RESEARCH ARTICLE

Cell Cycle Modulation of MCF-7 and MDA-MB-231 by a Sub-Fraction of *Strobilanthes crispus* and its Combination with Tamoxifen

Nik Soriani Yaacob1*, Nik Nursyazni Nik Mohamed Kamal1, Kah Keng Wong2, Mohd Nor Norazmi3

Abstract

**Background:** Cell cycle regulatory proteins are suitable targets for cancer therapeutic development since genetic alterations in many cancers also affect the functions of these molecules, *Strobilanthes crispus* (*S. crispus*) is traditionally known for its potential benefits in treating various ailments. We recently reported that an active sub-fraction of *S. crispus* leaves (SCS) caused caspase-dependent apoptosis of human breast cancer MCF-7 and MDA-MB-231 cells. **Materials and Methods:** Considering the ability of SCS to also promote the activity of the antiestrogen, tamoxifen, we further examined the effect of SCS in modulating cell cycle progression and related proteins in MCF-7 and MDA-MB-231 cells alone and in combination with tamoxifen. Expression of cell cycle-related transcripts was analysed based on a previous microarray dataset. **Results:** SCS significantly caused G1 arrest of both types of cells, similar to tamoxifen and this was associated with modulation of cyclin D1, p21 and p53. In combination with tamoxifen, the anticancer effects involved downregulation of ERα protein in MCF-7 cells but appeared independent of an ER-mediated mechanism in MDA-MB-231 cells. Microarray data analysis confirmed the clinical relevance of the proteins studied. **Conclusions:** The current data suggest that SCS growth inhibitory effects are similar to that of the antiestrogen, tamoxifen, further supporting the previously demonstrated cytotoxic and apoptotic actions of both agents.

**Keywords:** *Strobilanthes crispus* - tamoxifen - cell cycle - estrogen receptor - MCF-7 - MDA-MB-231

Introduction

Many cancer patients seek traditional therapeutic medicines with the hope of enhancing their cancer therapy while minimizing side effects. Others may seek such treatments due to the limited access to conventional pharmacological treatment. As such, interest in the therapeutic use of natural products has grown with many researchers now focussing on medicinal plants as a source of anticancer remedies or as adjuvants to enhance the efficacy of conventional chemotherapeutic drugs.

Cell proliferation is tightly regulated by a number of different molecules that control the balance between growth promoting and growth inhibiting signals. Dysregulation of normal cell division processes results in uncontrolled cell proliferation that may lead to cancer development. Cell cycle checkpoints monitor the cellular environment and control cell cycle transition. Cyclins and cyclin-dependent kinases (CDKs) accelerate cell cycle progression, while CDK inhibitors such as p21 and p27, and tumor suppressor proteins, p53 and RB, halt its progression (Garrett, 2001). Abnormalities of cell cycle regulators in cancers are commonly reported, making cell cycle regulators important potential targets for chemopreventive treatments (Stewart et al., 2003). In the current study, a bioactive subfraction of *Strobilanthes crispus* (SCS) that was previously shown to be potent inhibitors of breast and prostate cancer cell growth (Yaacob et al., 2010) was investigated for its ability to modulate cell cycle progression in MCF-7 and MDA-MB-231 breast cancer cells. SCS was recently found to synergise with tamoxifen to induce apoptosis of these cancer cell lines (Yaacob et al., 2014). Hence, the effect of SCS on tamoxifen-induced cell cycle-related activities was explored. Herein we showed that SCS alone and in combination with tamoxifen induced cell cycle arrest and modulated specific cell cycle regulatory proteins that are reported to be abnormally expressed in human breast adenocarcinoma.

Materials and Methods

**Plant material**

The leaves of *S. crispus* (L.) Blume were purchased...
from Agrodynamic Resources (Malaysia) that cultivated the plant in Tasek Gelugor, Pulau Pinang. A voucher specimen was deposited at the university herbarium (no. 11046) following plant authentication. SCS was prepared by the Centre for Drug Research, Universiti Sains Malaysia, as previously described (Yaacob et al., 2010) and kept at -20°C as a 10 mg/ml stock in DMSO (Sigma-Aldrich, USA).

**Antibodies**

Rabbit anti-human β-actin polyclonal IgG, rabbit anti-human cyclin A polyclonal IgG and goat polyclonal secondary antibody to rabbit IgG-H&L (HRP) were purchased from Abcam (UK). Rabbit anti-human cyclin B1 polyclonal antibody, rabbit anti-human cyclin D1 polyclonal IgG, rabbit anti-human cyclin E2 polyclonal IgG, rabbit anti-human p21 polyclonal IgG and rabbit anti-human p53 polyclonal IgG antibodies were purchased from Cell Signaling Technology (USA).

**Cell culture and treatment**

MCF-7 and MDA-MB-231 breast cancer cell lines (ATCC, USA) were maintained in Roselle’s Park Memorial Institute medium (RPMI-1640) and Dulbecco-modified Eagle’s medium (DMEM), respectively, with 10% fetal bovine serum (Hyclone, USA) and 50 units/ml penicillin/streptomycin (GIBCO BRL, UK) at 37°C in an atmosphere of 5% CO₂. Tamoxifen (Sigma-Aldrich, USA) was prepared as a 10 mM stock in ethanol. Cells were seeded up to 70% confluence and then treated with SCS at 8.5 or 10.0 μg/ml as previously reported (Yaacob et al., 2010), 5 μM tamoxifen or their combination for 24h (cell cycle analyses by flow cytometry) and up to 48h for Western blot analyses.

**Cell cycle analysis**

Prior to treatment for cell cycle analysis, the cells were serum starved (using serum-free medium) for 24 h to synchronize them in G₀ phase of the cell cycle. Cell cycle distribution was assayed using Cycle TESTPLUS DNA Reagent Kit (Becton Dickinson, USA) according to the manufacturer’s guideline and analyzed by the Becton Dickinson FACScan apparatus. In brief, both floating and adherent cells were harvested, followed by centrifugation and washing with ice-cold PBS. Cells (1x10⁶) were incubated with propidium iodide in the presence of RNase A for 30 min at 4°C in the dark. Data from at least 15,000 cells per sample were acquired and analyzed using the ModFit LTTM software (USA).

**Protein extraction and Western blotting**

Cell lysates were harvested by centrifugation at 12,000 rpm for 2 min following lysis in lysis buffer (50 mM of Tris-HCl, 150 mM of NaCl, 0.2% SDS, 1 mM PMSF, 2 μg/ml of leupeptin, 2 μg/ml of aprotinin and 1 mM of Na₂VO₃). The protein concentrations were determined spectrophotometrically (Thermoscientific, USA) and samples of 50-100 μg were resolved using 10% SDS-polyacrylamide gel followed by semi-dry transfer onto the PVDF membrane. The proteins were immunoblotted for cyclins A, B, D1 and E, p21, p53 and β-actin overnight at 4°C and subsequently reacted with horseradish peroxidase-conjugated secondary antibody. Antibody-bound proteins were detected by chemiluminescence using ECL™ Prime Western Blotting Detection reagent (GE Healthcare, UK) according to the manufacturer’s protocols and visualised using the Fluor chem FC2 image analyser (DKSH, US). The band density for each treatment compared to control was analyzed using ImageJ 1.46 software (http://imagej.nih.gov/ij/) and normalized to the β-actin band density.

**Microarray data-mining**

A microarray dataset (GEO accession number: GSE3744; Richardson et al., 2006) containing transcript values of CCNE1 (probeset ID: 213523_at), CCNA2 (203418_at), CCNB1 (228729_at), CCND1 (208711_at), ESR1 (215551_at), ESR2 (1569554_at), CDKN1A (202284_s_at) or TP53 (201746_at) in ductal breast carcinomas (n=40) and non-malignant breast tissues (n=7) of human origin were obtained from Oncomine (https://www.oncomine.org) and analyzed.

**Statistical analysis**

Statistical analyses for DNA content and protein expression profiles were performed using IBM SPSS Statistics 20 (Chicago, IL, USA) and significance levels were evaluated using ANOVA with Tukey’s multiple comparison test and p<0.05 was considered as significantly different from control. Bioinformatics analysis comparing the transcript values between malignant and non-malignant breast tissues was carried out using Mann-Whitney test.

**Results**

**Induction of cell cycle arrest in MCF-7 and MDA-MB-231 cells**

Following our recent findings that SCS synergises with tamoxifen to induce death of breast cancer cells (Yaacob et al., 2014), we hypothesized that this involved inhibition of cell cycle progression. The DNA content of cells treated with SCS, tamoxifen and their combination was first measured by flow cytometry. SCS significantly increased (p<0.05) the percentage of MCF-7 cells in G1 (73.4%) and G2/M phases (9.4%) with reduced percentage of cells in S phase (17.2%) compared to untreated controls (58.7, 5.4 and 35.9%, respectively; Figure 1A). Similarly, 5 μM tamoxifen either alone or in combination with SCS, caused accumulation of cells in G1 phase (72.8 and 70.6%, respectively) compared to untreated controls. These results suggest that SCS, tamoxifen and their combination exerted their growth suppressive activity in MCF-7 cells by arresting cell cycle progression at G1 phase. The combined SCS and tamoxifen however, caused similar levels of cell cycle arrest compared to either agent alone.

As with MCF-7 cells, SCS treatment resulted in significant accumulation of MDA-MB-231 cells in G1 phase (74.0%) while reducing the percentage of cells in S phase (19.4%; p<0.05) compared to control cultures (51.3 and 34.8%, respectively) (Figure 1C). However, there
was no significant change in the cell population in G2/M phase. Treatment with tamoxifen or the combination of tamoxifen and SCS also resulted in a significant increase in the population of MDA-MB-231 cells in G1 phase (63.5 and 70.2%, respectively) compared to controls. A corresponding decrease in the percentage of cells in the S phase was also evident following tamoxifen (26.4%) and SCS+tamoxifen (18.9%). The results thus far indicate that the mechanism of growth inhibitory effect of SCS is similar to that of tamoxifen in both types of breast cancer cells.

**Effects on cell cycle regulatory proteins in MCF-7 and MDA-MB-231 cells**

To further explore the mechanism of cell growth arrest by SCS and tamoxifen, we determined the expression of cyclins, p21 CDK inhibitor and p53 tumor suppressor protein, in 24 h- and 48 h-treated and untreated cells. We observed a three-fold increase in the expression of cyclin D1 after 24 h treatment of MCF-7 cells with SCS which was accompanied with increased p21 and p53 expression. This was then followed by a decrease in cyclin D1 expression at 48 h, similar to the control level (Figure 2A). Similarly, tamoxifen alone or in combination with SCS markedly decreased cyclin D1 expression while increased cyclin A and B expression at 48 h post-treatment compared to the control. The expression of cyclin E was found to be reduced by SCS and the combination treatment while that of cyclins A and B remained similar to control level.

In contrast to the observations in MCF-7 cells, SCS decreased cyclin D1 protein expression in MDA-MB-231 cells at both 24 h and 48 h (Figure 2B). Although tamoxifen alone had no effects on the expression of cyclin D1, its combination with SCS caused a reduction in this expression. The expression of cyclins E, A and B in these cells was not significantly modulated by SCS or tamoxifen alone. However, reduced expression of cyclin E was observed following the combination treatment while cyclin B expression was slightly increased at 48h. The expression of p53 protein was not significantly modulated by all treatments.

**Effects on the ER protein expression in both MCF-7 and MDA-MB-231 cells**

The ERα-positive MCF-7 cells depend on estrogens acting on ERα for proliferation (Helguero et al., 2005). However, our results showed that SCS or tamoxifen did not interfere with the constitutive expression of ERα nor ERβ protein in the hormone-dependent MCF-7 cells (Figure 3) indicating that cell growth inhibition of these cells by the individual agents does not involve ER regulation. Interestingly, treatment with the combination of SCS and tamoxifen for 48 h led to a four-fold reduction in ERα expression and a two-fold reduction in ERβ expression in MCF-7 cells compared to controls. Similarly, tamoxifen alone or in combination with SCS decreased ERα and ERβ expression in MDA-MB-231 cells.

**Figure 2. Cell Cycle-related Proteins in MCF-7 and MDA-MB-231 Cells Treated with SCS, Tamoxifen and SCS+Tamoxifen.** MCF-7 (A) and MDA-MB-231 (B) cells treated with SCS (8.5 and 10.0 μg/ml, respectively), tamoxifen (T5; 5 μM) or SCS+tamoxifen (C5) for 24 h and 48 h were harvested for preparation of total protein and Western blotting. DMSO (0.1% v/v) was used for the control cultures. The band density for each treatment compared to control was analyzed using ImageJ 1.46 software (http://imagej.nih.gov/ij/) and normalized to the β-actin band density.

**Figure 3. ERα and ERβ Expression in MCF-7 and MDA-MB-231 Cells Treated with SCS, Tamoxifen and SCS+Tamoxifen.** MCF-7 and MDA-MB-231 cells treated with SCS (8.5 and 10.0 μg/ml, respectively), tamoxifen (T5; 5 μM) or SCS+tamoxifen (C5) for 24 h and 48 h were harvested for preparation of total protein and Western blotting. DMSO (0.1% v/v) was used for the control cultures. The band density for each treatment compared to control was analyzed using ImageJ 1.46 software (http://imagej.nih.gov/ij/) and normalized to the β-actin band density.
Apoptosis is commonly associated with cell cycle arrest as both mechanisms involve common genes or molecules (Choi et al., 2001; Alenzi, 2004). For example, the tumor suppressor protein, p53, is known to affect both cellular growth arrest and apoptosis, primarily through transactivation of molecules such as p21, Gadd45, Mdm2, cyclin D1, Bax, Bcl-xL and FasL (Alenzi, 2004; Pfaum et al., 2014). Herein we show that SCS significantly induced cell cycle arrest of both ERα-positive MCF-7 and ERα-negative MDA-MB-231 cells at G1 phase, similar to the action of the commonly used antiestrogen, tamoxifen. A number of anti-cancer agents derived from plants are also reported to inhibit cancer cell growth by blocking cell cycle progression. For example, quercetin (a polyphenol) arrested breast cancer cells at G1 or G2/M phase (Choi et al., 2001), curcumin (derived from turmeric of Curcuma longa) caused G2/M arrest in bladder cancer cells (Park et al., 2006) and gambogic acid (isolated from Gambogia resin of Garcinia hanburyi) inhibited growth of gastric carcinoma cells by inducing G2/M arrest (Yu et al., 2006).

Microarray data mining of a gene expression dataset (Richardson et al., 2006) presented herein indicates that genes coding for cyclins were abnormally expressed in breast cancer suggesting that dysregulation of the cell cycle associated with abnormal expression of cell cycle-related proteins is an important mechanism in breast cancer pathogenesis. Cyclin D1 for example is commonly overexpressed in approximately 50% of breast cancer cases (Fu et al., 2004). Deregulation of cyclin D1 expression alters cell division cycle of normal cells to be less dependent on growth factors and therefore accelerates passage through the G1 phase (Alao, 2007). Herein, we determined the expression of cyclin D1, E, A and B proteins and found that cyclin D1 expression was modulated by SCS and tamoxifen in MCF-7 cells. Previous studies demonstrated that reduction in cyclin D1 protein expression is an early and critical event in antiestrogen action (Doisneau-Sixou et al., 2003; Butt et al., 2005). This is consistent with our findings of two-fold reduction in cyclin D1 protein level following 48 h tamoxifen treatment. Cyclin D1 complexes with and regulates the activity of CDK4 or CDK6 that mediates phosphorylation of the retinoblastoma gene product, pRb, required for G1/S transition (Lange and Yee, 2011).

Cyclin D1 expression can mimic the action of estrogen on cell cycle progression and re-initiate cell proliferation in antiestrogen-arrested cells (Butt et al., 2005). Therefore, overexpression of cyclin D1 may cause sustained ER signaling and development of endocrine resistance in breast cancer cells (Hui et al., 2002; Butt et al., 2005). In our study, cyclin D1 expression in MCF-7 cells was initially increased by SCS at 24 h but then declined to control level after 48 h treatment. This could suggest an initial response by the cancer cells to develop resistance against SCS treatment. This effect was however abrogated with longer exposure to SCS, or combined treatment of SCS and tamoxifen which resulted in a 2.5-fold reduction in cyclin D1 expression, similar to the effect of the antiestrogen alone. In addition, the increased cyclin D1 expression was accompanied with elevated p21 and p53 expression. The effects of cyclin D1 overexpression was proposed to be neutralized by a feedback mechanism between the positive (cyclin D1) and negative (p21) regulators of the cell cycle, which ultimately resulted in proliferation arrest (de Jong et al., 1999). Thus, p21 elevation in the current study may serve as a feedback response to the apparent increase in cyclin D1 protein level induced by SCS in MCF-7 cells. The increased p53 expression is in agreement with previous reports that p53 increases the transcription of several genes, including...
p21, followed by arrest of cells at G1 phase (Ichikawa et al., 2008; Jednak et al., 2008). Similarly, Chong and colleagues (Chong et al., 2012) demonstrated an increase in p53 protein level following S. crispus ethanol extract treatment of MCF-7 cells. It is therefore postulated that induction of p21 and p53 expression contributes to the G1 phase arrest and apoptotic effects of SCS in MCF-7 cells.

Interestingly, the initial upregulation of cyclin D1 protein was not observed in the ERα-negative MDA-MB-231 cells in which a 2-fold and 5-fold reduction were noted with SCS alone or in combination with tamoxifen, respectively. MDA-MB-231 cells contain mutant p53 and no concomitant increase in p53 or p21 expression was observed, suggesting that the inhibition of MDA-MB-231 cell growth arrest by SCS may involve a mechanism different from that of MCF-7 cells. Overexpression of mutant p53 would be undesirable as it could provide the cells with growth advantage. The current finding is also consistent with the reported ability of phenethyl isothiocyanate (derived from watercress plant) and conjugated linoleic acids (derived from linoleic acid) to selectively suppress mutant p53, which was followed by apoptotic cell death in MDA-MB-231 cells (Majumder et al., 2002; Wang et al., 2011).

Cyclins A and B that are associated with S phase and G2/M transition, respectively, were not affected by SCS treatment indicating insignificant role in SCS mechanism of action in both MCF-7 and MDA-MB-231 cells. Cyclin B expression was however, increased with the combined SCS and tamoxifen at 48 h. As activation of Cdc2/cyclin B complex is required for cell progression from G2 to M phase and binding of p21 to this complex inhibits its activity (Garrett, 2001), the increased expression of cyclin B could be a consequence of the lack of p21 upregulation in MDA-MB-231 cells which unlike MCF-7 cells, are p53-defective. On the other hand, the reduced expression of cyclin E by SCS alone or in combination with tamoxifen may cause disruption of the formation of active CDK2/cyclin E complex required for phosphorylation of pRb and activation of E2F transcription factor, which are crucial for G1/S transition (Garrett, 2001). This provides support for the G1 phase arrest induced by SCS above.

The effects of estrogen are mediated via two estrogen receptors, ERα that mediates proliferation and ERβ that exerts proapoptotic effects in cancer cells, such as with breast and prostate cancer cell lines (Helguero et al., 2005). ERβ is co-expressed with ERα in many human breast cancers (Kurebayashi et al., 2000) but is expressed at low levels compared to normal tissues (Freund et al., 2003). ERα and ERβ have similar DNA-binding domains but differ in the ligand-binding domains especially in the N-terminal transactivation domain (Katzenellenbogen et al., 2000). Studies suggest that ERβ negatively regulates some of ERα functions (Lindberg et al., 2003; Paruthiyil et al., 2004). The opposing action of ERβ on cell proliferation potentially occurs via repression of cyclin D1 and cyclin A expression and upregulation of growth inhibitory molecules like p21 and p27 (Paruthiyil et al., 2004). SCS alone did not alter the expression of ERα in MCF-7 cells but caused a four-fold decrease in ERα protein expression when combined with tamoxifen.

Although upregulation of p21 was observed in MCF-7 cells, no significant changes in ERβ expression were observed following all treatments of MCF-7 cells as well as the ERα-negative MDA-MB-231 cells. We could therefore conclude that SCS anticancer activities involved downregulation of ERα protein in MCF-7 cells but are independent of ER-mediated mechanism in MDA-MB-231 cells. Steigerova et al. (2010) showed that brassinosteroids (plant hormones) caused cell cycle arrest and apoptosis of MCF-7 and MDA-MB-435 cancer cells via modulation of ERα but not ERβ expression, which is in agreement with our current findings.

In conclusion, our data suggest that the growth inhibitory effects of SCS is similar to that of the antiestrogen, tamoxifen which provides further support to their synergistic cytotoxic and apoptotic action reported earlier (Yaacob et al., 2014). Its growth inhibitory action either alone or in combination with tamoxifen, is partly attributed to suppression of ERα and induction of p21 and p53 leading to G1 phase arrest of MCF-7 cells. In the ERα-negative MDA-MB-231 cells, the mechanism involves downregulation of cyclin D1 and upregulation of p21 as well as suppression of the mutated p53 protein. The specific components of SCS that possess the antiestrogenic and anticancer effects have yet to be determined.

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