Scutellarin Sensitizes Drug-evoked Colon Cancer Cell Apoptosis through Enhanced Caspase-6 Activation

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Abstract. Background: We have reported that resveratrol (RSV) and 5-fluorouracil (5-FU) evoked apoptosis through caspase-6 activation in wild-type (p53⁺/⁺) and knockout (p53⁻/⁻) HCT116 human colon cancer cells. In this study, we investigated the sensitization effects of scutellarin (SC), a compound isolated from the traditional Chinese herb Erigeron breviscapus, on RSV and 5-FU-evoked apoptosis of these cancer cells. Materials and Methods: The drug-induced apoptosis was qualified by TUNEL staining under fluorescence microscopy, before being quantified by propiodium iodide staining through flow cytometric assay. Results: SC (100 μM) sensitized RSV- (200 μM) and 5-FU (500 μM)-evoked apoptosis in p53⁺/⁺ but not p53⁻/⁻ cells. RSV- and 5-FU-elicited caspase-6 activation was promoted by SC in a time-dependent manner. SC itself did not trigger apoptosis or caspase-6 activation at the concentration tested. Conclusion: SC is a novel sensitizing agent for both RSV- and 5-FU-evoked apoptosis, through the enhancement of caspase-6 activation in a p53-dependent manner.

Colorectal carcinoma is amongst the commonest types of cancer worldwide and is often diagnosed at advanced stages that require adjuvant chemotherapy (1). Contemporary pharmacological agents for colon cancer include 5-fluorouracil (5-FU) as the principle component of a multidrug regimen, which enhances treatment efficacy and tumor response compared to the use of 5-FU alone. However, advancements in treatment options in the past 40 years since 5-FU have been hampered by the development of tumor cell resistance, with response rates of colon cancer chemotherapy remaining as low as 10-20% (2). There is therefore an urgent need for novel chemotherapeutic drugs and new strategies to overcome the issue of chemoresistance.

A potential strategy that has been utilized for colon cancer treatment is through the induction of cancer cell apoptosis, a mechanism of programmed cell death characterized by rapid loss of plasma membrane integrity, DNA fragmentation and caspase activation (3). Existing drugs in use such as 5-FU have been shown to evoke colon cancer cell apoptosis through both mitochondrial and death receptor pathways (4). Similarly, investigative agents including resveratrol (RSV) (3,5,4'-trihydroxystilbene), a natural plant product, have been discovered to elicit apoptosis in colon cancer cells. In particular, our previous studies suggest that caspase-6 activation may be an important signalling event in human colon cancer cell apoptosis induced by both RSV (5) and 5-FU (6). The addition of an enhancer of apoptosis signalling mechanisms is consequently a candidate method to overcome 5-FU-resistance in tumor cells (7). Indeed, newer clinical drugs such as irinotecan have been shown to enhance 5-FU cytotoxicity by promoting its apoptotic effects (8).

Recently, compounds isolated from traditional Chinese herbs have been recognized as valuable resources for the development of novel and improved cancer therapeutics (9). One of the prospective compounds is a flavone glycoside known as scutellarin (SC) (scutellarein 7-O-β-D-glucuronide) (Figure 1A), which is a major active component of the traditional Chinese herb Erigeron breviscapus (10). It has been reported that SC, while being relatively non-toxic in humans, is useful in the therapy of various ailments, such as cardiovascular diseases, sleep disorders, depression, migraine, pain and memory impairment (11). Interestingly, plant extract containing SC has been shown to induce cell death in the human colon cancer cell line LoVo (12). The actual anticarcinogenic properties of SC, however, are currently unknown. In the present study, we therefore investigated SC as a potential chemosensitization agent in RSV and 5-FU-elicited colon cancer cell apoptosis.
Materials and Methods

Cell culture. Colon cancer cell line HCT116 p53+/+ (wild-type) and p53–/– (knockout) cells were kindly provided by Dr. B. Vogelstein (School of Medicine, the John Hopkins University, Baltimore, MD, USA). Cells homozygous for p53 (p53+/+) were the parental cells for p53–/–. All cells were maintained in modified McCoy’s 5A medium supplemented with 10% fetal bovine serum, 1% L-glutamine and 1% penicillin-streptomycin in a humidified incubator with 5% CO2 at 37°C. Modified McCoy’s 5A, penicillin-streptomycin and L-glutamine were purchased from Gibco (Invitrogen Singapore PTE, Singapore). Fetal bovine serum was a product of HyClone (Logan, UT, USA). Cell cultures at approximately 70% confluence were used for all experimental treatments. For the experimental treatments involving SC, cells were pre-incubated for 30 minutes with medium containing SC (100 μM), washed once with fresh medium before being subjected to treatment with medium containing RSV (200 μM) or 5-FU (500 μM) for 10 h, 15 h or 24 h.

Chemicals and antibodies. RSV and 5-FU were purchased from Sigma (Sigma-Aldrich, Singapore). Scutellarin was purchased from the Chinese Institute of Biological Products (Beijing, China). Antibodies for β-actin, cleaved lamin-A and cleaved caspase-6 were purchased from Cell Signalling (Beverly, MA, USA). Secondary antibodies and SuperSignal Kit for Western blot analysis were provided by Pierce Biotechnology (Rockford, IL, USA). Lysis buffer and fluorogenic substrate for caspase activity assay were purchased from BD Biosciences (San Jose, CA, USA).

Determination and quantification of apoptosis. Cell viability was quantified using the Cell Proliferation Kit II (XTT assay) from Roche Diagnostics (Penzberg, Germany), following the manufacturer’s protocol (5). Apoptosis was evidenced and quantified using In Situ Cell Death Detection Kit (TUNEL assay) from Roche Diagnostics, followed by confocal microscopy. Briefly, HCT116 cells were grown in Labtek Chamber Slides (Rochester, NY, USA), washed once with phosphate-buffered saline (PBS) and fixed in 100% methanol at −20°C for 10 min. The fixed cells were blocked in blocking buffer (1% BSA, 0.05% Tween-20 in 1xPBS) for 1 h, followed by three washes with 1xPBS. The cells were then incubated with the TUNEL stain provided by the manufacturer, washed three times with 1xPBS before being mounted with UltraCruz Mounting Medium (Santa Cruz, CA, USA). The cells were promptly viewed under confocal microscopy for TUNEL positivity and DNA fragmentation. The cells were regularly checked to ensure that the cells remained adherent to the slides. Proof of apoptosis was also provided by flow cytometric cell cycle analyses of sub-G1 fractions using propidium iodide (PI) staining for DNA fragmentation (5).

SDS-PAGE and Western blot analysis. Western blot analysis was performed as described elsewhere (5). Briefly, cell lysates were subjected to 15% PAGE then transferred onto PVDF membranes. After blocking 1 h with 5% dry milk in TBST solution (50 mM Tris/HCl pH 7.4, 150 mM NaCl, 0.1% Tween-20), membranes were washed three times using TBST, exposed to primary antibody overnight at 4°C with gentle shaking, washed with TBST three times, exposed to secondary antibody for 1 h, washed three times again in TBST, and eventually subjected to chemiluminescence detection using the SuperSignal Substrate Western Blotting Kit.

Determination of caspase-6 activity. Caspase-6 activity assay was performed as described previously (5). Briefly, cells were harvested and lysed with 1x cell lysis buffer (BD Biosciences Pharmingen, San Diego, CA, USA). Cell lysate was added to a 2x reaction buffer (10 mM HEPES, 2 mM EDTA, 10 mM KCl, 1.5 mM MgCl2, 6 mM dithiothreitol). Fluorogenic caspase-6 specific substrate VEID-AFC (50 nM) was then added followed by 1 h of incubation at 37°C. The protease activity was determined by measuring the relative fluorescence intensity at 505 nm following excitation at 400 nm using a spectrophotometer (TECAN Spectrofluor Plus, Austria). Caspase activities were normalized with protein concentration and expressed as fold increase in activity relative to the enzymatic activity obtained from untreated control cells.

Statistical analysis. Results are presented as mean±standard deviation (SD) of data collected from three independent experiments. Statistic analysis of mean values was performed through t-tests using the statistical software package SPSS (version 14.0; Chicago, USA). A p<0.05 was considered to be significant.

Results

Scutellarin sensitizes both RSV and 5-FU-elicited apoptosis in HCT116 p53+/+ colon cancer cells. Combination chemotherapy regimens based on the addition of newer agents such as oxaliplatin or irinotecan to 5-FU have only managed to increase objective response rates to 40-50% in advanced colon cancer. In this study, we investigated a novel anticancer drug candidate SC (Figure 1A) in the promotion of drug-evoked colon cancer cell apoptosis. As shown by XTT cell viability assay, SC (up to 100 μM) did not decrease the cell viability of HCT116 colon cancer cells, regardless of their p53 status (Figure 1B). We proceeded to examine effects of SC on RSV and 5-FU-evoked apoptosis by exposing the cancer cells either to RSV (200 μM) or 5-FU (500 μM) alone, or together with SC (100 μM) before analyzing DNA fragmentation in the cells using TUNEL staining. Pre-incubation with SC increased the degree of DNA fragmentation and TUNEL staining induced by RSV or 5-FU at 24 h treatment (Figure 1C); however, this effect was not evident in p53–/– cells. To confirm that the added cytotoxicity of SC on RSV or 5-FU-evoked cancer cell death was mediated through apoptosis, quantification of apoptosis by PI staining and flow cytometry were performed. As shown in Figure 1D, SC in combination with RSV produced a marked increase in the sub-G1 fraction of p53+/+ cells after 10 h, 15 h and 24 h of treatment (18.8%, 26.6%, 41.6%, respectively), in comparison to the sub-G1 fraction in the cells treated with RSV alone (8.5%, 18.3%, 24.6%, respectively). On the other hand, SC in combination with 5-FU resulted in a significant increase in sub-G1 fraction only after 24 h of treatment (41.6%, in comparison to the sub-G1 fraction in the cells treated with 5-FU alone (33.4%, p<0.05) (Figure 1E). SC alone did not result in significant apoptosis of the colon cancer cells compared to untreated controls at any of the time points tested (10 h, 15 h and 24 h).
Figure 1. Scutellarin (SC) sensitizes both RSV- and 5-FU-elicited apoptosis in HCT116 p53<sup>+/+</sup> colon cancer cells. A, Chemical structure of SC. B, SC (up to 100 μM) does not decrease cell viability of HCT116 colon cancer cells, regardless of p53 status. Cell viability was assessed using XTT assays. C, SC increased the degree of DNA fragmentation and TUNEL staining induced by RSV or 5-FU in p53<sup>+/+</sup> but not p53<sup>−/−</sup> cells at 24 h treatment. Only a representative figure is shown here. D, SC (100 μM) in combination with RSV (200 μM) produced a marked increase in the sub-G<sub>1</sub> fraction of the p53<sup>+/+</sup> cells after 10 h, 15 h and 24 h of treatment, compared to the sub-G<sub>1</sub> fraction in the cells treated with RSV alone. E, SC in combination with 5-FU (500 μM) resulted in a significant increase in the sub-G<sub>1</sub> fraction only at 24 h of treatment as compared to that of cells treated with 5-FU alone.
Scutellarin enhances both RSV and 5-FU-elicited caspase-6 activation. Our previous studies suggest that caspase-6 activation is a major signaling event in both RSV and 5-FU-evoked apoptosis in HCT116 colon cancer cells (5-6). In line with this, we investigated if SC promoted both RSV and 5-FU-elicited apoptosis through caspase-6 activation. As shown by caspase activity assay, SC (100 μM) promoted RSV (200 μM)-elicited caspase-6 activation in the p53+/− colon cancer cells after 24 h of treatment (7.6-fold) compared to RSV alone (5.2-fold) (p<0.05). Similarly, SC enhanced the 5-FU (500 μM)-elicited caspase-6 activation in the cancer cells at 24 h of treatment (13.2-fold) compared to 5-FU alone (6.5-fold) (Figure 2A) (p=0.001). As further evidence of the involvement of caspase-6 in SC-enhanced RSV or 5-FU-evoked apoptosis, cleavage of caspase-6 and lamin-A were demonstrated by Western blot analysis, which corroborated results from caspase activity assays. Combination of SC (100 μM) with either RSV or 5-FU increased levels of cleaved caspase-6 and cleaved lamin-A compared to treatments with RSV or 5-FU alone (Figure 2C and 2D, respectively). This finding strengthened the importance of caspase-6 activation in the apoptotic signaling mechanism in HCT116 colon cancer cells. At the sensitizing dose, SC alone exhibited little toxicity and did not cause significant apoptosis or caspase-6 activation in the cancer cells.

Discussion

In our previous studies, we had investigated the signaling mechanisms that underlie the apoptotic-triggering machinery of RSV and 5-FU in human colon cancer cells. To enhance the chemotherapeutic effect of RSV and 5-FU, we investigated the novel use of SC in the promotion of colon cancer cell apoptosis by both agents. SC, a flavonoid, is the major active ingredient extracted from Erigeron breviscapus, a plant used in Chinese herbal medicine. In vivo, breviscapine and its preparations have been shown to protect against cerebral ischemia-reperfusion injury. In China, SC is used for the clinical treatment of cerebrovascular disorders and is currently under clinical trials for vascular protection. However, there is insufficient information on its molecular properties, apart from it being a known calcium-channel blocking agent and an antioxidant. Its pharmacokinetic and anticarcinogenic properties are virtually unknown (10, 11). Interestingly, SC has been speculated to exert its neuroprotective effects by inhibiting oxidative stress-induced apoptosis in neuronal cells. It has been shown that in phaeochromocytoma PC12 cells, SC reduced caspase-3 activity, increased Bcl-XL expression, inhibited p38 phosphorylation and attenuated production of reactive oxygen species (13, 14). Our results, however, show that SC promoted drug-evoked apoptosis of HCT116 colon cancer cells. SC may exhibit cell type-specific or trigger-
specific effect on apoptotic sensitivity, although the underlying mechanism remains unknown.

Our previous reports (5, 6) showed that caspase-6 activation is a crucial signaling mechanism in both RSV and 5-FU-evoked apoptosis of colon cancer cells and is enhanced by the presence of p53. In this study, our results demonstrated that SC promoted both RSV- and 5-FU-evoked apoptosis only of the cancer cells wild-type for p53. It should be noted that SC at the dose tested was unable to enhance the drug-evoked apoptosis in p53 knockout HCT116 cells. Previously, p53 was shown to transactivate caspase-6 by acting at the third intron of the caspase-6 gene, which is believed to decrease the cellular apoptotic threshold (15). Interestingly, the sensitization effect of SC on 5-FU was observed only after 24 h of treatment. This observation may be due to SC acting as an enhancer of apoptosis at its downstream pathway(139,246),(321,505). At the potent sensitizing doses, SC alone exhibited little toxicity and did not cause significant apoptosis. In support of this notion, we showed that SC promoted the drug-evoked apoptosis through enhanced caspase-6 activation, a downstream effector proteolytic enzyme responsible for nuclear lamina breakdown via lamin-A cleavage. This finding also strengthened the importance of caspase-6 activation in the apoptotic signaling mechanism of these cancer cells. At the potent sensitizing doses, SC alone exhibited little toxicity and did not cause significant apoptosis or caspase-6 activation in the cancer cells. Taken together, we speculate that SC may sensitize RSV- and 5-FU-evoked apoptosis of these cancer cells through a p53-regulated caspase-6 activation mechanism. On the other hand, the sensitizing doses of SC required may be different in the absence of p53, and a higher dose or exposure time could be needed to elicit the sensitization effect.

In conclusion, we have demonstrated a novel chemosensitizing ability of SC for both RSV- and 5-FU-evoked HCT116 colon cancer cell apoptosis, through the enhancement of caspase-6 activation. Our results would benefit the pharmacological development of SC, RSV and 5-FU as agents for combination chemotherapy, as well as caspase-6 as a plausible molecular target for colon cancer therapy.

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References