Anti-proliferation effects of trifolirhizin on MKN45 cells and possible mechanism

XINGANG LU1*, JIANXIA MA2*, HONGFU QIU1, LIU YANG3, LEI CAO4 and JIE SHEN5

Departments of 1Traditional Chinese Medicine and 2Gastroenterology, Huadong Hospital, Fudan University, Shanghai 200040; 3Department of Oncology, Baoshan Hospital of Integrated Traditional Chinese and Western Medicine, Shanghai University of Traditional Chinese Medicine, Shanghai 201999; 4Department of Nursing, The 19th Ward, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai 200032; 5Department of Pharmacy, Huadong Hospital, Fudan University, Shanghai 200040, P.R. China

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Correspondence to: Dr Jie Shen, Department of Pharmacy, Huadong Hospital, Fudan University, 221 West YanAn Road, Shanghai 200040, P.R. China
E-mail: shj421@126.com

*Contributed equally

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Abstract. Trifolirhizin is a compound isolated from Sophora flavescens. It has been shown to exert cytotoxicity on several cancer cell lines. However, the underlying mechanism remains unknown. MKN45 cells were used as a research model. We assessed the cytotoxicity of trifolirhizin to MKN45 by MTT. Hoechst staining and TUNEL method were used to demonstrate apoptosis. Flow cytometry was used to determine cell cycle and ratio of apoptosis. Caspase activity assay was used to examine the activation of caspase cascade pathways. Western blot assay manifested that trifolirhizin activated the EGFR-MAPK signaling pathways. This study indicated that trifolirhizin may be a therapeutic application in human gastric cancer therapy.

Introduction

Gastric cancer is one of prevailing cancers worldwide accounting for nearly 8-10% of cancer mortality (1). According to published reports, there are approximately one million gastric cancer cases with 73,800 deaths in 2008 (2). In recent years, many advances have taken place in diagnosis and treatment (3). However, the mortality of gastric cancer remains 70% especially with late stage patients (4). Pharmacotherapy remains the effective strategy for treatment of gastric cancer (5). Screening effective agents from herb plants have been proved efficient (6). Many constituent have been demonstrated with antitumor effects both in vitro and in vivo, for instance: celastrol (7), bigelovin (8), and parthenolide (9). Other natural compounds also demonstrated many functions leading to cell proliferation, cell differentiation, apoptosis and cell cycle changes (10,11).

Sophora flavescens have been widely planted in East Asian countries as herbal plants and cosmetics (12). The root of Sophora flavescens was frequently used for treatment of mental health, inflammation, asthmas, and microbial activities (13-15). Trifolirhizin is a natural pterocarcan flavonoid extracted from Sophora flavescens (16). Published reports demonstrated its chemical structure (Fig. 1A) (17). Trifolirhizin has many pharmacological activities, such as hepatoprotective (18), anti-inflammatory (19), anti-proliferation (20), and skin-whitening (21). Trifolirhizin is also a biologically active constituent of Zeng-Sheng-Ping, a commercial Chinese traditional medicine for cancer prevention (22). Accumulating reports manifested that trifolirhizin had anti-proliferative activities in melanoma B16 cells, human A2780 ovarian and H23 lung cancer cells in vitro (19,21). Trifolirhizin also induced apoptosis in human leukemia HL–60 cells in vitro (23). Despite reports on the trifolirhizin cytotoxicity effect on various cells, the intrinsic mechanism of cytotoxicity and apoptosis triggered by trifolirhizin remains unknown. Herein, it is demonstrated that trifolirhizin induce apoptosis and cell cycle arrest while we explored also the mechanism of activation.

Materials and methods

Reagents and trifolirhizin preparation. Trifolirhizin standard preparation (purity >98%) were purchased from Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). All other chemicals used were provided from Sigma-Aldrich (St. Louis, MO, USA). Trifolirhizin was dissolved in DMSO. Cells were treated with increasing concentrations of trifolirhizin for designated hours. Control groups were exposed to the same volumes of DMSO under the same conditions.
and control were incubated for 30 min and then tested with a RNase A (10 µg/ml) and exposed to trifolirhizin under desired concentrations. MKN45 cells were harvested, diluted to desired concentrations (5x10⁶) and injected intraperitoneally into the right flank of each mouse. Then, trifolirhizin was injected into the BALB/C nude mice when tumor volume achieved a mean group size of 100 mm³. The saline was injected into control group. All mouse groups were injected every 3 days. BALB-nu mice were sacrificed at 24 h when tumor volume achieved average group size of 1,000 mm³. MKN45 tumor xenografts were evaluated and fixed for further experiments. Tumor volumes were evaluated as (W² x L)/2 per 3 days using micrometer calipers, and tumor weights were calculated.

Immunohistochemistry. The xenografted MKN45 tumor tissue samples were removed and cut to desired small pieces. samples (5 µm) were fixed and stained by cleaved-caspase-3 and Ki67 antibodies, respectively, for immunohistochemistry. We observed the visual images using an inverted fluorescence microscope (D5100, Nicon, Tokyo, Japan).

Statistical analysis. We performed triplicate experiments using independent samples. Results were expressed as mean values ± standard deviation (mean ± SD). Statistical significance of our present results was determined using the one-way ANOVA. All statistical analyses were performed with commercial SPSS software (SPSS190, Chicago, IL, USA).

Results
Trifolirhizin exerts anti-proliferation effects in human gastric cancer cells. Published reports have manifested that trifoli-
rhizin is cytotoxic on cancer cells. MTT assay was used to assess the numbers of viable MKN45 cells. Exposure under trifolirhizin results in a concentration- and time-dependent suppression of MKN45 cell viability (Fig. 1B). The trifolirhizin exposure time was 48 h in the present examination. The IC50 value of trifolirhizin on MKN45 cells was 33.27±2.06 µg/ml. We also conducted MTT assay to evaluate the efficacy of trifolirhizin on a human normal liver cell line and human normal kidney cell line in vitro. To our satisfaction, >60% tested cells were viable when treated with increasing trifolirhizin dose ≤250 µg/ml. The apparently higher IC50 value of trifolirhizin on two normal human cell lines exhibited more safety and further implication possibility (Fig. 1C).

**Trifolirhizin induces apoptosis in human stomach cancer cells in vitro.** MKN45 cells were treated with desired concentrations of trifolirhizin as above at 48 h. Hoechst staining exhibited visual images with cell shrinkage, nuclear fragmentation and chromatin compaction of apoptosis (Fig. 4A). Images indicated that trifolirhizin lead to increasing population of apoptotic cells. Consistently, TUNEL staining showed a significant difference between trifolirhizin treated cells vs. the vehicle control, the ratio of green staining (apoptotic) vs blue staining (normal) arise with increasing trifolirhizin (20-40 µg/ml, shown in Fig. 4B). Annexin V/PI assay was performed to evaluate the apoptotic population quantitation after trifolirhizin exposure. In this study, trifolirhizin significantly triggered apoptosis. The apoptotic population in trifolirhizin (20-40 µg/ml) induced MKN45 cells increased (Fig. 2). The JC-10 assay was used to indicate the mitochondrial outer membrane permeabilization after trifolirhizin treatment in early phase. Fig. 1D demonstrated that red fluorescence (normal cells) was decreased while green fluorescence (apoptotic cells) increased with trifolirhizin treatment (20-40 µg/ml) at 24 h, so the

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**Figure 1.** The inhibition effect of trifolirhizin on different cells. Cells were exposure for 48 h with increasing trifolirhizin or combined with EGF. (A) The chemical structure of trifolirhizin. (B) MTT assay of MKN45 cells treated with increasing trifolirhizin for 48 h. (C) Effect of trifolirhizin on normal HEK293 and L02 cells using MTT assay for 48 h. (D) Collapse of MMP in MKN45 cells induced with trifolirhizin for 24 h. (E) The body weights of BALB-nu MKN45 model. (F) The suppressed tumor growth effect of trifolirhizin in MKN45 BALB-nu models. Statistical differences were compared between trifolirhizin treatment group vs. control group at the levels of *P<0.05, **P<0.01 or ***P<0.001.
Figure 2. Trifolirhizin induced apoptosis in MKN45 cells. Cells were induced with increasing trifolirhizin or combined with EGF for 48 h. Flow cytometry analyses were used to assess the apoptosis of MKN45 cells with multiple dose of trifolirhizin treatment or combined with EGF for 48 h. Statistical differences between trifolirhizin treatment group vs. control group were considered significant at the levels of *P<0.05, **P<0.01 or ***P<0.001. Statistical differences between trifolirhizin treatment group vs. combination group were considered significant at the levels of #P<0.05, ##P<0.01, ###P<0.001.

Figure 3. Trifolirhizin induced cell cycle arrest in MKN45 cells. Cells were treated with increasing trifolirhizin or combined with EGF for 48 h. Flow cytometry analyses were used to evaluate the cell cycle distributions of MKN45 cells with trifolirhizin treatment or combined with EGF for 48 h. Statistical differences between trifolirhizin treatment group vs. control group were considered significant at the levels of *P<0.05, **P<0.01 or ***P<0.001. Statistical differences between trifolirhizin treatment group vs. combination group were considered significant at the levels of #P<0.05, ##P<0.01, ###P<0.001.
upgrade ratio of green/red fluorescence reflected that the MMP ($\Delta \Psi_m$) in MKN45 after trifolirhizin treatment (20-40 µg/ml) was decreased.

The expression levels of the epidermal growth factor receptor (EGFR) signaling pathways and its downstream mitogen activated protein kinase (MAPK) signaling pathway were shared involving multiple anticancer agent molecular activities. The EGFR levels, containing RAS/RAF/MEK/ERK are downregulated with the increasing trifolirhizin, Fig. 4A indicates that trifolirhizin (20-40 µg/ml) triggered the increase of p38 and elicited no impact on p-JNK. Bcl-2 and Bax family members are the critical regulators in mitochondrial apoptosis pathways. Trifolirhizin leads to disorder of expression ratio of Bcl-2 and Bax. The induction of apoptosis

Figure 4. The visual effect of trifolirhizin on MKN45 cells and the underlying mechanism. Cells were exposed with increasing trifolirhizin for 48 h. (A) The Hoechst staining of MKN45 cells exposed with various doses of trifolirhizin for 48 h. Scale bar, 10 µm. (B) The TUNEL staining on glass cover of the MKN45 cells were imaged by immunofluorescence for DAPI (blue), TUNEL FITC (green) and their merging. Scale bar, 50 µm. (C) The graphical abstract of mechanism of trifolirhizin action on MKN45 cells. (D) IHC staining effect of trifolirhizin on the expression levels of cleaved-caspase-3 and Ki67 in vivo. Scale bar, 100 µm.
by trifolirhizin was subsequently mediated through activities of c-Myc, caspase-9, and caspase-3.

**Trifolirhizin causes cell cycle arrest in human gastric cancer cells in vitro.** PI was employed to determine the cell cycle distribution under trifolirhizin exposure. Fig. 3 shows that trifolirhizin exposure (20-40 µg/ml) resulted in increasing accumulation in G2/M phase in MKN45 cells. Furthermore, trifolirhizin exhibited impact on the cell cycle through regulation of cell cycle related proteins. There are an appreciable downregulation of cyclin B as well as Cdc2 in trifolirhizin treatment (20-40 µg/ml). The P53 protein, which is located upstream of cyclin complex and downstream of EGFR signaling pathways, is a key point between signaling pathway
Table I. The inhibitory effect of trifolirhizin on MKN45 implantation tumor growth in BALB/C-nu mice.

<table>
<thead>
<tr>
<th>Groups (mg/kg)</th>
<th>No. of animals survived</th>
<th>Body weights (g)</th>
<th>Tumor weight (g)</th>
<th>Inhibition rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>Start</td>
<td>End</td>
<td></td>
</tr>
<tr>
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<td></td>
<td>20.35±0.68</td>
<td>24.6±0.98</td>
<td>1.42±0.42</td>
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<td>24.58±0.74</td>
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<tr>
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<td>24.28±1.30</td>
<td>0.61±0.24</td>
</tr>
<tr>
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<td>20.73±0.42</td>
<td>23.38±0.56</td>
<td>0.34±0.22</td>
</tr>
</tbody>
</table>

\*P<0.05 vs. the vehicle control. \*P<0.01 vs. the vehicle control. \*P<0.001 vs. the vehicle control.

and cellular response in cell cycle arrest. Trifolirhizin treatment (20-40 µg/ml) elicited P53 activation (Fig. 5A).

**Anti-proliferation effect of trifolirhizin on MKN45 xenograft tumors.** Efficacy of trifolirhizin was also confirmed because there were few changes in the mouse body weight in the experiment (Fig. 1E). These results certified the relative safety of trifolirhizin in vivo. After 21 administration days, xenograft tumors treated with trifolirhizin (1-3 mg/kg) were smaller than control treated with saline (Table I and Fig. 1F). The significantly decreasing levels of Ki67-positive cells were found in the study, which hinted the anti-proliferation effect of trifolirhizin (1-3 mg/kg) in vivo. Cleaved-caspase-3 positive cells were increased which reflected the trifolirhizin apoptotic effect (Fig. 4D). These results also manifested the antitumor effects of trifolirhizin.

**Discussion**

A previous published study indicated that trifolirhizin had cytotoxicity in several cancer cells (19). Despite the cytotoxicity on human cells by previous reports, the markedly higher IC50 in our finding manifested the tissue specific toxicity on human cells by previous reports, the markedly cytotoxicity in several cancer cells (19). Despite the cytotoxicity in several cancer cells, the underlying apoptotic mechanism remains unclear. Thus, it was chosen as one candidate agent in our large scale natural anticancer compound screening for further efforts in future. In 2004, it was indicated that trifolirhizin induces apoptosis in HL-60 cells (23). However, the underlying apoptotic mechanism remains unclear. Thus, it was chosen as one candidate agent in our large scale natural anticancer compound screening for further efforts. The MKN45 cell is a typical gastric cancer cell commonly conducted as an anti-tumor model (24,25). Our assay indicated that trifolirhizin inhibited the proliferation of MKN45 gastric carcinoma cells in a time- and dose-dependent manner. Apoptosis hypothesis was further confirmed by flow cytometry data and visual contact under a microscope.

Many studies have indicated that EGFR-MAPK pathway has the ability to affect the Bcl-2 family (26). For instance, JNK can inactivate Bcl-2 while eliciting mitochondrial translocation of Bax (27). The Bcl-2 protein family is a crucial regulator of apoptosis (28). Bcl-2 protein family contains two main types of members: pro-apoptotic members Bax, anti-apoptotic members Bcl-2. The ratio of Bax/Bcl-2 leads to impaired apoptosis (29). In this study, imbalance in expression level of Bcl-2/Bax was clearly detected in the trifolirhizin treated MKN45 cells. MMP decreased significantly in trifolirhizin treated MKN45 cells. Loss of MMP contributes to the caspase pathway cascade (30). Caspases, a family of cysteine proteases, are important proteins in regulating the apoptotic response (31). Caspase-9 represents the intrinsic cascade pathway, while caspase-3 is the key executioner in apoptosis (32). Based on our western blot assay results, trifolirhizin induced the activation of caspase-9, caspase-3, and cleavage of PARP. Then apoptosis triggered cells to death. On the other hand, most cytotoxic agents usually inhibit cell proliferation via arresting the cycle in G1/S or G2/M phase (33,34). This arrest process is mediated with cyclin-dependent protein (Cdc) with specific cyclin proteins. Trifolirhizin treatment leads to mediating accumulation in blocking of Cdc2 and inhibition of cyclin B resulting in a G1/M phase arrest.

We used the EGF (100 ng/ml) to re-activate EGFR signal pathway for the mechanism validation (35). The apoptosis ratio in trifolirhizin (30 µg/ml) combined with EGF using flow cytometry assay was decreased compared with trifolirhizin (30 µg/ml, Fig. 2). The cell cycle distributions were also transformed (30 µg/ml, Fig. 3). The EGFR protein levels were upregulated in the combination group (30 µg/ml, Fig. 5B). The examination manifested that EGFR signaling pathway may be paramount in the effect of trifolirhizin on MKN45 cells.

In conclusion, trifolirhizin induced apoptosis in MKN45 cancer cells in vitro and in vivo mediated via EGFR-MAPK pathways. Trifolirhizin also arrest G1/M cycle through impact on Cdc2/cyclin B complex (Fig. 4C). The results of this study implied that trifolirhizin may a feasible agent for anti-stomach tumor treatment, further scientific and therapeutic development should be established.

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