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Inhibitory effect of desoxyrhaponticin and rhaponticin, two natural stilbene glycosides from the Tibetan nutritional food *Rheum tanguticum* Maxim. ex Balf., on fatty acid synthase and human breast cancer cells

Ping Li, a Weixi Tian, a Xiaoyan Wang*b and Xiaofeng Ma*a

Fatty acid synthase (FAS) has attracted more and more attention as a potential target for cancer treatment. Natural FAS inhibitors are emerging as potential therapeutic agents to treat cancer. *Rheum tanguticum* Maxim. ex Balf. (rhubarb) is a traditional Chinese nutritional food and has been reported to possess a variety of biological activities, including the ability to induce the apoptosis of cancer cells. This study indicates that desoxyrhaponticin (DC) and rhaponticin (RC), two stilbene glycosides from rhubarb, could be considered as promising FAS inhibitors. We found that both DC and RC could inhibit intracellular FAS activity and downregulate FAS expression in human breast cancer MCF-7 cells. In addition, the apoptotic effect of DC on human cancer cells was announced for the first time. Since FAS plays a key role in the biosynthesis pathway of fatty acids in cancer cells, these findings suggest that DC has potential applications in the prevention and treatment of cancer.

**Introduction**

*Rheum tanguticum* Maxim. ex Balf., a perennial herb from the Polygonaceae family, is mainly distributed in the Qinghai and northeastern Tibetan areas of China. The root and rhizome of *R. tanguticum* Maxim. ex Balf. is commonly called rhubarb, which is a widely used traditional Chinese nutritional food and one of the important ingredients in Chinese traditional prescriptions.1-3 The extract of *R. tanguticum* Maxim. ex Balf. exhibits various bioactivities, such as anti-oxidant, antibacterial, anti-pyretic, anti-inflammatory, and anti-cancer effects. Phytochemical studies have demonstrated that anthraquinones, anthracenes and stilbene glycosides are major bioactive constituents of rhubarb.4,5 Among these constituents, stilbene glycosides including desoxyrhaponticin (DC) and rhaponticin (RC) are the main active compounds. Although the exact amount of DC and RC in *R. tanguticum* Maxim. ex Balf. has not yet been reported, researchers have conducted quantitative studies of the DC and RC in some other rhubarbs, such as *R. hotaoense* and *R. franzenbachii*. The contents of DC and RC in these plants are about 4% and 6%, respectively.6,7 They have recently attracted a great deal of attention due to their anti-HIV, anti-oxidant, anti-malarial, and anti-allergy effects.8-14

Although the anti-cancer activity of the crude extract of rhubarb and its anthraquinones are widely described,15-18 the literature concerning the role of rhubarb stilbene glycosides in the prevention and treatment of cancer is limited. It was reported that RC could induce the apoptosis of human stomach cancer KATO III cells.19 Another study found that RC could inhibit proliferation and differentiation of the human hepatoma cell line SMMC-7721.20 However, the mechanisms involved remain unclear.

Fatty acid synthase (FAS, EC 2.1.3.85) is a key enzyme which catalyzes the synthesis of long-chain fatty acids by using acetyl-CoA as a primer, malonyl-CoA as a two-carbon donor, and NADPH as a reducing equivalent.21,22 An increased FAS expression level has been observed in various types of cancers, while it has been undetectable in normal cells.23-24 Inhibition of FAS activity, which is selectively cytotoxic to cancer cells, blocks tumor cell development, survival, aggressiveness and metastasis, and induces cell apoptosis in human cancer cells both *in vitro* and *in vivo* with minimal effect on normal cells.21-26 Thus, searching for FAS inhibitors would open a new window for combating cancer.

In the present study, we investigated not only the inhibitory effect of DC and RC on FAS expression and activity, but also the apoptotic effect of DC and RC on FAS over-expressed human breast cancer MCF-7 cells.

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**Abstract**

Fatty acid synthase (FAS) has attracted more and more attention as a potential target for cancer treatment. Natural FAS inhibitors are emerging as potential therapeutic agents to treat cancer. *Rheum tanguticum* Maxim. ex Balf. (rhubarb) is a traditional Chinese nutritional food and has been reported to possess a variety of biological activities, including the ability to induce the apoptosis of cancer cells. This study indicates that desoxyrhaponticin (DC) and rhaponticin (RC), two stilbene glycosides from rhubarb, could be considered as promising FAS inhibitors. We found that both DC and RC could inhibit intracellular FAS activity and downregulate FAS expression in human breast cancer MCF-7 cells. In addition, the apoptotic effect of DC on human cancer cells was announced for the first time. Since FAS plays a key role in the biosynthesis pathway of fatty acids in cancer cells, these findings suggest that DC has potential applications in the prevention and treatment of cancer.
Materials and methods

Reagents
Acetyl-CoA, malonyl-CoA, NADPH, and DMSO were purchased from Sigma (St. Louis, MO, USA). Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Beijing, China). FAS antibody was obtained from BD Pharmingen (San Diego, CA, USA). PARP and GAPDH were purchased from Cell Signaling Technology (Denvers, MA, USA).

Preparation of DC and RC
The whole plants were collected from the mountainous areas on the Qinghai–Tibetan Plateau (3500–4400 m above sea level) in September, 2010. The plants were identified by Honglun Wang (Professor in phytochemistry, Northwest Plateau Institute of Biology, Chinese Academy of Sciences, Xining, China) as R. tanguticum Maxim. ex Balf.

The air-dried rhizomes of R. tanguticum Maxim. ex Balf. were ground into powder in a FZ102 plant disintegrator. Thereafter, 50 g of powder was extracted with 250 ml of 50% ethanol for 3 h at 40 °C combined with ultrasonication. The extraction procedure was repeated twice. All supernatants of the ethanol extracts were collected together and evaporated under reduced pressure at 45 °C. The dried crude extract (7.6 g) was obtained and stored at 4 °C before use. This sample was then separated by high speed counter current chromatography to yield pure compounds of DC and RC.

Preparation of FAS and substrates
The FAS used was obtained from chicken liver, since the amino acid sequence of chicken FAS has 63% identity with that of humans.27 The FAS from chicken liver was purified, stored, and applied as described previously.28 The preparation was homogeneous on PAGE in the presence and absence of SDS. The enzyme and substrate concentrations were determined by absorption measurements using the extinction coefficients according to a method previously described.29

FAS activity assay
The overall reaction of FAS was determined with an Amershams Pharmacia Ultrospec 4300 pro UV-Vis spectrophotometer at 37 °C by following the decrease of NADPH at 340 nm. The overall reaction mixture contained potassium phosphate buffer, 100 mM, pH 7.0; EDTA, 1 mM; DTT, 1 mM; acetyl-CoA, 6 μM; malonyl-CoA, 12 μM; NADPH, 37.5 μM and chicken liver FAS 10 μg in a total volume of 2.0 ml.27

Assay of FAS inhibition activity
FAS inhibition was determined by adding the inhibitor into the reaction system before FAS initiated the reaction. This inhibition is generally caused by the non-covalent loading on the enzyme, and is fast and reversible. The final concentration of ethanol did not exceed 0.2% (v/v) in the reaction mixture, so the ethanol did not affect the FAS activity. The extent of inhibition by the addition of inhibitor was measured by reference to the half inhibitory value (IC50), which was obtained from a plot of residual activity versus inhibitor concentration.

Cell line and cultures
The human breast epithelial cell line MCF-7, an estrogen receptor-positive cell derived from an in situ carcinoma, was used in the study. The cells were grown in DMEM supplemented with 10% fetal bovine serum. Cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO2.

Cell viability assay
Cell viability was assessed by a Cell Counting Kit (CCK-8; Dojindo Laboratories, Kumamoto, Japan) assay as previously described.29 Briefly, cells were seeded at a concentration of 1 × 104 cells per 200 μl well into 96-well plates, and allowed an overnight period for attachment. The medium was removed, and fresh medium along with various concentrations (0, 12.5, 25, 50, 100, 200, 400 μM) of DC and RC were added to cultures in parallel. Following treatment, a drug-free medium (100 μl per well) and 10 μl CCK-8 solution were added and cells, which were then incubated for 1 h at 37 °C. The optical density (OD) value (absorbance) was measured at 450 nm by a microplate spectrophotometer (Multiskan, MK3). All experiments were performed in quadruplet on three separate occasions.

Analysis of apoptosis
Cell apoptosis detection was performed using an Annexin-V-FITC Apoptosis Detection Kit (BD company, US) according to the manufacturer’s protocol. Briefly, cells were collected after 24 h treatment with DC and RC. The cells were washed twice with cold PBS then resuspended in 1 × binding buffer at a concentration of 1 × 106 cells per ml. Then 500 μl of the cell suspension was incubated with 5 μl annexin-V-FITC and 10 μl PI for 15 min in the dark and analyzed by a FACSscalibur instrument (Becton Dickinson, San Jose, US) within 1 h. Apoptotic cells are those stained with annexin V+/PI− (early apoptotic cell) plus annexin V+/PI+ (late apoptotic cell).

Intracellular FAS activity assay
After 24 h exposure to DC and RC, cells were harvested by treatment with trypsin-EDTA solution, pelleted by centrifugation, washed twice, and resuspended in cold phosphate buffered solution (PBS). Cells were sonicated at 4 °C and centrifuged at 13 000 rpm for 30 min at 4 °C to obtain particle-free supernatants. The FAS activity was determined spectrophotometrically at 37 °C by measuring the decrease of absorption at 340 nm due to oxidation of NADPH, as previously described.30 125 μl particle-free supernatant, 25 mM KH2PO4–K2HPO4 buffer, 0.25 mM EDTA, 0.25 mM dithiothreitol, 30 μM acetyl-CoA, 350 μM NADPH (pH 7.0) in a total volume of 500 μl were monitored at 340 nm for 1 min to measure background NADPH oxidation. After the addition of 100 μM malonyl-CoA, the reaction was assayed for an additional 1 min to determine the FAS-dependent oxidation of NADPH. The FAS activity was expressed in nmol NADPH oxidized per min per mg protein.
Immunoblot analysis

Following treatment of breast cells with DC and RC at the corresponding concentration and for the indicated time, cells were harvested using trypsin-EDTA, washed twice with PBS, and stored at −80 °C. Cells were lysed in lysis buffer (1 mM EDTA, 150 mM NaCl, 100 μg ml⁻¹ phenylmethylsulfonyl fluoride 50 mM Tris–HCl, pH 7.5) for 30 min on ice and then a particle-free supernatant solution was obtained by centrifugation at 14 000 g for 15 min. All operations were at 0–4 °C. A sample was taken for the measurement of protein content by a bicinchoninic acid (BCA) assay (Pierce). Equal amounts of protein were heated in sodium dodecylsulphate (SDS) sample buffer (Laemmli) for 15 min at 95 °C, separated on a 10%–12% SDS-polyacrylamide gel and transferred onto polyvinylidene fluoride membranes. Membranes were blocked with 5% non-fat milk powder (w/v) in TBST (10 mM Tris, 10 mM NaCl, 0.1% Tween 20) for 2–4 h at room temperature to prevent nonspecific antibody binding, and incubated with the corresponding primary antibody diluted in blocking buffer overnight at 4 °C. After 3 × 10 min washes in TBS-T, blots were incubated for 1 h with corresponding peroxidase conjugated secondary antibody and developed employing a commercial kit (West Pico chemiluminescent substrate). Blots were reprobed with an antibody against GAPDH as the control of protein loading and transfer.

Results

The inhibitory effect of DC and RC on FAS activity

The activities for the FAS overall reaction were assayed to determine the inhibitory capabilities of DC and RC. The enzymatic determination showed that both DC and RC possessed an inhibitory function on FAS activity with IC₅₀ values of 172.6 μM and 73.2 μM.

DC and RC inhibited FAS expression and activity in MCF-7 cells

We next identified whether DC and RC could inhibit FAS expression. Incubation of MCF-7 cells with DC and RC separately resulted in an incubation dose-dependent reduction of FAS expression (Fig. 1A). We further checked the activity of FAS in cells treated with the two compounds for 24 h, as described in the Material and methods section. Compared with the control, DC and RC significantly inhibited the intracellular FAS activity in a dose-dependent manner. As shown in Fig. 1B, MCF-7 cells were treated with DC and RC at the concentration of 100, 200 and 400 μM for 24 h. Intracellular FAS activities were reduced to 13% and 51% at the concentration of 400 μM DC and RC, respectively. These results suggested that both DC and RC could inhibit FAS expression and intracellular FAS activity in MCF-7 cells.

Effect of DC and RC on the viability of MCF-7 cells

To evaluate the cytotoxicity of DC and RC, MCF-7 cells were incubated with various concentrations of DC and RC (0, 12.5, 25, 50, 100, 200, 400 μM) for 24 and 48 h, followed by a CCK-8 assay. As shown in Fig. 2, DC showed a dose- and time-dependent inhibitory effect in MCF-7 cells with IC₅₀ values of 380 μM in 24 h and 326 μM in 48 h. However, compared with the same concentrations of DC, RC showed no obvious cytoxicity in MCF-7 cells.

Apoptotic effect of DC and RC on MCF-7 cells

The apoptotic effect of DC was analyzed and quantified by flow cytometry using the annexin V-FITC Apoptosis Detection Kit. As shown in Fig. 3A, DC induced MCF-7 cell apoptosis in a dose-dependent manner, reaching 22.47% at 400 μM (11.67% early apoptosis plus 10.80% late apoptosis). However, no significant apoptosis was found when treated with 400 μM RC. The apoptotic activity of DC was also confirmed by Western blotting analysis, which showed cleavage of PARP. Treatment of MCF-7 cells with DC for 24 h induced a marked increase in the levels of the PARP cleavage product (89 kDa band) in a dose-dependent manner (Fig. 3B).
Discussion

Breast cancer is one of the largest causes of cancer-related death among women worldwide.\textsuperscript{31} The development of breast cancer involves gene amplification and protein overexpression of certain oncogenes. The differential expression of FAS between breast cancer cells and normal cells has made FAS a potential molecular target for anti-cancer drug development.\textsuperscript{32–34} Given the widespread high expression of FAS in many types of human cancer,\textsuperscript{22} combinations of novel drugs directed against FAS dependent endogenous fatty acid biosynthesis may provide increased efficacy for common human cancer. However, no FAS inhibitors have been developed as anti-cancer drugs so far. It is necessary to exploit more safe and effective FAS inhibitors that may be applied practically in the treatment of cancer.

As a traditional Chinese medicinal herb, rhubarb is known to have a variety of pharmacological activities. It is worth mentioning that rhubarb roots have been used as a laxative for thousands of years in China. Stilbene glycosides are active compounds of rhubarb. However, no previous studies have evaluated the influence of stilbene glycosides on fatty acid biosynthesis. In the present work, we found that DC and RC could inhibit FAS activity. Moreover, both DC and RC could inhibit FAS expression and intracellular FAS activity in MCF-7 cells. Compared with known FAS inhibitors, such as cerulenin, C75, EGCG, theaflavin, and flavone, DC and RC are compounds with a distinct structure and character. To the best of our knowledge, it is the first time that the FAS inhibitory activity of the stilbene glycosides has been announced.

The anti-cancer activity of rhubarb stilbene glycosides has rarely been noticed before. Considering DC and RC could inhibit FAS both for the pure enzyme and in FAS over-expressed cancer cells, we further determined their effect on cancer cell viability. It is reported that most normal human tissues, except liver and adipose tissues, exhibit low levels of FAS expression.\textsuperscript{35,36} However, the expression of FAS is surprisingly high in a variety of human cancers, such as cancer of the breast, prostate, ovary and lung.\textsuperscript{37–40} The activity of FAS in cancer cells was closely related to the amount of intracellular fatty acids, because it plays the key role of \textit{de novo} fatty acid biosynthesis. In the present study, we found that DC could inhibit MCF-7 cell viability while RC could not. This difference was consistent with their inhibition activity on intracellular FAS. The reduction of intracellular FAS activity (Fig. 1B) and FAS expression (Fig. 1A) in MCF-7 cells revealed that FAS was the target that DC acted on.

Most of the current cytotoxic anticancer drugs have been shown to induce apoptosis in susceptible cells. We investigated the apoptotic effect of DC and RC. We found that DC enhanced the cleavage of PARP, the hallmark feature of apoptosis, in a dose-dependent manner. We also demonstrated that DC could induce MCF-7 cell apoptosis by flow cytometry. RC shares a very similar structure with DC, however, no significant apoptosis...
was found in MCF-7 cells when treated with 400 µM RC. This was consistent with DC possessing superior cytotoxicity to MCF-7 cells compared to RC. The subtle difference in structure between DC and RC was critical for their evident distinction in bioactivities. To inhibit intracellular enzyme activity, it is necessary for the inhibitor to pass through the cell membrane. Although the chemical structures of DC and RC are similar, RC has an additional hydroxyl group compared with DC. The lipid-water partition coefficient of DC and RC are totally different, thus we hypothesize that DC could pass through the cancer cell membrane more easily than RC.

In conclusion, we demonstrated that DC and RC are novel FAS inhibitors. They could reduce intercellular FAS expression and inhibit intracellular FAS activity in MCF-7 cells. In addition, DC could induce MCF-7 cell apoptosis. Since its potent inhibition on the proliferation of MCF-7 cells, DC has the potential to be a drug candidate for treating human cancers.

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