Original Article

Grandinin down-regulates phosphorylation of epidermal growth factor receptor

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Abstract: Aims: Grandinin (C_{46}H_{34}O_{30}) is a compound found in *Melaleuca quinquenervia* leaves and in oaks. This study is to determine effects of grandinin on malignant lung cells and the related molecular mechanisms. Methods: Malignant cells were treated with grandinin with various concentrations. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays and apoptosis assays were performed to determine effects of grandinin on cell viability and apoptosis. Western blotting and real time-PCR were used to determine if grandinin affects levels of phosphorylated EGFR (p-EGFR) and phosphorylated AKT (p-AKT), as well as their mRNA transcript levels. Results: It was found that grandinin treatments reduce viability of malignant lung cells and induces apoptosis. When treated with grandinin (16 µM), the apoptosis of the three lung cancer cell lines MS-1, A549, and LK-2 were increased by 8-9 folds, in comparison with the cells treated with DMSO only (the control condition). Furthermore, grandinin treatments lead to down-regulation of levels of p-EGFR and p-AKT in three malignant lung cell lines. However, grandinin does not affect mRNA levels of EGFR and AKT. Conclusions: These experimental results indicated grandinin significantly reduce malignant cell viability and effectively induces apoptosis of malignant lung cells by mediating phosphorylation down-regulation of cellular signaling proteins EGFR and AKT. It is suggested that grandinin treatments might be an effective therapeutic strategy of lung malignancies upon further studies in the future.

Keywords: Grandinin, phosphorylation, epidermal growth factor receptor, down-regulates phosphorylation

Introduction

Epidermal growth factor receptor (EGFR) is an important cell surface receptor, which is activated by binding of its specific ligand such as epidermal growth factor and transforming growth factor [1, 2]. Upon binding of the ligands, EGFR elicits a transition from an inactive monomeric form to its active homodimer form [2]. Dimerization of EGFR triggers its intrinsic intracellular protein-tyrosine kinase activity, resulting in autophosphorylation of several tyrosine residues in the C-terminal domain of EGFR [2]. This autophosphorylation elicits activation of downstream signaling pathways, such as the AKT-mTOR pathway [3-5]. These pathways are involved in multiple cell processes including cell proliferation, apoptosis, cell migration, and so on [3, 4].

Inhibition of EGFR activity is an important chemotherapy of various tumors. The effective chemotherapy for lung cancers includes gefitinib that has significant anti-tumor activities [6-8]. Gefitinib function as EGFR tyrosine-kinase inhibitors. In addition, a number of polyphenols has been found to target receptor tyrosine kinases, including EGFR [9-14].

Grandinin (C_{46}H_{34}O_{30}), a compound found in *Melaleuca quinquenervia* leaves [15] and in oaks [16] is reported to inhibit the phosphorylation of EGFR in human colon carcinoma cells [17]. However, it is unclear if grandinin affect phosphorylation of EGFR in other types of malignancies. We have previously determined combined treatments of an Hsp90 inhibitor and TNF treatments on multiple cell resulted in synergistic killing of malignant lung cells [18]. Such effects were confirmed by the apoptosis determination using a fluorescence microscopic assay following staining of the drug-treated cells with Hoescht 33258. Moreover, the experimental results indicated that the synergistic killing due to Hsp90 inhibitor and TNF treat-
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<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences</th>
<th>Targets</th>
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<tbody>
<tr>
<td>EGFR_F</td>
<td>5’ GCGTCTCTGCGCGGAATGT</td>
<td>EGFR</td>
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<tr>
<td>EGFR_R</td>
<td>5’ CTTGCTTTTCCCCAGAAG</td>
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<tr>
<td>AKT_F</td>
<td>5’ AAGCAAATCAGGAGAGCCTG</td>
<td>AKT</td>
</tr>
<tr>
<td>AKT_R</td>
<td>5’ AAAGAAAAGGAGCTGAGGAG</td>
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ments may be related to the reduced IKKβ levels upon treatments [18].

Phosphorylated EGFR (p-EGFR) has been identified to correlate with progression of NSCLC [19, 20]. AKT is active in most NSCLC cells [21] and high level of phosphorylated AKT (p-AKT) is often correlated with lung cancers [22]. In this paper, we investigated the effects of grandinin on malignant cells. It was found that treatment of grandinin significantly reduces cell viabilities of three malignant lung cell types in vitro. Furthermore, grandinin inhibits levels of p-EGFR and p-AKT of the three cell lines. We also found that grandinin does not affect mRNA levels of EGFR and AKT.

Materials and methods

Reagents and cell lines

Grandinin (C_{46}H_{34}O_{30}) with a purity of greater than 99% was purified from Melaleuca quinquenervia leaves and provided by the Department of Chemistry in College of Life Sciences, Ocean University of Qingdao. Two small cell lung cancer (SCLC) cell lines (SBC3 and MS-1), an adenocarcinoma cell line (A549), and a squamous-cell carcinoma cell line (LK-2) were provided by Shanghai Cell Biology Institute (Shanghai, China). The cells were maintained in RPMI-1640 medium (Sigma-Aldrich Co. Ltd, Irvine, CA) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin/streptomycin at 37°C with 5% CO₂ and 100% humidity.

Cell treatments and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Briefly, cells at a density of 1 × 10⁵ cells/well were seeded into 6-well plates in RPMI-1640 supplemented with 10% FBS and were cultured for 24 h. The cells were then treated with vehicle control (DMSO, 0.1%, v/v), grandinin (0 µM, 2 µM, 4 µM, 8 µM, 16 µM). At the end of each experiment, cells were incubated with 0.5 mg/ml MTT for 4 h according to the protocol of manufacturer. Viability of treated cells was expressed relative to the control cells treated with DMSO. The relative viability was calculated.

Apoptosis assay

Cells at a density of 1 × 10⁶ cells/well were cultured in six-well plates in RPMI-1640 supplemented with 10% FBS for 48 h, followed by addition of DMSO (0.016%, v/v), grandinin (2 µM, 4 µM, 8 µM, 16 µM). After 48 h, cells were pelleted by centrifugation, washed once with PBS, fixed by incubation in 4% paraformaldehyde for 30 min at room temperature, and then washed again with PBS to remove the fixative. The fixed cells were resuspended in PBS that contained Hoescht 33258 (5 µg/ml), followed by an incubation at room temperature for 15 min in the dark. Aliquots of cells were placed on glass slides and examined for cells with apoptotic morphology (nuclear condensation and chromatin fragmentation) via fluorescence microscopy. To quantify the apoptosis, 250 nuclei from random microscopic fields were analyzed. Data are presented as the mean percentages of apoptotic cells.

Western blot assay

Total proteins were harvested from cells, separated on 10% SDS/PAGE gels, and then subjected to western blot analyses. The primary antibodies against the p-EGFR (about 180 kDa), p-AKT (about 60 kDa), and β-actin were purchased from Santa Cruz, USA (anti-p-EGFR, cat # sc-81489, 1:200; anti-p-AKT, cat # sc-33437, 1:200; anti-β-actin, cat # sc-130301, 1:10,000). Secondary antibodies were horse-radish-peroxidase-conjugated secondary antimouse IgG (cat # 1:10,000; Pierce Biotechnology) and anti-rabbit IgG (cat # 1:5,000; Pierce biotechnology). Bound antibodies were detected using the ECL system (Pierce Biotechnology). The experiments were repeated for at least 3 times. The mean normalized optical density (OD) of detected protein bands relative to the OD of β-actin band was calculated.

Real time-PCR

Quantitative RT-PCR analyses of EGFR and AKT mRNA levels in cells were performed. Briefly, total RNAs were harvested from cells using the RNeasy Kit (Qiagen, USA) according to the manu-
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The experimental data are expressed as mean ± SD. Statistical software (SPSS10.0) was used for independent sample t tests, followed by one-way variance analysis. In all analyses, \( P < 0.05 \) was considered statistically significant.

Figure 1. Cell treatments with DMSO (0.1%, v/v) or grandinin. Two SCLC cell lines (SBC3 and MS-1), an adenocarcinoma cell line (A549), and a squamous-cell carcinoma cell line (LK-2) were treated with either vehicle control (DMSO) or grandinin (2 µM, 4 µM, 8 µM, 16 µM) for 72 h. (A) SBC3 cells; (B) MS-1 cells; (C) A549 cells; (D) LK-2 cells. Cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay immediately before (day 0) and after 1, 2, or 3 days of incubation with the compounds. Values are means ± SD for three experiments. It is considered not significant, when \( P > 0.05 \) vs. control (DMSO) cell viability of each treatment. *, it is considered as a significant difference, when \( P < 0.05 \) vs. corresponding control.

Results

Grandinin treatments reduce viability of malignant cells

To investigate if grandinin affects proliferation of lung tumor cells in vitro, two SCLC cell lines (SBC3 and MS-1), an adenocarcinoma cell line (A549), and a squamous-cell carcinoma cell line (LK-2) were treated with either vehicle control DMSO (grandinin, 0 µM) or grandinin (2 µM, 4 µM, 8 µM, 16 µM) for 72 h. The treatment with DMSO served as a drug vehicle control (grandinin, 0 µM). The cells were analyzed for differences in cell killing upon various treatments via number counting of living cells in the presence or absence of the above compounds.

As shown in Figure 1, the cell treatment results showed that the treatments with the drug vehicle control (DMSO) did not significantly affect cell viability of all of these four types of cells (Figure 1). Grandinin with a concentration of 2 µM or 4 µM had slight effects, if any, on cell viability of all of these four types of cells. Grandinin (8 µM) decreased viabilities of the three lung cancer cell lines MS-1, A549, and LK-2 to approximately 50-60% at day 3, but no obvious decreases for SBC3 cells, when compared to the cells treated with DMSO only (grandinin, 0 µM). When the concentration of grandinin was increased to 16 µM, the viabilities of the three lung cancer cell lines MS-1, A549, and LK-2 to approximately 50-60% at day 3, but no obvious decreases for SBC3 cells, when compared to the cells treated with DMSO only (grandinin, 0 µM). When the concentration of grandinin was increased to 16 µM, the viabilities of the three lung cancer cell lines MS-1, A549, and LK-2 were reduced to 42%, 12%, and 18%, respectively, when compared with the cells treated with DMSO only. The viabilities of SBC3 cells were reduced to 85% only. These results suggest that such dosages (8 µM and 16 µM) of grandinin effectively reduce viability of malignant cells MS-1, A549, and LK-2.

Grandinin induces apoptosis of the malignant lung cells

Since grandinin effectively reduced viability of malignant cells, the effects of the compound on apoptosis in all of these 4 types of cells were...
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Figure 2. Detection of phenotype-dependent apoptosis induced by treatments with DMSO or grandinin. The SBC3, MS-1, A549, and LK-2 cells were treated with either vehicle control (DMSO) or grandinin (2 µM, 4 µM, 8 µM, 16 µM). Cells were harvested 48 h later. Hoechst 33258-stained cells were examined for apoptotic characteristics (nuclear margination and chromatin condensation) using a fluorescence microscope. Apoptotic incidence was calculated. Data were expressed as means ± SD for three independent experiments.

Figure 3. Grandinin decreases expression of p-EGFR and p-AKT. SBC3, MS-1, A549, and LK-2 cells were treated with either vehicle control (DMSO) or grandinin (16 µM) for 48 h. Whole-cell extracts were prepared and western blot analyses were performed to analyze the expression of p-EGFR, p-AKT, and β-actin. The cellular β-actin served as a loading control. Representative blots are given. The experiments were repeated for at least three times.

Further determined. The cells were treated with either vehicle control DMSO (grandinin, 0 µM) or grandinin (2 µM, 4 µM, 8 µM, or 16 µM) for 48 h. To quantify the apoptotic incidence, we used a fluorescence microscopic assay following staining of the drug treated cells with Hoechst 33258.

As shown in Figure 2, the treatments with the drug vehicle control (DMSO) did not significantly affect apoptosis of all of these four types of cells. Grandinin with a concentration of 2 µM or 4 µM had slight effects, if any, on apoptosis of all of these four types of cells. Grandinin (8 µM) increased viabilities of the three lung cancer cell lines MS-1, A549, and LK-2 by approximately 6-7 folds, but no obvious increases in apoptosis incidences of SBC3 cells, when compared to the cells treated with DMSO only (grandinin, 0 µM). When the concentration of grandinin was increased to 16 µM, the apoptosis of the three lung cancer cell lines MS-1, A549, and LK-2 were increased by 8-9 folds, when compared with the cells treated with DMSO only. The apoptosis incidences of SBC3 cells were not affected significantly. These results suggest that such dosages (8 µM and 16 µM) of grandinin effectively increase apoptosis incidences of malignant cells MS-1, A549, and LK-2.

Grandinin treatments lead to down-regulation of levels of p-EGFR and p-AKT

Since the p-EGFR and p-AKT levels are often correlated with lung cancers, we investigated the effects of grandinin on p-EGFR and p-AKT levels. The SBC3, MS-1, A549, and LK-2 cells were treated with either vehicle control (DMSO) or grandinin (16 µM) for 48 h. The total proteins were extracted and the levels of p-EGFR and p-AKT were determined by western blot analysis, with the cellular β-actin protein serving as a loading control. The mean normalized OD of p-EGFR and p-AKT bands relative to the OD of β-actin band from the same condition was all calculated and subjected to statistical analyses.
Grandinin inhibits EGFR phosphorylation

As shown in Figure 3, treatment with grandinin did not significantly affect (or slightly affect, if any) p-EGFR and p-AKT levels in SBC3 cells. However, treatments with grandinin decreased levels of p-EGFR and p-AKT levels in MS-1, A549, and LK-2 cells, when compared with the levels in the conditions treated with DMSO only. These results indicated that grandinin treatments lead to down-regulation of levels of p-EGFR and p-AKT in MS-1, A549, and LK-2 cells.

Grandinin does not affect mRNA levels of EGFR and AKT

To determine if grandinin affects mRNA levels of EGFR and AKT, the SBC3, MS-1, A549, and LK-2 cells were treated with either vehicle control (DMSO) or grandinin (16 µM) for 48 h. The total RNAs were isolated for all conditions and the EGFR and AKT mRNA transcript levels in the SBC3, MS-1, A549, and LK-2 cells were determined by quantitative RT-PCR. The levels of the mRNA transcripts in cells treated with DMSO only were assigned a value of 100.

As shown in Figure 4, the mean levels of EGFR and AKT mRNA transcripts in all 4 types of cells treated with grandinin were similar to those treated with DMSO. These results suggest that grandinin does not affect mRNA levels of EGFR and AKT.

Discussion

Grandinin was found to have cytotoxic functions to some malignancies [17, 23]. However, it is unknown that if grandinin has effects on lung tumor cells. In this study, we have determined the effects of grandinin on several lung cancer cell lines. The cell viability results show that grandinin with dosages of 8 µM and 16 µM effectively reduce viability of malignant cells MS-1, A549, and LK-2, but the effects on another cell line SBC3 are relatively smaller. It is necessary to continue study if the effect of grandinin on lung cancer cells is cell type-dependent. If so, this compound may have different effects on different pathological types of lung cancers. The smaller effects of grandinin on SBC3 may be related to the receptors on different types of cells.

The reduced cell viabilities may result from various reasons, such as increased apoptosis. The apoptosis assays show that this compound significantly increased the apoptosis rates of malignant cells MS-1, A549, and LK-2. The effective dosages are 8 µM and 16 µM, which are safe to normal human cells according to previous reports [17]. To achieve much higher efficacies, the combined uses of grandinin with other drugs such as gefitinib [6-8] or drug candidates such as 17-DMAG [18] should be investigated in the future.

EGFR, as a major mediator of development of many tumors, can induce activation of tumor proliferation and growth, and inhibit apoptosis [24, 25]. Activation of Akt is one of the mechanisms that mediate the effects of EGFR [26]. The roles of EGFR and AKT in tumor development are mediated by their phosphorylation [27, 28]. Therefore, we determined if the effects of grandinin on malignant lung cells are related to levels of their phosphorylated forms.
Grandinin inhibits EGFR phosphorylation

Our results clearly show that treatments with grandinin decreased levels of p-EGFR and p-AKT in MS-1, A549, and LK-2 cells, when compared with the levels in the conditions treated with DMSO only. These results indicated that grandinin treatments lead to down-regulation of levels of p-EGFR and p-AKT in MS-1, A549, and LK-2 cells. Moreover, these results are consistent to the inhibitory effects of grandinin on these cell types. Altogether, our results suggest that grandinin significantly reduce malignant cell viability and effectively induces apoptosis of malignant lung cells by down-regulating phosphorylation of cellular signaling proteins EGFR and AKT.

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Disclosure of conflict of interest

None.

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