Curcuma wenyujin Extract Induces Apoptosis and Inhibits Proliferation of Human Cervical Cancer Cells In Vitro and In Vivo

Chuan-Bian Lim, BS,1 Nung Ky, BS,1 Hui-Min Ng, BS,1 Mohamed Sabry Hamza, PhD,2 and Yan Zhao, MD, PhD1

Abstract
An essential oil extract, derived from the rhizome of Curcuma wenyujin (CWE), possesses antioxidative, antimicrobial, and anti-inflammatory properties. However, it remains unknown how exactly CWE inhibits tumor growth. In this study, using human cervical cancer HeLa cells, the authors postulated that CWE has the ability to inhibit tumor growth. The study shows that CWE dose-dependently suppressed colony formation and inhibited the proliferation of HeLa cells through blockade of cell cycle progression at G1 phase and apoptosis. CWE-induced G1 arrest was associated with retinoblastoma protein dephosphorylation and reduced amounts of cyclins D1 and D3, and cyclin-dependent kinase 4 and 6 proteins. CWE treatment resulted in apoptosis in HeLa cells as evidenced by morphological changes, caspase activation and PARP cleavage, which can be reversed by a pan-caspase inhibitor. It was observed that CWE treatment activated the mitochondrial apoptotic pathway indicated by a decrease in Mcl-1 and Bcl-xL levels, resulting in mitochondrial membrane potential loss and caspases 9 activation. CWE-treated cells displayed reduced PTEN, AKT, and STAT3 phosphorylation and downregulation of NFκB signaling, providing a mechanism for the G1 arrest and apoptosis observed. Furthermore, CWE inhibited tumor growth of HeLa in a xenograft mouse tumor model, suggesting that CWE inhibited tumorigenesis by inhibiting cell proliferation and inducing apoptosis. These findings are the first to reveal the molecular basis for the anticervical cancer action of CWE. The results suggest that CWE could be developed as a drug for the management of cervical cancer.

Keywords
Curcuma wenyujin, Ezhu, extract, apoptosis, G1 arrest, cervical cancer, HeLa

Introduction
Cervical cancer is the second most commonly diagnosed cancer in women.1 Globally, cervical cancer is also the third leading cause of cancer death among women, where 273 505 women died out of an estimated 493 243 cases diagnosed in 2002, the majority of which occur in developing countries.2 It also poses an important public health threat in Singapore with an age-standardized rate of 10.6 per 100 000 women per year recorded for the period from 1998 to 2002, which is higher than most of Europe and the United States.3 Although its incidence rate has been declining consistently over the past 3 decades, cervical cancer is still the most prevalent gynecological cancer in Singapore.3 The majority of these cases are believed to be the result of infection of the cervical epithelium by oncogenic human papillomavirus (HPV).4 Although there are more than 200 types of HPV virus, HPV-16 and HPV-18 are associated with 50.5% and 13.1% of cervical cancers, respectively.5 Current treatment strategies in managing preinvasive cervical lesions involve one of the following approaches: electrocoagulation, cryotherapy, laser ablation, or local surgery whereas invasive carcinomas involve a combination of surgery, chemotherapy, and radiotherapy.5 Unfortunately, despite advances in detection and treatment of this cancer, survival rates remain alarmingly low (vary widely from <30% in Africa to 70% in developed countries) and it is clear that a different treatment approach is much needed.1 As such, research into potential novel therapies for

1Nanyang Technological University, Singapore
2Schering-Plough Technologies Pte Ltd, Singapore

Corresponding Author:
Yan Zhao, Division of Chemical Biology and Biotechnology, School of Biological Sciences, Nanyang Technological University, 60 Nanyang Drive, 637551 Singapore
Email: zhaoyan@ntu.edu.sg
cervical cancer with higher efficacy and lesser toxicity are currently being pursued.

Curcuma, a member of the Zingiberaceae or ginger family, is a medicinal plant that is widely distributed all over the world. At present, the dried rhizome of this perennial herb is used as a popular Chinese, Japanese, and Korean herbal medicine. Chinese Pharmacopoeia states that the dried rhizome of 5 species (Curcuma phaeocaulis, Curcuma kwangsiensis, Curcuma wenyujin, Curcuma zedoaria, and Curcuma aromatica), collectively known as Ezhu to the Chinese, are used for treating blood stasis and alleviating pain.7 8 Although about 20 Curcuma species could be found in China, pharmacological studies of other species are very few, mainly because botanical origins of Curcuma drugs are not easily identified because of similarity in plant morphology and differences in nomenclature derived from used parts and producing areas. Currently 4 Curcuma drugs are recorded in the Chinese Pharmacopoeia: Yujin (the tubers of C wenyujin, C longa, C phaeocaulis, or C kwangsiensis), Jianghuang (the rhizome of C longa), Pian-Jianghuang (the rhizome of C wenyujin), and Ezhu (the rhizomes of C phaeocaulis, C kwangsiensis, or C. wenyujin).8 The essential oil extract of Ezhu (herein referred to as Ezhu oil) has been reported to possess antimicrobial,9 anti-inflammatory,10,11 antiproliferative,12 and antitumor9,13,14 activities. Not only does Ezhu oil have antitumor effects, a query of the NAPRALERT database for plants, maintained by the Program for Collaborative Research in the Pharmaceutical Sciences, University of Illinois, reported Ezhu oil to have been used ethnomedically against a plethora of other human conditions, such as fever, wounds, diarrhea, and snake bite.

Although studies have demonstrated that Ezhu oil exhibits antitumor activities,7 these reports did not show convincing proof of its mechanistic mode of action. Clinically, Ezhu oil has been used for the treatment of early-stage cervical cancer.7 Either 1% Ezhu oil alone or a mixture of 1% Ezhu oil and 0.5% curcuminol (or curzerenone) were directly injected into the tumor sites with concomitant local application and intravenous infusion of Ezhu oil alone or in combination with curcuminol or curzerenone.7 Results from clinical trials conducted in the late 1970s reported that Ezhu oil is well tolerated with aggregate effective rate of about 70%,7 but the mechanisms by which it brings about antitumor effect are unclear. This knowledge gap compromises the medicinal value of Ezhu oil and can even jeopardize the safety of patients. These reasons, therefore, emphasize the pressing need to investigate thoroughly the pharmacological and biological actions responsible for the antitumor activity of Ezhu oil. Additionally, given that cervical cancer ranks high on the list of lethal cancers that plague women worldwide, there is a tremendous interest in drugs such as Ezhu oil that possess antitumor activity.

In this study, we present evidence that treatment of HeLa cells with C wenyujin essential oil extract (CWE) leads to a dose-dependent reduction in cell proliferation and apoptosis. Intratumoral delivery of CWE to mice bearing HeLa xenografts resulted in growth suppression and induction of apoptosis. The data suggest that blocking PTEN (phosphatase and tensin homolog deleted on chromosome 10)/phosphatidylinositol 3-kinase (PI3K)/AKT (also known as protein kinase B)/nuclear factor kappa B (NFkB) signaling pathway with CWE may represent a novel approach to the treatment of cervical cancer.

Materials and Methods

Reagents

Ezhu oil was purchased from Guangzhou Endless Trading Co, Ltd (China), which was delivered under the name of the species C wenyujin. Dimethyl sulfoxide (DMSO) and propidium iodide (PI) were purchased from Sigma (St Louis, MO). Stock solutions of CWE were prepared at 5% in DMSO and DMSO was used throughout the experiment as the vehicle control.

Cell Lines and Culture Conditions

HeLa cells (ATCC CCL-2) were cultured in Dulbeccos’ modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (all from GIBCO Invitrogen, Carlsbad, CA) at 37°C and 5% CO2.

Cell Proliferation Assay

Inhibition of cell proliferation by CWE was assessed using the MTT assay (Roche, Indianapolis, IN). Briefly, HeLa cells were plated in 96-well culture plates (5 × 104 cells/well) and treated the following day with CWE or DMSO vehicle as described in the Results section. Following CWE treatment, cells were incubated with MTT labeling reagent for 4 hours, solubilized in 10% SDS, and the MTT metabolite formazan crystals were quantitated at 575 nm on a microplate reader (Tecan, Männedorf, Switzerland).

Clonogenic Assay

To determine the growth suppression effect of CWE treatment, HeLa cells were treated with CWE or DMSO vehicle for 24 hours as described in the Results section. After treatment, cells were replated in complete DMEM and allowed to grow for 14 days to form colonies that were then stained with crystal violet (Sigma).
Flow Cytometry Analysis

HeLa cells (1 × 10^5 cells/mL) were seeded in 6-well dishes in complete DMEM and incubated for 48 hours, whereupon the cells were treated with CWE at the concentration and for the time indicated in the appropriate figure legend. For cell cycle analysis, cells were fixed in 70% ethanol, stained with PI and analyzed using a FACScalibur flow cytometer (BD Biosciences, San Jose, CA). The data were analyzed using CellQuest software (BD Biosciences).

Apoptosis Assay

For measurement of apoptosis, cells were treated with CWE at the concentration indicated in the appropriate figure legend for 24 hours and double stained with FITC-conjugated annexin V and PI using Annexin V-FITC Apoptosis Detection kit (BD Pharmingen) according to manufacturer's instructions. To determine the effects of caspase inhibitor on apoptosis, cells were treated with CWE with and without the pan-caspase inhibitor z-VAD-fmk (final concentration 50 μM; Santa Cruz Biotechnology, Santa Cruz, CA).

Mitochondrial Membrane Potential Assay

Changes in mitochondrial membrane potential after treatment with CWE were determined by staining cells with the indicator fluorescent dye JC-1 (BD Pharmingen) and measured by flow cytometry, as recommended by the manufacturer.

Immunoblot Analysis

Immunoblot analyses were performed as described.15 HeLa cells were lysed in Cellytic M Cell Lysis Reagent (Sigma). Whole cell lysates were separated by SDS-PAGE and transferred onto nitrocellulose membrane (Bio-Rad, Hercules, CA). The membranes were immunoblotted with primary antibodies against Cdk 2, 4, 6, Cyclin B1, A, Bcl-2, p15INK4b, p16INK4a, p21\(^{\text{WAF1/Cip1}}\), p27\(^{\text{Kip1}}\), Rb, Cdc25A, Cdc25C, Bcl-2, Mcl-1, and Bcl-x\(_L\) (Santa Cruz Biotechnology). The remaining primary antibodies were purchased from Cell Signaling Technology (Danvers, MA). Blots were incubated with horseradish peroxidase–conjugated goat anti-rabbit, goat anti-mouse, or rabbit anti-goat (Santa Cruz Biotechnology) and visualized using ECL Plus Western blotting detection kit (Amersham Biosciences, Piscataway, NJ).

In Vivo Tumor Xenograft Study

Female SCID mice (5 to 7 weeks old) were purchased from the Animal Resources Centre (Canning Vale, Australia) and housed in negative pressure isolators with 12-hour light/dark cycles, with free access to sterilized food and water. HeLa cells (2 × 10^6 cells) were injected subcutaneously into the flanks of SCID mice. Tumors were allowed to develop until they reached ~200 mm^3, when treatment was initiated. Ten mice were randomly divided into 2 groups. The mice in the treatment group were intratumorally injected with CWE in DMSO (625 μg/kg body weight) in a 50 μL volume. The control group was treated with an equal volume of DMSO vehicle. After transplantation, tumor size was measured using vernier calipers, and tumor volume was calculated as \(L \times W^2/2\), where \(L\) is the length and \(W\) is the width. At the end of the study, the mice were sacrificed with body and tumor weights being recorded, and the tumors were harvested for analysis.

Immunohistochemical Analysis

To assess cell proliferation, freeze sections were incubated with mouse monoclonal anti-Ki67 (1:400 dilution; Novocastra, Newcastle upon Tyne, UK) after blocking endogenous peroxidase activity and nonspecific staining. This was followed by incubation with a secondary goat anti-mouse EnVision-HRP (undiluted; Dako, Glostrup, Denmark) and then visualization of Ki67-positive cells using 3,3′-diaminobenzidine (Dako) as the chromogen. To assess cell death, apoptotic cells were identified by terminal deoxynucleotidyl transferase–mediated dU TP nick end labeling (TUNEL) staining using ApopTag Plus peroxidase in situ apoptosis detection kit (Chemicon, Rosemont, IL) according to manufacturer’s instructions. Sections were viewed with an Eclipse 90i upright microscope using a 20× objective (Nikon, Tokyo, Japan). Images were analyzed using Image-Pro Plus software (Media Cybernetics, Bethesda, MD). Ki67- and TUNEL-positive cells were quantified by counting brown-stained cells within the total number of cells at 10 randomly selected fields.

Statistical Analysis

Data were expressed as means ± standard error. Statistically significant differences between samples means were determined by 2-tailed Student’s \(t\) test for unpaired data. \(P < .05\) was considered significant.

Results

CWE Inhibits Cell Proliferation and Clonogenic Survival in HeLa cells

To investigate the effect of CWE on cell proliferation and clonogenic survival, human cervical cancer HeLa cells were treated with CWE. The effects of CWE on cell proliferation of HeLa cells were determined using MTT assay.
HeLa cells were cultured in the presence of increasing doses of CWE (0 to 125 μg/mL) for 24 hours. As shown in Figure 1A, our data clearly demonstrate that CWE inhibited cell proliferation in a dose-dependent fashion in HeLa cells. Figure 1B shows marked morphologic signs of apoptosis that included changes such as membrane blebbing and apoptotic body formation in a dose-dependent manner after exposure to CWE. To further investigate the growth suppression effect of CWE, we performed in vitro clonogenic assay. Figure 1C shows the effects of CWE on the clonogenic potential of the control and the CWE-treated HeLa cells. CWE suppressed clonogenicity of HeLa cells in a dose-dependent manner, where maximal clonogenic inhibition was observed at the maximum concentration used of 50 μg/mL. No clonogenic growth was observed after treatment with 100 μg/mL CWE, indicating that all cells were eliminated (data not shown).

**Induction of G1 Phase Arrest in HeLa Cells by CWE**

To elucidate the mechanism of CWE-induced proliferation inhibition, we examined the effect of CWE on cell cycle distribution by flow cytometry. As shown in Figure 2A, concomitant with the growth inhibitory effect, CWE treatment induced a weak G1 phase arrest in a dose-dependent manner after 24 hours of treatment. For instance, cell populations in the G0/G1, S, and G2/M phases were 59.98%, 18.76%, and 21.01%, respectively, in control HeLa cells. However, after 24 hours of incubation with 10 and 50 μg/mL CWE, the G1 population was noticeably enhanced by 2.48% and 6.28%, respectively. This increase in the G1-phase cell population was accompanied by a concomitant decrease in the S and G2/M phase cell populations. Interestingly, the G2/M population of HeLa was markedly reduced after treatment with 50 μg/mL CWE, indicating the blockage of the S-G2 transition in HeLa cells. Meanwhile, the subG1 population was slightly increased in cells exposed to 50 μg/mL CWE. After treatment, however, with CWE at 100 μg/mL for 24 hours, the percentage of subG1 cells, that is, apoptotic cells containing hypodiploid amount of DNA, was increased to 47.45%, compared with cells treated with 50 μg/mL CWE (Figure 2A). We also observed that the percentage of G1 cells decreased with 100 μg/mL CWE (66.26% at 50 μg/mL vs 40.67% at 100 μg/mL). These data indicated that exposure to CWE blocked or delayed the progression of cells from G1 phase into S phase, and induced cells toward apoptosis.

As another index for G1 phase arrest, we measured the expression levels of cyclins D1 and D3, and cyclin-dependent kinases CDK4 and CDK6, because the cyclin D-CDK4/6 complex is the primary regulator of the G1/S phase restriction point. As shown in Figure 2B, HeLa cells exposed to CWE for 24 hours exhibited downregulation of cyclin D1,
Figure 2. *Curcuma wenyujin* essential oil extract (CWE) induces cell cycle arrest in G1 phase and modulates cell cycle regulators.

A, $1 \times 10^5$ HeLa cells were seeded onto 6-well plates and incubated for 24 hours. Various concentrations of CWE were added to the culture medium and incubated for an additional 24 hours. Cells were then harvested and analyzed by flow cytometry. The cell cycle phase distribution was determined using CellQuest software. B and C, HeLa cells were treated with increasing concentrations of CWE for 24 hours, following which cells were harvested, cell lysates prepared and subjected to immunoblot analysis to detect the levels of cell cycle regulators and β-actin (loading control).
cyclin D3, CDK4, and CDK6 expressions, which coincided with the CWE-induced cell cycle arrest at the G1 phase.

**Effect of CWE on the Expression of Cell Cycle Regulators**

On the basis of an observed G1 arrest in HeLa cells induced by CWE, we assessed the levels of cell cycle regulators that may modulate this effect. As shown by immunoblot analysis in Figure 2C, CWE treatment caused a dose-dependent decrease in the expression levels of cyclin A, cyclin B1, and Cdk2. CWE treatment also led to the dephosphorylation of retinoblastoma protein (Rb), which may contribute to the cell cycle progression from G0/G1 to S phase. Binding of cyclins to CDKs would form active kinase complexes, which are regulated and inhibited by various CDK inhibitors. Next, we assessed the effect of CWE treatment on the expression levels of CDK inhibitors p15INK4b, p16INK4a, p21WAF1/Cip1, and p27Kip1 in HeLa cells. As shown by immunoblot analysis in Figure 2C, the expression levels of p16INK4a and p27Kip1 were downregulated at 100 μg/mL CWE treatment, without any noticeable changes in p15INK4b levels. Interestingly, the levels of p21WAF1/Cip1 dramatically decreased at 100 μg/mL CWE treatment with the concomitant appearance of a 15-kDa fragment. This cleavage was specifically inhibited by addition of a pan-caspase inhibitor z-VAD-fmk in accordance with a previous report that caspases are regulated and inhibited by various CDK inhibitors. In addition to CDKs, cyclins, and CDK inhibitors examined above, cdc25 phosphatases also play an important role in cell cycle regulation. Based on our findings showing that CWE caused G1 phase arrest, we next assessed its effect on the expression levels of Cdc25A and Cdc25C under identical experimental conditions as for other cell cycle regulatory molecules. As shown in Figure 2C, CWE treatment of HeLa cells resulted in a dose-dependent decrease in Cdc25C and Cdc25A expression levels at 100 μg/mL CWE. Together, these results clearly show a decrease in the protein levels of CDKs, cyclins, CDK inhibitors and Cdc25 phosphatases by CWE in HeLa cells, suggesting their possible roles in the observed cell cycle arrest response of CWE.

**Induction of Apoptosis in HeLa Cells by CWE**

During the cell proliferation assay, we observed that CWE caused a significant decrease in HeLa cell growth and proliferation, where higher doses were more effective. As shown in Figure 1A, CWE induced HeLa cell death in a dose-dependent manner. Cell cycle analysis showed that treatment of HeLa cells with CWE for 24 hours led to a dose-dependent increase in the number of cells in the sub-G1 population, suggesting that CWE was inducing apoptosis (Figure 2A). At the highest concentration (100 μg/mL), levels of apoptosis were 47.45% in HeLa cells. Similar results were obtained in human HCT116 colon and A2780 ovarian cancer cells (supplementary data). To investigate the mechanism of apoptosis, cells were treated with 10, 50, and 100 μg/mL CWE for 24 hours, and we confirmed that apoptosis was the major mode of cell death induced by CWE in HeLa cells, by measuring 3 different apoptotic events, including caspase-3/6/7/9 activation, PARP cleavage, and annexin V binding (Figure 3A and data not shown). Consistent with the effect on caspase-3/6/7/9 activation, CWE also caused a strong cleavage of PARP, yielding an 89-kDa fragment in cells exposed to CWE for 24 hours (Figure 3A). As another index of apoptotic cell death, we measured the apoptotic cell population by annexin V/PI assay, which showed that CWE induced a dose-dependent increase in the apoptotic cell population after 24-hour treatment with 10, 50, and 100 μg/mL CWE (data not shown). Evidence for CWE-induced apoptosis through the intrinsic mitochondrial pathway was shown by a loss of mitochondrial membrane potential (MMP) as measured by loss of JC-1 staining (Figure 3C). To study the mechanism involved in MMP loss, we analyzed changes in the expression of pro-apoptotic proteins involved in the control of mitochondrial permeability transition. Although CWE treatment did not alter total cellular level of Bcl-2 and Bax, Bcl-xL, Bak, Bid, Bim, Bik, Bad, and PUMA displayed a relatively large decrease in expression level as compared with untreated controls (Figure 3B). The regulation of these proteins seems to occur independently of p53 expression (Figure 3B). To confirm CWE-induced apoptosis was dependent on caspases, we treated cells with zVAD-fmk (50 μM) for 2 hours before treatment with increasing concentrations of CWE (24 hours). As shown in Figure 4, CWE-induced apoptosis was blocked by zVad-fmk pretreatment, evidenced by immunoblot analysis and confirmed by annexin V apoptosis assay.

**CWE Targets Survival Pathways**

The PI3K/Akt pathway is a pivotal survival pathway that protects cells from apoptosis induced by various types of stresses. To determine the effects of CWE on PI3K/Akt survival pathway, we looked at the expression of Akt. Figure 5A shows that downregulation of Akt expression was evident after 100 μg/mL CWE treatment. Similarly, the phosphorylation of AKT at Thr308 residue was significantly reduced by 100 μg/mL CWE. CWE-mediated deactivation of Akt was additionally confirmed by determining phosphorylation of one of its substrates, Bad. As shown in Figure 5A, in comparison with the control, the phosphorylation of Bad at Ser112 and Ser136 decreased after HeLa cells were exposed to 100 μg/mL CWE. To
Figure 3. *Curcuma wenyujin* essential oil extract (CWE) induces apoptosis through the intrinsic mitochondrial pathway

A and B, HeLa cells were treated as described in Figure 2 and subjected to immunoblot analysis for the proteins indicated. C. Treatment of HeLa cells with CWE induces loss of mitochondrial membrane potential. HeLa cells were treated with increasing concentrations of CWE (50 and 100 μg/mL) for 24 hours, harvested and stained with JC-1 dye and analyzed by flow cytometry. The percentage of the cells that emit only green fluorescence indicates the depolarized mitochondrial membrane as shown in the lower right quadrant of the histogram.
verify the possible role of PI3K in CWE-induced apoptosis, HeLa cells were pretreated for 2 hours with the specific inhibitor for PI3K, LY294002. Subsequently, the LY294002-treated cells were exposed to CWE, and then apoptosis was determined as evaluated by annexin V/PI staining (Figure 5B). CWE did not induce a significant increase of apoptosis in HeLa cells at 10 or 50 μg/mL (Figure 5B), in keeping with the previous findings obtained in the cell cycle analysis. As shown in Figure 5C, HeLa cells preincubated with 20 μM LY294002 and exposed to CWE inhibited the Thr308 phosphorylation of Akt and promoted HeLa cell apoptosis with respect to HeLa cells exposed to CWE in the absence of LY294002 (Figure 5B). Because the PI3K/Akt pathway is negatively regulated by PTEN,22 we presumed that the inhibition of Akt by CWE might correspond to an activation of PTEN via dephosphorylation at Ser380. Hence, we determined the changes in the activation of PTEN in HeLa cells treated with CWE. We observed a decrease in Ser380-phosphorylated PTEN levels in a dose-dependent manner (Figure 5A). Thus, it appears that PTEN/PI3K/AKT signaling is involved in the observed protection of HeLa cells against low dose CWE-induced apoptosis, suggesting that HeLa can be sensitized to CWE-mediated apoptosis by blocking this pathway.

We also assessed the activity of the signal transducer and activator of transcription 3 (STAT3) pathway, a major pathway involved in cellular proliferation and apoptosis.23 The levels of Thr705- and Ser727-phosphorylated STAT3 are downregulated by CWE treatment suggesting that the induction of apoptosis by CWE in HeLa cells results, at least in part, from an inhibition of the STAT3 pathway (Figure 5D).

Finally, we investigated the influence of CWE on the activity of the NFκB pathway as NFκB activation blocks apoptosis and promotes cell proliferation.24 We showed a decrease of expression of NFκB p65 subunit with CWE treatment (Figure 5D). NFκB is present in the cytosol as an inactive precursor complexed with the inhibitory IkB protein.25 Activation occurs via phosphorylation of IkBα at Ser32 and Ser36 residues, resulting in the release and nuclear translocation of NFκB.26 To further confirm CWE-induced downregulation of the activity of NFκB pathway, we performed immunoblot analysis for Ser32-phosphorylated IkBα. CWE treatment leads to a decrease in IkBα phosphorylation (Figure 5D), suggesting that CWE blocks IkBα phosphorylation, preventing its ubiquitination and further degradation thus preventing nuclear translocation of NFκB.

CWE Inhibits Tumor Growth in SCID Mouse Model of Cervical Cancer

To determine whether CWE can inhibit tumor growth in vivo, equal numbers of HeLa cells were injected subcutaneously into the flanks of SCID mice and treated intratumorally with 625 μg/kg CWE on days 0, 3, and 6. There was significant suppression of tumor growth (55% reduction in tumor size) in the CWE treatment group compared with the
Figure 5. *Curcuma wenyujin* essential oil extract (CWE) affects the PI3K/AKT/NFκB signaling pathway

A, HeLa cells were treated as described in Figure 2 and probed with the indicated antibodies. B, HeLa cells were treated with increasing concentrations of CWE for 24 hours after preincubation with 20 μM LY294002 (PI3Ki) for 2 hours, stained with FITC-annexin V and PI and analyzed by flow cytometry. C, HeLa cells were preincubated with 20 μM PI3Ki for 2 hours, followed by CWE treatment for 24 hours before p-AKT levels were subjected to immunoblot analysis.
vehicle group ($P < .05$; Figures 6A and 6B). Mean values for tumor size at day 9 were 1667.4 ± 422.8 mm$^3$ in the control group and 850.2 ± 135.9 mm$^3$ in the CWE treatment group.

No sign of toxicity, as judged by parallel monitoring of body weight, was observed in CWE-treated mice (Figure 6C). To gain insight into the mechanism of CWE’s inhibition of
tumor growth in vivo, we subjected the HeLa tumor xenografts from vehicle-treated and CWE-treated mice to immunohistochemistry after treatments and assessed proliferation and apoptosis by Ki67 and TUNEL analyses, respectively. As shown in Figures 6D and 6E, the human cervical tumor tissues with CWE treatment in vivo displayed decrease in Ki67 expression and tumor cell apoptosis, as determined by TUNEL assay, at day 9 after CWE treatment compared with vehicle group in vivo.

Discussion

Because of unsatisfactory treatment options for cervical cancer, there is a pressing need to develop novel therapies for this malignancy. One such approach is through discovery of anticancer compounds from natural sources. CWE, a commercially available natural product, has been tested in China as a treatment for women with cervical cancer.7

The main bioactive constituents of CWE are mainly sesquiterpenes, which include curcumenol, curcumol, curzerene, curzerenone, curdione, β-elemene, γ-elemene, isocurcum enol, furanodiene, furanodienone, germacrene D, germacrone, and neocurzidene.27,28 Numerous studies have shown that curdione, curzerene, β-elemene, and furanodiene exhibit significant anticancer effects.9,29-34 Most notably, furanodiene was found to inhibit both in vitro and in vivo xenograft growth of cervical cancer cells.29 However, it remains unclear how CWE modulates intracellular signal(s) to lead to the inhibition of cell proliferation and the subsequent induction of the apoptotic pathway, further studies into the anticancer mechanisms of CWE are necessary for better clinical application. In this regard, detailed investigations were undertaken here to define the mechanism by which CWE plays a role in the induction of apoptosis. Using HeLa cells, we demonstrated that CWE effectively inhibits tumor cell growth in vitro, concomitant with induction of cell cycle arrest and apoptosis, and inhibits tumor cell growth in SCID mice. Furthermore, CWE induces in vivo tumor growth inhibition in the cervical cancer with no severe signs of toxicity; this suggests that CWE possesses selectivity between normal and cancer cells.

In the present study, we showed that CWE induced growth inhibition and apoptosis in HeLa cells, however, cytotoxicity resulted mainly from the induction of apoptosis. Apoptosis was indicated by the appearance of cells with membrane blebbing and low DNA content, exposure of phosphatidylserine on the outer leaflet of the cell membrane, increase in cleaved caspases 3, 6, 7, and 9 levels and activation of PARP cleavage by caspases. Induction of apoptosis by CWE was previously demonstrated in human hepatoma HepG2 cells,13 as well as in A2780 and HCT116 cells (in the present study), pointing to the broad-spectrum antitumor activity of CWE against various histologic types of human cancers. Moreover, the pretreatment of HeLa cells with zVd-fmk, a pan-caspase inhibitor, effectively inhibited caspase activity and prevented CWE-induced cell death.

The apoptotic pathways induced on CWE treatment in HeLa cells remain to be uncovered. Previous studies have reported that human hepatoma HepG2 cells undergo apoptosis through the activation of caspase 3 and dissipation of mitochondrial membrane potential via the release of cytochrome c from mitochondria into the cytosol.13 To gain insight into the apoptotic signaling pathways involved in CWE action in HeLa cells, we examined the changes in the levels of Bcl-2 family of proteins and prosurvival transcription factors after treatment with CWE. CWE caused reduction in the levels of antiapoptotic Mcl-1 and Bcl-xL but did not alter the expression levels of Bcl-2 in HeLa cells. The proapoptotic Bcl2 family members have a major role in controlling apoptosis by regulating mitochondrial membrane permeability and cytochrome c release.35 In addition to the well-defined function of Bax and Bak in induction of apoptosis, a critical role of Bax and Bak in autophagic cell death has also been uncovered recently.36 It has been observed that deficiency in Bax and Bak proteins led to autophagic cell death in response to hypericin-mediated photodynamic therapy.36 The observed decrease in proapoptotic Bcl-2 proteins Bak, Bid, Bim, Bik, Bad, and PUMA levels on CWE incubation in HeLa cells could possibly explain the induction of cell death response. Moreover, these effects seem to be independent of p53. Additional studies are needed to establish the implication of proapoptotic Bcl-2 protein degradation induced by CWE in HeLa cells.

Inhibition of deregulated cell cycle progression in cancer cells is an effective strategy to halt tumor growth.37 Our results further demonstrate that treatment of HeLa cells with CWE induces G1 phase arrest of cell cycle progression indicating that one of the mechanisms by which CWE inhibits the proliferation of HeLa cells is inhibition of cell cycle progression. Further molecular mechanistic studies demonstrate a marked dose-dependent decrease in the expression of cyclins A, B1, D1, D3 and CDK2, CDK4, and CDK6 in HeLa cells after CWE treatment, which suggests disruption of the uncontrolled cell cycle progression of human cervical cancer cells and that the CWE-induced G1 arrest is mediated through the reduction of Rb phosphorylation and upregulation of total Rb proteins, which regulates the restriction point at the G1/S transition of the cell cycle.16 Cyclin-dependent kinase inhibitors, such as p21WAF1/Cip1, are frequently upregulated in response to anti-proliferative signals.38 This is, however, inconsistent with our results, which showed that CWE decreases the expression of p16INK4a, p21WAF1/Cip1, and p27Kip1. We also have found that CWE causes caspase-mediated cleavage of p21WAF1/Cip1. Recently, it was found that cleaved p21WAF1/Cip1...
could switch from promoting cell cycle arrest to inducing apoptosis. Similarly, downregulation of p16^{INK4A} and p27^{kip1} was shown to contribute to apoptosis induced by DNA damage and growth factor deprivation, respectively. In addition, treatment of HeLa with CWE also decreases the expression of Cdc25A, Cdc25C, and phospho-Cdc2, which suggests that CWE might be capable of inducing G2/M arrest, as previously reported. In this regard, it is important to emphasize that we did not observe any cells arrested in G2/M phase of the cell cycle. More studies, however, are needed in the future to resolve this issue, as well as to provide mechanistic insights. Taken together, these mechanistic observations were in accord with an overall efficacy of CWE in inducing a G1 arrest in the cell cycle followed by the inhibition of cell growth and apoptosis.

The PI3K/AKT pathway regulates many vital cellular processes such as cell proliferation, survival, angiogenesis, and glucose metabolism. Recent studies have shown that the PI3K/AKT signaling pathway is deregulated in human cervical cancer. Thus, these observations suggest that inhibition of PI3K/AKT in cervical cancer is an attractive chemotherapeutic approach. We found that CWE can suppress activation of AKT in HeLa cells. Compatible with a role for the PI3K/AKT pathway in the pathophysiology of cervical cancer, the blockade of PI3K/AKT signaling by the selective inhibitor LY294002 inhibited the phosphorylation of Akt. This inhibition was associated with the inhibition of cell proliferation and induction of apoptosis in HeLa cells. Our data showed that CWE-induced apoptosis was accompanied by dephosphorylation of AKT at Thr308, and downregulation of AKT kinase expression. We also observed that CWE treatment leads to the Ser380-dephosphorylation of PTEN, an upstream inhibitory modulator of PI3K/AKT activity. Our data further showed that inhibition of PI3K/AKT signaling by LY294002 renders HeLa cells susceptible to low dose CWE-induced apoptosis, which suggests that PI3K/AKT activation is likely to be involved in protecting HeLa cells from CWE-induced apoptosis.

Consistent with the reported prosurvival roles of STAT3, CWE treatment markedly reduced the levels of Tyr705- and Ser727-phosphorylated STAT3, the 2 phosphorylation sites required for maximal STAT3 activation. Notably, the activity of NFκB, another important antiapoptotic transcription factor, is markedly reduced as well. CWE decreases the expression of p65 subunit of NFκB and reduces phosphorylation of IκBζ at Ser32 consistent with downregulation of the NFκB pathway. Taken together, our results indicated that CWE modulates multiple regulatory molecules important in apoptosis and survival.

A number of recent studies have demonstrated therapeutic benefits from the use of inhibitors that co-target multiple simultaneously activated signaling pathways in the treatment of cancer. Multimodal inhibitors in this regard include sorafenib (BAY 43-9006), midostaurin (PKC412A), motesanib (AMG 706), and PU-H71. Sorafenib is by far the most promising of these multimodal inhibitors. The agent is currently approved for the treatment of advanced renal cell carcinoma (RCC) and unresectable hepatocellular carcinoma (HCC). Sorafenib is a small molecule inhibitor with activity against Raf kinase and several receptor tyrosine kinases including vascular endothelial growth factor receptor (VEGFR)-1/2/3, VEGF-3, platelet-derived growth factor receptor β, fibroblast growth factor receptor 1, c-Kit, Flt-3, and RET. In advanced RCC and HCC models, sorafenib exhibited antitumor activity that appeared to be antiproliferative and antiangiogenic in nature. With botanical extracts such as CWE, on which we have reported in this study, and emerging multimodal inhibitors, there is finally a real possibility of an antitumor agent capable of eradicating cancer.

We have demonstrated CWE efficacy as an anticancer agent not only in cell culture but also in the mouse model. SCID mice injected subcutaneously with HeLa cells and subsequently injected intratumorally with CWE were found to develop significantly smaller tumors, as compared with vehicle controls. It should be noted that the mice received 3 doses of CWE over 9 days and showed no outward signs of toxicity. In conclusion, this study is the first to report the effectiveness of CWE as an inhibitor of cervical cancer proliferation both in vitro and in vivo. Future clinical studies are warranted to examine the therapeutic effect of this chemotherapy.

Acknowledgments

We thank Dr Jie Shi (National Institute on Drug Dependence, Peking University, China) for assistance with the in vivo animal xenograft studies. We are also grateful to Keith Rogers, Hassan Siaka Hall, and Xiao Yong (Histopathology Facility, Institute of Molecular and Cell Biology, Singapore) for assistance with histological and image analysis. We also thank Dr Jerold M. Ward (Global Vet Pathology) for the valuable histopathological consultation.

Declaration of Conflicting Interests

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

Funding

This work was supported by the Academic Research Fund (AcRF), Tier 1 (RG78/07), Ministry of Education, Singapore, to ZY.

References


34. Zhang HY, Hu CX, Liu CP, et al. Screening and analysis of bioactive compounds in traditional Chinese medicines using...