Inhibition of Lung Cancer Proliferation by Wogonin is Associated with the Activation of Apoptosis and Generation of Reactive Oxygen Species

Wang and Cui. Inhibition of lung cancer proliferation by wogonin

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GRAPHICAL ABSTRACT
Background: Lung cancer has very high incidence rate and is one among the commonly diagnosed tumors in the developed countries.

Aims: The present study was designed to investigate the effect of wogonin on A549 and A427 lung cancer cells and explore the mechanism involved.

Study Design: In the present study effect of wogonin was investigated in vitro on A549 and A427 lung cancer and BEAS-2B normal cells and normal lung cells.

Methods: The cytotoxicity effect of wogonin on A549 and A427 lung cancer and BEAS-2B cells was assessed by MTT assay. The apoptosis onset was assessed by flow cytometry using Annexin V-FITC/PI staining.

Western blotting was used for determination of changes in apoptotic protein expression.

Results: Wogonin treatment exhibited cytotoxicity effect selectively on A549 and A427 cells without affecting BEAS-2B normal lung cells. The viability of A549 and A427 cells was reduced to 31 and 34%, respectively on treatment with 50 µM of wogonin. There was no significant reduction in BEAS-2B cell viability on treatment with 50 µM of wogonin. The percentage of apoptotic A427 cells showed a significant (P<0.049) increase on treatment with wogonin. Treatment of A427 cells with wogonin led to a marked increase in the activation of caspase-3/-8/-9. Wogonin treatment of A427 cells caused a marked increase in the production of reactive oxygen species at 72 h. Digital tomosynthesis (DTS) studies showed marked reduction in tumor development on treatment with wogonin.

Conclusion: In summary, present study demonstrated that wogonin treatment specifically exhibits cytotoxicity effect on lung cancer cells. Moreover, the cytotoxicity effect of wogonin is associated with the activation of apoptosis and generation of ROS. Therefore, wogonin can be developed for the treatment of lung cancer.

Keywords: Cytotoxicity, tomosynthesis, apoptosis, tumor recurrence, caspases

Lung cancer has very high incidence rate and is one among the commonly diagnosed tumors in the developed countries (1). In USA alone more than 15 million patients with lung cancer were detected (1). The treatments used for lung cancer include primary tumor radical resection followed by the use of adjuvant chemotherapy (2). Despite development of modern techniques lung cancer has very poor prognosis and in majority of patients tumor recurrence has been observed (2-4). The poor response of patients to currently available drugs demands discovery of novel and effective chemotherapeutic agents for lung cancer treatment. Reactive oxygen species (ROS) formed from various cellular metabolites play a vital role in suppression of cancer growth (5). Disturbance of equilibrium between anti-oxidants and ROS in the cells leads to oxidative stress (6). The ROS overproduction acts as signalling pathway for carcinoma cell apoptosis through DNA damage (5). It has been recognised that ROS serves as anti-tumor molecule (6).

Plants play a vital role in drug discovery programme since many of the secondary metabolites have shown pharmacological activities (7-9). Scutellaria belongs to Labiatae family which is consisting of around 400 species of annual and perennial herbs (7). The Scutellaria extract has been used for the treatment of inflammation, allergy and hepatitis in traditional system of medicine (8). Phytochemical investigation lead to the isolation of flavonoid and terpenoid compounds from this plant. The major compounds identified from this plant are baicalin, baicalein and wogonin (9). In consistence with the reported activity of flavonoid compounds these molecules also showed radical quenching potential, anti-oxidant activity and anti-tumor property (10). The wogonin molecules has flavonoid structure which may bestow anti-proliferative activity to it. Wogonin has been found to inhibit inflammatory activity of microglial cells by decreasing the generation of nitric oxide and cytokines (11). The present study was devised to investigate the effect of wogonin on lung cancer cell growth and proliferation. The study showed that wogonin is specific in inhibiting lung cancer cell proliferation without any toxicity against normal pulmonary cells.

Materials and methods

Cell culture
The A549 and A427 lung cancer cells and BEAS-2B normal cells were obtained from the Chinese Academy of Sciences, Shanghai, China. The cell lines were maintained for 24 h in RPMI-1640 medium. The medium was supplemented with 10% FBS and antibiotics (100 U/ml penicillin/streptomycin). The cell culture was performed under humid atmosphere of 95% air and 5% CO2 at 37°C.

Cell viability assay
The changes in A549, A427 and BEAS-2B cell proliferation on exposure to 5, 10, 15, 20, 25, 30 and 50 µM of wogonin were assessed using MTT assay. The cells at 2 x10^4 cells/well density were distributed 96-well plates and cultured for 24 h. Then fresh medium mixed with 5, 10, 15, 20, 25, 30 and 50 µM of wogonin was added to the wells and plates were incubated for 72 h. After 72 h, 20 µl of MTT (0.5 mg/ml) solution was added to the wells and incubation was performed for 4 h more. Then medium was removed and 120 µl of DMSO was added to each wells. The microplate reader was used for measurement of absorbance of each plate at 490 nm.

EdU proliferation assay
The A549, A427 and BEAS-2B cells at 2 x 10^4 cells/well density were distributed in 96-well plates. The cells were treated with 5, 10, 15, 20, 25, 30 and 50 µM of wogonin for 72 h. The changes in cell proliferation were
determined by EdU proliferation assay kit (Guangzhou RiboBio Co., Ltd., Guangzhou, China). The fluorescence microscope (Olympus IX51; Olympus Corporation, Tokyo, Japan) was used for observing the EdU stained cells.

Analysis of cell morphology using Hoechst 33342 staining
The A427 cells at 2 x 10^5 cells/well concentration were distributed in 12-well plates and cultured for 24 h. The cells were incubated with 25, 30 and 50 µM of wogonin for 72 h followed by PBS washing two times. The cells were fixed with 4% paraformaldehyde for 20 min at room temperature, washed with PBS three times and subsequently stained for 20 min with Hoechst 33342 (10 µg/ml). The fluorescence microscope (Olympus Corporation) was used for detection of morphological changes in the cells.

Detection of apoptosis using Annexin V-FITC/PI staining
Apoptosis in A427 cells on treatment with wogonin was detected by flow cytometry using Annexin V-FITC/PI kit. The cells at 72 h of treatment with 25, 30 and 50 µM of wogonin were harvested and then washed using ice cold PBS. The cells at 2 x 10^5 cells/ml density were suspended in 1X binding buffer. The cells were stained for 20 min with Annexin V-FITC and PI solution at room temperature under complete darkness. The flow cytometry (Beckman Coulter, Inc., Brea, CA, USA) was used for analysis of the stained cells for apoptosis induction.

ROS production detection
The 2',7'-dichlorofluorescein-diacetate (DCFH-DA), was used for detection of ROS production in A427 cells. The cells were treated with 25, 30 and 50 µM of wogonin for 72 h followed by washing with cold PBS three times. The cells were then re-suspended in serum free culture medium mixed with 10 µM DCFH-DA. The stained cells were detected for ROS generation by flow cytometry.

Western blot analysis
The cells after 72 h of treatment with 25, 30 and 50 µM of wogonin were lysed using RIPA lysis buffer. The lysate was centrifuged at 12,000 x g for 15 min at 4°C to collect supernatants. The protein concentration in the cell lysates was analyzed using a Bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.). The 30 µg protein samples were resolved on SDS-PAGE (8-12%) and subsequently transferred onto PVDF membranes. The non-specific sites in the membranes were blocked on incubation with 5% non-fat milk. The membranes were incubated overnight at 4°C with primary antibodies: anti-LC3, anti-RIP3, anti-caspase-3, anti-LC3I, anti-LC3II, anti-caspase-8, ant-caspase-9 and anti-PARP. After PBS washing two times the membranes were subjected to incubation with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody at room temperature for 2 h. The ECL detection system (Pierce; Thermo Fisher Scientific, Inc.) was used for visualization of the protein bands.

Statistical analysis
The data presented are the mean ± standard deviation of triplicate experiments. The data comparison was performed using ANOVA followed by Tukey's or Dunnett's test. The P<0.05 was taken to indicate a statistically significant differences. The data were analysed statistically using SPSS 17.0 software (IBM Corp., Armonk, NY, USA).

Results
Wogonin inhibits A549 and A427 lung cancer cell viability without affecting BEAS-2B normal cells
The proliferation of BEAS-2B normal lung cells was not affected on exposure to 5, 10, 15, 20, 25, 30 and 50 µM of wogonin (Figure 1). However, A549 and A427 cell viability showed a significant (P<0.048) decrease on treatment with wogonin for 72 h in dose based manner. Treatment of A549 cells with wogonin at 5, 10, 15, 20, 25, 30 and 50 µM reduced viability to 89, 83, 72, 60, 48, 39 and 31%, respectively. The viability of A427 cells was decreased to 86, 83, 76, 65, 51, 40 and 34%, respectively on treatment with at 5, 10, 15, 20, 25, 30 and 50 µM of wogonin.

Wogonin reduces A427 cell count
The microscopic observation showed A427 cell rounding on treatment with wogonin (Figure 2). The A427 cell count decreased significantly on treatment with 25, 30 and 50 µM of wogonin at 72 h.

Wogonin induces apoptosis of A427 cells
Annexin V-FITC/PI staining of control A427 cells showed normal nuclear shape and weak blue fluorescence (Figure 3). Wogonin treated A549 and A427 cells showed bright blue fluorescent granules and chromosomal condensation. The extent of bright blue fluorescence increased markedly in A427 cells on increasing the dose of wogonin from 25 to 50 µM.

Effect of wogonin on the expression of apoptotic proteins in A427 cells
The expression of procaspases-8/9/3 and cleaved PARP in A549 and A427 cells on treatment with wogonin was assessed by western blotting (Figure 4). A significant increase in caspases-8/9/3 expression was caused in A427 cells on treatment with 25, 30 and 50 µM of wogonin. The expression of cleaved PARP was increased by 25, 30 and 50 µM of wogonin in A427 cells.
Wogonin induces ROS production in A427 cells

The ROS production in A427 cells on treatment with wogonin was analysed using DCFH-DA probe by flow cytometry (Figure 5). The ROS production was promoted significantly by wogonin at 25, 30 and 50 µM in A427 cells at 72 h. Thus, wogonin promoted generation of ROS in lung cancer cells in dose based manner.

Wogonin induces autophagy in A427 cells

The acidic autophagic vacuole formation in A427 cells on treatment with wogonin was detected by fluorescence microscopy (Figure 6A). The formation of acidic autophagic vacuoles showed a significant increase in A427 cells on treatment with 25, 30 and 50 µM doses of wogonin for 72 h. In A427 cells treatment with wogonin enhanced the expression of LC3II in dose based manner (Figure 6B). The level of LC3I was decreased markedly in A427 cells on treatment with 25, 30 and 50 µM doses of wogonin.

Discussion

The present study demonstrated that wogonin exhibits inhibitory effect on A549 and A427 carcinoma cell proliferation in dose based manner. Moreover, wogonin did not exhibit any toxicity effect on BEAS-2B normal lung cells. Therefore, the present study suggests that wogonin specifically exhibits toxicity against lung cancer cells without affecting the normal epithelial cells.

Apoptosis, a complex process associated with the elimination of unwanted cells from body is controlled by several genes (12). The signalling pathway involved in apoptosis induction is linked to the activation of pro-caspases (13). In the present study wogonin treatment reduced proliferation of A549 and A427 carcinoma cells markedly in comparison to the control. The study investigated the mechanism of lung cancer cell proliferation inhibition on treatment with wogonin. Flow cytometry showed that wogonin treatment markedly promoted apoptosis onset in A549 and A427 cells. Therefore, wogonin suppressed lung cancer cell proliferation by the activation of apoptotic signalling pathway. It has been well established that caspases play a vital role in arresting carcinoma growth by inducing cell apoptosis (14). The members of caspase family like caspase-2, -8, -9 and -10 (initiators) are involved in activation of apoptotic cascade while as caspase-3, -6 and-7 (executers) execute the process of apoptosis(15). In the present study wogonin treatment enhanced expression of both initiator as well as executer caspases in A549 and A427 cells. The expression of initiator caspase-8/9 and executer caspase-3 in A549 and A427 cells was markedly higher on treatment with wogonin. These findings proved that wogonin caused apoptosis induction in A549 and A427 cells through caspase dependent pathway. The level of cleaved PARP in A549 and A427 cells was also promoted on treatment with wogonin. Increased production of reactive oxygen species also acts as signalling pathway for activation of cell apoptosis (16). The higher concentration of ROS leads to DNA damage, oxidative stress followed by cell apoptosis (17). Studies have shown that up-regulation of ROS formation in cells suppresses cell proliferation by inducing apoptosis (18). In the present study the level of ROS was promoted in A549 and A427 cells markedly higher on treatment with wogonin. In both A549 and A427 cancer cells wogonin treatment promoted production of ROS markedly compared to the control cells. Overproduction of ROS in the cancer cells has also been found to linked with the autophagy (19,20). In the present study wogonin promoted acidic autophagic vacuole formation in A549 and A427 cells. There was a marked up-regulation of LC3IIexpression in A549 and A427 cells on treatment with wogonin.

In summary, the present study demonstrates anti-cancer potential of wogonin against lung cancer cells without any toxic effect on normal cells. The toxic effect of wogonin involved apoptosis induction, activation of caspases and increased formation of ROS in A549 and A427 cells. Therefore, wogonin can be used to develop an effective treatment strategy for the lung cancer.

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References

Figure 1. Effect of wogonin on lung cell viability. A549, A427 cancer cells and BEAS-2B normal cells were exposed to different doses of wogonin. The cell viability changes by wogonin were assessed by MTT assay. *P<0.048, **P<0.019 and ***P<0.011 vs. control cells.

Figure 2. Effect of wogonin on lung cancer cell morphology. (A) The A427 cells were exposed to 25, 30 and 50 µM doses of wogonin. The cell morphological changes by wogonin were examined by microscopy. Images were taken at magnification, x200. The arrows indicate apoptotic cells. (B) Quantification of cell apoptosis. *P<0.05 and **P<0.02 vs. control cells.
Figure 3. Wogonin treatment of lung cancer cells causes apoptosis induction. The A427 cells exposed to 25, 30 and 50 µM of wogonin were examined by flow cytometry after Annexin V-FITC/PI staining. Images were taken at magnification x200.
Figure 4. Expression of apoptotic proteins in A427 cells on treatment with wogonin. The cells were treated with 25, 30 and 50 µM of wogonin for 72. The protein concentration was determined by western blot assay using β-actin as internal control.

Figure 5. Effect of wogonin in ROS production in lung cancer cells. (A) The cells exposed to different doses of wogonin were labelled with DCFH-DA and then examined by flow cytometry for ROS production. The arrows indicate ROS production. (B) Quantification of the ROS level. *P<0.05 and **P<0.02 vs. control cells.
Figure 6. Effect of wogonin on autophagy induction in A427 cells. (A) The cells treated with different doses of wogonin were examined for acidic autophagic vacuole formation by fluorescence microscopy. Magnification, x200. The arrows indicate apoptotic autophagic vacuoles. (B) The levels of LC3I and LC3II in A427 cells treated with different doses of wogonin was analysed by western blotting.