1

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Table 1

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Origin</th>
<th>IC50 (μM)</th>
<th>Jaceosidin</th>
<th>Cisplatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>HES</td>
<td>Human endometrial stroma cells</td>
<td>52.68</td>
<td>22.99</td>
<td></td>
</tr>
<tr>
<td>HESC</td>
<td>Human endometrial epithelial cells</td>
<td>55.10</td>
<td>21.66</td>
<td></td>
</tr>
<tr>
<td>Hec1 A</td>
<td>Human endometrial cancer cells</td>
<td>70.54</td>
<td>225.63</td>
<td></td>
</tr>
<tr>
<td>KLE</td>
<td>Human endometrial cancer cells</td>
<td>147.14</td>
<td>186.30</td>
<td></td>
</tr>
</tbody>
</table>

*IC50 is defined as the concentration that results in a 50% decrease in the number of cells compared to that of the control cultures in the absence of inhibitor. The values represent the means of results from three independent experiments with similar patterns.*
and Chk2, we examined whether jaceosidin treatment resulted in ATM phosphorylation. Jaceosidin stimulated the activation of ATM as part of a DNA damage response, as indicated by an increase in histone H2AX (γH2AX) phosphorylation (Fig. 6A and B). Additionally, pretreatment with the ATM inhibitor, Ku-55933, partially reduced the growth inhibitory effect of jaceosidin (Fig. 6C). These data implicate the ATM-Chk1/2-Cdc25C pathway in the cell cycle arrest induced by jaceosidin.

3.5. ERK1/2 is involved in the jaceosidin-induced G2/M cell cycle arrest

It has been demonstrated that jaceosidin-induced apoptosis is mediated by regulation of ERK1/2 activation as well as ROS accumulation (Kim et al., 2007). In this regard, we investigated the involvement of the ERK signaling in growth-inhibitory effect by jaceosidin in endometrial cancer. Interestingly, jaceosidin increased ERK1/2 phosphorylation (Fig. 7A). Additionally, jaceosidin-induced growth inhibition of Hec1A cells was significantly abrogated in the presence of the ERK1/2 inhibitor, PD98059 (Fig. 7B). These findings are consistent with previous reports showing that ERK1/2 activation may contribute to the activation of the ATM, Chk1/2, and Cdc25C pathway (Kuo et al., 2006; Wei et al., 2010; Yan et al., 2007).

4. Discussion

Jaceosidin, a naturally occurring dietary flavonoid isolated from Artemisia princeps, has anti-inflammatory, anti-oxidative, and anti-

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**Fig. 2.** Effects of jaceosidin on the cell cycle in Hec1A cells. DNA fluorescence flow cytometry histograms of Hec1A cells were generated after treatment with jaceosidin for the indicated times (8, 16, and 32 h) and concentrations (100 or 150 μM). Flow cytometry (FACS) determined the cell cycle distribution profiles of the cells. Results were highly reproducible in three independent experiments.

**Fig. 3.** Effects of jaceosidin on the expression of G2/M phase-related proteins in the Hec1A cells. Cells were treated with jaceosidin (100 μM) for the indicated times (8, 16, and 32 h). The expression of cyclin B1 and Cdc2 proteins and the phosphorylation of Cdc2 (Tyr 15) was measured by Western blotting using specific antibodies. These pictures are representatives of three individual experiments.

**Fig. 4.** Effects of jaceosidin on the expression of p21 and p27 in Hec1A cells. (A) The expression of p21 and p27 was measured by Western blotting using specific antibodies. These pictures are representatives of three individual experiments. (B and C) Hec1A cells were transiently transfection with p21 siRNA (B), p27 siRNA (C) or control (con) siRNA and treated with jaceosidin (100 μM) for 32 h. The assay was performed to investigate cell viability. *p < 0.05 vs. the control group, #p < 0.05 vs. con siRNA transfected group.
tumor properties (Kim et al., 2007; Lee et al., 2005, 2007; Lv et al., 2008; Min et al., 2009; Park et al., 2008). Jaceosidin has been reported to have growth-inhibitory effects in several cancer cell lines including breast, ovarian, cervical, prostate, colon cancer, and glioblastoma cells (Khan et al., 2011; Kim et al., 2007; Lee et al., 2005; Lv et al., 2008; Woerdenbag et al., 1994). However, the mechanism of action of jaceosidin on the cell growth inhibition in cervical, prostate, and colon cancer has not been elucidated (Lee et al., 2005; Woerdenbag et al., 1994). On the other hand, the growth-inhibitory effect of jaceosidin was associated with the induction of apoptosis in ras-transformed human breast epithelial cells (Kim et al., 2007) and CAOV-3 ovarian cancer cells (Lv et al., 2008). The proapoptotic activity of jaceosidin in ras-transformed human breast epithelial cells was mediated by ROS accumulation and inhibition of ERK1/2 and JNK activation (Kim et al., 2007). In CAOV-3 cells, jaceosidin-induced apoptosis involved activation of caspase-3 via mitochondrial pathway (Lv et al., 2008). In addition, jaceosidin induced apoptosis in U87 glioblastoma cells through G2/M phase arrest (Khan et al., 2011). In the present study, we found that jaceosidin induces growth inhibition in human endometrial cancer cells by increasing the population of cells in the G2/M phase of the cell cycle. These data suggested that jaceosidin has cell line-specific growth-inhibitory effect in various cancer cells. The exact mechanism of the different responses of cancer cells to jaceosidin remains to be further investigated.

Cell cycle blockade is regarded as an effective strategy for eliminating cancer cells (Buolamwini, 2000; Hajduch et al., 1999). Indeed, agents that inhibit cell cycle progression, such as mifepristone and histone deacetylase inhibitors, have been reported as potential therapeutics for endometrial cancers (Nava et al., 2008; Takai and Narahara, 2007). Regulation of the cell cycle occurs at two major checkpoints, one at the G1/S transition and one at the G2/M transition. The G2/M checkpoint plays a key role in the maintenance of chromosomal integrity by allowing cells to repair DNA damage before entering mitosis. Cdc2 (cell division cycle 2), a cyclin-dependent kinase, is a key regulator of the cell cycle at this checkpoint (Nurse, 1990). Cdc2 activity is regulated through phosphorylation and dephosphorylation by Wee1 kinase and Cdc25C phosphatase, respectively. Cdc2 binds to cyclin B to form a complex that is activated at the onset of mitosis by dephosphorylation of the inhibitory sites on Cdc2 by a functional Cdc25C. In this study, jaceosidin modulated the levels of cyclin B and p-Cdc2, which are involved in the G2/M phase transition in Hec1A cells.

It is well documented that p21WAF1/CIP1, a member of the cyclin-dependent kinase inhibitor (CDKI) family, plays a role in both the G1 and G2 checkpoints (Ando et al., 2001; Harper et al., 1993). Cyclin B-Cdc2 complexes are bound by p21WAF1/CIP1, rendering the complex inactive. In addition, Kim et al. reported that jaceosidin increases p21 expression in ras-transformed human breast epithelial cells (Kim et al., 2007). In this regard, we first investigated whether p21 plays a role in the jaceosidin-induced G2/M arrest in Hec1A cells. Jaceosidin was shown to increase p21 expression in Hec1A cells. Additionally, jaceosidin-induced inhibition of cell growth was partially attenuated by p21 siRNA. Earlier studies reported that p21 is involved in G2/M arrest (Wong et al., 2008). For example, over-expression of p21 caused a number of different cancer cells to arrest at G2 (Bates et al., 1998; Medema et al., 1998; Niculescu et al., 1998). In contrast, ionizing radiation failed to induce G2 arrest in cells lacking p21 (Bunz et al., 1998).

Because p21 was shown to only be partially involved in jaceosidin-induced cell growth, it is possible that additional mechanisms are involved in jaceosidin-induced G2/M arrest. It has been reported that jaceosidin induces apoptosis in ras-transformed human breast epithelial cells through generation of reactive oxygen species (ROS) (Kim et al., 2007). Thus, we have investigated whether jaceosidin induces the formation of ROS and whether the antioxidant NAC can attenuate the growth inhibitory effect of jaceosidin in Hec1A cells. As shown in Supplementary Fig. 4, jaceosidin did not affect the level of ROS, and NAC failed to abrogate jaceosidin activity, suggesting that ROS are not involved in jaceosidin’s effects on Hec1A cells. Next, we determined the activation status of the ATM and Chk1/2 checkpoint kinases. ATM and Chk1/2 are activated by phosphorylation of Ser 216 and subsequently, inactivated by the Cdc25C phosphatase. In turn, Cdc2 is inactivated by phosphorylation at Tyr 15, leading to G2/M cell cycle arrest. We demonstrated that ATM plays a central role in mediating the jaceosidin-induced cell cycle arrest. Our conclusions are based on several findings. First, jaceosidin induces ATM phosphorylation in a time-dependent manner at 30 min (Fig. 6A). Second, the specific ATM kinase inhibitor Ku55933 partially antagonised jaceosidin-induced cell growth inhibition (Fig. 6C). Third, jaceosidin treatment results in the phosphorylation of the Chk1 and Chk2 downstream proteins, which, in turn, phosphorylate and inactivate Cdc25C (Fig. 5). Inactivation of Cdc25C leads to inhibition of Cdc2 through phosphorylation of Tyr 15 and, subsequently, to G2/M cell cycle arrest. We also demonstrated that H2AX, a well-described substrate of ATM kinases, is phosphorylated in a time-dependent manner (Fig. 6B). H2AX phosphorylation is a major and early response to double-stranded DNA breaks (DSBs) (Fernandez-Capetillo et al., 2003). ATM kinase induces phosphorylation of Ser 139 on the histone H2AX tail and the subsequent rapid formation of H2AX foci at the DSB sites (Redon et al., 2002). These observations suggest that jaceosidin may induce a DNA damage response, which results in the anti-tumor effect of jaceosidin in human endometrial cancer cells. These findings are consistent with previous findings suggesting that some flavonoids are likely to induce strand breaks in human DNA and inhibit cell growth (Park et al., 2009; Shin et al., 2011).

Current evidence suggests that ERK1/2 and Akt regulate the ATM/Chk1/2 pathway during the G2/M arrest (Kuo et al., 2006; Ouyang et al., 2009; Wei et al., 2010; Yan et al., 2007). For example, Wei et al. reported that the ERK1/2 kinase promotes G2/M arrest in etoposide-treated MCF-7 cells by facilitating ATM activation (Wei et al., 2010). G2/M arrest induced by irradiation involves the activation of ERK1/2 and the subsequent activation of ATM in MCF-7 cells (Yan et al., 2007). Suppression of Akt by plumbagin enhanced the activation of Chk2, resulting in G2/M arrest in human breast cancer cells (Kuo et al., 2006). Treatment with genistein induced G2/M arrest and affected the phosphorylation state of ERK1/2 and Akt in human leukaemia and ovarian cancer cells (Ouyang et al., 2009; Sanchez et al., 2009). We demonstrated that jaceosidin increases the phosphorylation level of ERK1/2 (Fig. 7A) and decreases the phosphorylation level of Akt (Supplementary
Additionally, jaceosidin-induced inhibition of cell growth was significantly attenuated by the ERK inhibitor, PD98059. In a parallel experiment, Akt overexpression did not result in a significant change in jaceosidin-induced cell growth inhibition (Supplementary Fig. 5B). These findings demonstrate that ERK activation, but not Akt inactivation, may contribute to the activation of the ATM pathway by jaceosidin in endometrial cancer cells. A study showed that jaceosidin induced apoptosis and inhibited the activation of ERK1/2 in ras-transformed human epithelial cells (Kim et al., 2007). The inhibition of ERK1/2 by jaceosidin in the previous study (Kim et al., 2007) is different from our present study that jaceosidin induced G2/M arrest by activating the ERK1/2 signaling in the endometrial cancer cells. These data suggested that jaceosidin has cell type-specific regulatory effect on signaling pathway in various cancer cells. The exact mechanism of the different responses of cancer cells to jaceosidin remains to be further investigated. It is noteworthy that cAMP/PKA has opposite effects on ERK1/2 activation in different cell lines (Frodin et al., 1994; McKenzie and Pouysségur, 1996; Young et al., 1994). Activation of ERK1/2 by cAMP/PKA in a cell-type seemed to be mediated by B-RAF, while cAMP/PKA-induced inhibition of ERK1/2 in another cell-type involved RAF-1 (Qiu et al., 2000; Stork and Schmitt, 2002). In this regard, it will be of interest to further investigate whether cAMP/PKA and/or RAF isoforms are involved in the opposite effects of jaceosidin for ERK1/2 activation in breast and endometrial cancer cells.

In this study, we demonstrated that jaceosidin was more potent than cisplatin in inhibiting cell growth in the human endometrial cancer Hec1A and KLE cells. Jaceosidin showed relatively low cytotoxicity in normal human endometrial HES and HESC cells when compared to cisplatin. The IC50 values of cisplatin for Hec1A and KLE cells (IC50 = 225.63 and 186.3 μM, respectively) revealed in this study were a bit different from that in other previous studies (Chon et al., 2012; Rabik et al., 2008). The variations in IC50 value for cisplatin in several studies might be due to the different experimental conditions such as passage of the cell lines, cell seeding density, serum percentage in the medium, and incubation time.

Development of novel therapeutic agents is urgently required for the treatment and survival of those patients with late-stage and recurrent endometrial cancer (Garai et al., 2006). The findings reported herein provide a firm molecular basis for the effects underlying the use of Artemisia princeps (Japanese mugwort).
and jasacin B as potential therapeutic agents for endometrial cancer.

Conflict of Interest

The authors have declared no conflict of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fct.2012.01.026.

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